RNases J1 and J2 are critical pleiotropic regulators in Streptococcus mutans

Xi Chen,1 Nan Liu,1† Sharukh Khajotia,2 Fengxia Qi1,3 and Justin Merritt1,3†

1Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA
2Department of Dental Materials, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA
3Division of Oral Biology, University of Oklahoma Health Sciences Center, OK 73104, USA

Correspondence
Justin Merritt
merrittj@ohsu.edu

Received 22 December 2014
Accepted 16 January 2015

In recent years, it has become increasingly evident that post-transcriptional control mechanisms are the principal source of gene regulation for a large number of prokaryotic genetic pathways, particularly those involved in virulence and environmental adaptation. Post-transcriptional regulation is largely governed by RNA stability, which itself is determined by target accessibility to RNase degradation. In most Firmicutes species, mRNA stability is strongly impacted by the activity of two recently discovered RNases referred to as RNase J1 and RNase J2. Little is known about RNase J1 function in bacteria and even less is known about RNase J2. In the current study, we mutated both RNase J orthologues in Streptococcus mutans to determine their functional roles in the cell. Single and double RNase J mutants were viable, but grew very slowly on agar plates. All of the mutants shared substantial defects in growth, morphology, acid tolerance, natural competence and biofilm formation. However, most of these defects were more severe in the RNase J2 mutant. Phenotypic suppression results also implicate a role for RNase J2 as a regulator of RNase J1 function. Unlike Bacillus subtilis, RNase J2 is a major pleiotropic regulator in S. mutans, which indicates some fundamental differences from B. subtilis in global gene regulation. Key conserved residues among the RNase J2 orthologues of lactic acid bacteria may hint at a greater role for RNase J2 in these species.

INTRODUCTION

There is an emerging body of literature implicating post-transcriptional regulation as a substantially more important aspect of gene regulation than previously assumed (Storz et al., 2011). For a continually increasing number of genetic pathways, post-transcriptional mechanisms are proving to be the predominant mode of regulatory control, especially for highly dynamic pathways such as those involved in virulence or environmental adaptation (Hoe et al., 2013; Repoila et al., 2003). Despite this, surprisingly little is understood about the mechanisms controlling mRNA stability in most prokaryotes. Recent studies suggest that the degradation of prokaryotic mRNAs is primarily controlled by either the RNase E pathway commonly found among the enterobacteria or the RNase J/RNase Y pathway best characterized in Bacillus subtilis (Lehnik-Habrink et al., 2012). While the RNase E pathway was first identified several decades ago, amazingly, RNases J and Y were only discovered within the last decade (Even et al., 2005; Shahbabian et al., 2009).

Most Firmicutes species do not encode RNase E orthologues, rather, they typically encode RNase Y along with two copies of RNase J paralogues referred to as RNase J1 and RNase J2 (Even et al., 2005; Merritt et al., 2014). In B. subtilis, RNase Y likely provides much or most of the endoribonuclease activity, which is analogous to the role of RNase E (Bechhofer, 2011). RNase J, however, has the intriguing ability to degrade RNA as a 5′–3′ exoribonuclease (Mathy et al., 2007). Until recently, this ability was thought to be an exclusively eukaryotic degradative pathway. Both RNase J1 and RNase J2 also possess endoribonuclease activity in in vitro assays, but there is still debate as to the importance of this activity in vivo (Condon, 2010; Lehnik-Habrink et al., 2012). In B. subtilis, RNases J1 and J2 form heterotetramers, and oligomerization plays a central role in determining the cleavage specificity of the enzymes (Mathy et al., 2010). Interestingly, most of the exonuclease activity derived from the RNase J1/J2 complex can be attributed to RNase J1. RNase J2 exonuclease activity is at least two orders
of magnitude weaker in in vitro cleavage assays (Mathy et al., 2010). Consequently, it is doubtful whether RNase J2 serves as a major RNase in _B. subtilis_. However, in _Streptococcus pyogenes_, RNase J2 depletion affects the mRNA stability of a diverse post-transcriptional regulon and the enzyme is even produced in higher abundance than RNase J1 (Bugrysheva & Scott, 2010). RNase J2 has also been implicated in the regulation of _Enterococcus faecalis_ pilin production (Gao et al., 2010). Thus, there is reason to suspect that highly disparate levels of intrinsic enzymic activity exist among RNase J2 orthologues.

Overall, there is very limited information available regarding the phenotypes of RNase J1 mutants in species other than _B. subtilis_ and almost nothing is known about RNase J2 in general. Phenotypically, RNase J1 mutants have thus far exhibited the greatest consistency. Mutant strains in several species have each exhibited severe growth deficiencies or lethality (Bugrysheva & Scott, 2010; Figaro et al., 2013; Linder et al., 2014). In _B. subtilis_, this growth deficiency is accompanied by a wide range of other pleiotropic effects such as defects in sporulation, competence and morphology, in addition to greatly increased sensitivities to various antibiotics (Figaro et al., 2013). Interestingly, _B. subtilis_ RNase J2 mutants do not exhibit these defects, which is consistent with the suggestion that its RNase J2 orthologue serves primarily as a regulator and/or scaffold for RNase J1/J2 complexes (Figaro et al., 2013). In contrast to _B. subtilis_, _S. aureus_ RNase J1 and J2 mutants both exhibit major growth defects and a pronounced sensitivity to high and low temperatures (Linder et al., 2014). Likewise, in _S. pyogenes_, RNase J1 and J2 mutations both exhibit lethality, at least on standard growth medium (Bugrysheva & Scott, 2010). Thus, RNase J2 is likely to be a much more important regulator in some species compared with _B. subtilis_.

Surprisingly little is known about RNase J1 or J2 among streptococci, which provided the impetus for the current study. In contrast to _S. pyogenes_, RNase J1, J2 and J1/J2 double mutants were all viable in _Streptococcus mutans_. Similar to _B. subtilis_, the _S. mutans_ RNase J1 orthologue is likely a pleiotropic regulator of a wide variety of genetic pathways. However, unlike _B. subtilis_, an RNase J2 mutation triggers a broader range of pleiotropic effects compared with RNase J1. Therefore, our results suggest that the _S. mutans_ RNase J2 orthologue is likely to be a major RNase with a post-transcriptional regulon that is at least partially distinct from that of RNase J1. Furthermore, the RNase J1/J2 double mutant phenotypes also support a role for RNase J2 as a regulator of RNase J1 function.

**METHODS**

**Bacterial strains and culture conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. All _Streptococcus_ strains were grown anaerobically in an atmosphere consisting of 85% N2, 10% CO2 and 5% H2 at 37 °C. For natural transformation experiments, cells were maintained in Todd–Hewitt medium (Difco) supplemented with 0.3% (w/v) yeast extract (THYE). For the selection of antibiotic-resistant colonies of _S. mutans_, brain heart infusion (BHI) plates were supplemented with 800 µg ml−1 kanamycin, 12 µg ml−1 tetracycline, 900 µg ml−1 spectinomycin or 12.5 µg ml−1 erythromycin. The competence-stimulating peptide used for _S. mutans_ transformation was custom synthesized by AnaSpec.

**Construction of _rnjA_ and _B_ mutant strains.** The primers used in this study are listed in Table 2. The ΔrnjA (J1KO) and ΔrnjB (J2KO) allelic replacement mutants were previously constructed (Liu et al., 2015). We also constructed an _rnjA/B_ double mutant (J1J2KO) via double-crossover homologous recombination. Two DNA fragments were amplified from the genomic DNA of strains J1KO and J2KO using the primers (SMU368upF/SMU368dnR and SMU144upF/SMU144dnR), which bind upstream and downstream of _rnjA_ and _rnjB_, respectively. Next, the two DNA fragments were transformed into WT strain UA159 and then selected on agar plates containing kanamycin. Transformants were then patched onto agar plates containing erythromycin to identify double mutant strains.

**Construction of _rnjA_, _rnjB_ and _rnjA/B_ complemented mutant strains.** To complement the _rnjA_, _rnjB_, and _rnjA/B_ mutations, we constructed three plasmids containing WT copies of the deleted genes. Each of the plasmids was created using a previously described cloning-independent methodology (Xie et al., 2013). Firstly, PCR amplifications of the shuttle vector pDL278 and the target inserts were generated using primers that also add complementary sequences to the amplicon 5’ termini. For the _rnjA_ complementation vector, the primers PDLF_J1/PDLR_J1 were used to amplify pDL278 and the primers J1F_PDL/J1R_PDL were used to amplify _rnjA_. For the _rnjB_ complementation vector, the primers PDLF_J2/PDLR_J2 were used to amplify pDL278 and the primers J2F_PDL/J2R_PDL were used to amplify _rnjB_. The _rnjA/B_ complementation vector, the primers PDLF_J1/J2R_PDL were used to amplify _rnjA_ and _rnjB_, respectively. Secondly, concatemers were generated by prolonged overlap extension PCR (POE–PCR) using the vector and insert amplicons generated in the first-round PCRs. Lastly, the POE–PCR amplicons were directly transformed into the WT UA159 via natural transformation and selected on BHI agar plates containing spectinomycin. After confirming the presence of the expected plasmids in several isolates for each construct, the corresponding WT copies of the _rnjA_, _rnjB_ or _rnjA/B_ genes were mutated by transforming the appropriate allelic replacement constructs described previously. This resulted in the final complemented mutant strains J1c (_rnjA_), J2c (_rnjB_) and J1J2c (_rnjA/B_).

**Growth curve measurements.** Overnight cultures of UA159 WT and derivative strains (except J2KO and J2c) were first grown to an OD600 of 0.2 and subsequently diluted 1:40 in fresh pre-reduced BHI medium. The cultures were then transferred to a 96-well plate and sealed under anaerobic conditions. OD measurements were then monitored at 37 °C in 20 min intervals using a Bioscreen C analyser version 2.4 (Oy Growth Curves).

**Natural competence measurements.** Genetic competence was determined by a transformation efficiency assay using donor genomic DNA containing a tetracycline marker. Overnight cultures of WT UA159 and the derivative strains were diluted 1:30 in THYE medium and incubated until the optical density reached an OD600 of approximately 0.2. Due to the extremely slow growth rate of the _rnjB_ mutant, its culture was still in exponential phase after overnight growth and was therefore diluted 1:10 in THYE. It was also necessary to estimate its optical density due to aggregation within the ΔrnjB cultures. After incubation, 2.5 µg of transforming DNA and 1 µg ml−1 final concentration competence-stimulating peptide were added to each 500 µl cell culture and incubated until the optical density reached an OD600 of approximately 0.6. Cultures were plated on both
selective and nonselective media. Transformation efficiency was determined by calculating the ratio of transformants to total viable cells.

**Acid tolerance measurements.** Overnight cultures of WT UA159 and derivative strains were diluted in BHI and incubated until the cultures reached exponential phase. Cultures were then diluted to an OD_{600} of 0.2 and serially diluted on both untreated BHI plates of 0.2 before diluting 1:500 into BHI supplemented with 0.1 mM NaHCO₃. We previously determined this to be the minimum concentration of NaHCO₃ required to achieve a terminal pH >6.5 in stationary phase BHI cultures. No measurable differences in growth rate were detectable as a result of NaHCO₃ supplementation.

**BacLight fluorescent viability staining.** Stationary phase cultures were diluted 1:20 in fresh BHI medium and grown to exponential phase. The samples were then briefly vortexed and stained using a BacLight LIVE/DEAD staining kit (Life Technologies) according to the manufacturer’s protocol. Cells were visualized by epifluorescence microscopy using an Olympus BX61 fluorescence microscope with image capture. All images were captured using a 100 × oil immersion lens and Spot camera software v3.5.

**Biofilm measurements.** Static biofilms were grown in six-well polystyrene cell culture dishes (Cellstar). Overnight cultures were diluted 1:50 into fresh BHI and grown to exponential phase. Cultures were adjusted to an OD_{600} of 0.2 before diluting 1:500 into BHI medium supplemented with 1% (w/v) sucrose. The biofilms were incubated anaerobically at 37°C for 24 h for the WT and complemented strains and 48 h for the mutant strains. After the incubation period, the spent culture medium was removed by aspiration and the wells were gently rinsed with 1 × PBS solution. Fresh BHI was then added to each well and incubated at 37°C for 1 h before staining with a BacLight LIVE/DEAD viability kit (Life Technologies). Biofilms were imaged at the University of Oklahoma Health Sciences Center Laboratory for Molecular Biology and Cytometry Research core facility using a Leica SP2 MP confocal microscope. 3D reconstructions of biofilm images were generated by importing confocal microscopy z-stacks of the biofilms into Volocity software (version 5.0.2, Improvision/Perkin-Elmer). The reconstructions were produced in HR Opacity format using a previously published protocol.

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UA159</td>
<td>WT <em>Streptococcus mutans</em> genome reference strain</td>
<td>Ajdić et al. (2002)</td>
</tr>
<tr>
<td>J1KO</td>
<td>UA159 ΔrnjA, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Liu et al. (2015)</td>
</tr>
<tr>
<td>J2KO</td>
<td>UA159 ΔrnjB, Em&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Liu et al. (2015)</td>
</tr>
<tr>
<td>J12KO</td>
<td>UA159 ΔrnjA ΔrnjB, Km&lt;sup&gt;+&lt;/sup&gt;, Em&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>J1c</td>
<td>UA159 ΔrnjA::pCompJ1, Km&lt;sup&gt;+&lt;/sup&gt;, Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>J2c</td>
<td>UA159 ΔrnjB::pCompJ2, Em&lt;sup&gt;+&lt;/sup&gt;, Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>J12c</td>
<td>UA159 ΔrnjA ΔrnjB::pCompJ1J2, Km&lt;sup&gt;+&lt;/sup&gt;, Em&lt;sup&gt;+&lt;/sup&gt;, Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pDL278</td>
<td>E. coli-<em>Streptococcus</em> shuttle vector, Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Chen &amp; LeBlanc (1992)</td>
</tr>
<tr>
<td>pCompJ1</td>
<td>pDL278::rnjA, Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pCompJ2</td>
<td>pDL278::rnjB, Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pCompJ1J2</td>
<td>pDL278::rnjA, rnjB, Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

### Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ → 3′)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMU1444upF</td>
<td>GCTACGGTTCAGTTAACAATGG</td>
<td>rnjA/B allelic replacement</td>
</tr>
<tr>
<td>SMU1444dnR</td>
<td>TGCTCTCTGCCTTTCTCC</td>
<td>rnjA/B allelic replacement</td>
</tr>
<tr>
<td>SMU368upF</td>
<td>GACCGTTTGCTCGCATTACAC</td>
<td>rnjA/B allelic replacement</td>
</tr>
<tr>
<td>SMU368dnR</td>
<td>ATTAATATTTGCATTTCATTGAAACTCCGTTAATTG</td>
<td>rnjA/B allelic replacement</td>
</tr>
<tr>
<td>J1F_PDL</td>
<td>ACGAGGCTAGTCTCCTACGTTTCTTTCTTCTTTACGGTATAG</td>
<td>ΔrnjA complementation</td>
</tr>
<tr>
<td>J1R_PDL</td>
<td>CACGAACGAAATCGATCTCCTCAAGGTTTGGAGGTTGTC</td>
<td>ΔrnjA complementation</td>
</tr>
<tr>
<td>PDLF_J1</td>
<td>GACGAAGCCTCAAAAGCTTGGAGAGATGTTAAGAGATGGTACCTTTCGTTAATTG</td>
<td>ΔrnjA complementation</td>
</tr>
<tr>
<td>PDLR_J1</td>
<td>CTTATCCGTTAGAAGAGAGAGGAAAACTTAGATAGCTCATTGAAACTAGGCTCGT</td>
<td>ΔrnjB complementation</td>
</tr>
<tr>
<td>J2F_PDL</td>
<td>ACGAGGCTAGTCTCCTACGTTTCTTTCTTCTTTACGGTATAG</td>
<td>ΔrnjB complementation</td>
</tr>
<tr>
<td>J2R_PDL</td>
<td>CACGAACGAAATCGATCTCCTCAAGGTTTGGAGGTTGTC</td>
<td>ΔrnjB complementation</td>
</tr>
<tr>
<td>PDLF_J2</td>
<td>GTGTTGCGTTTCTCAGCAGAAGATGTTAAGAGATGGTACCTTTCGTTAATTG</td>
<td>ΔrnjB complementation</td>
</tr>
<tr>
<td>PDLR_J2</td>
<td>CAACCTCCCCTGCAGTTTCTTTCTATAGTTAAGGATACTACGCTCCTTG</td>
<td>ΔrnjB complementation</td>
</tr>
<tr>
<td>J1R_J2</td>
<td>CAACCTCCCCTGCAGTTTCTTTCTATAGTTAAGGATACTACGCTCCTTG</td>
<td>ΔrnjA/B complementation</td>
</tr>
<tr>
<td>J2F_J1</td>
<td>GACGAAGCCTCAAAAGCTTGGAGAGATGTTAAGAGATGGTACCTTTCGTTAATTG</td>
<td>ΔrnjA/B complementation</td>
</tr>
</tbody>
</table>
(Khajotia et al., 2013). For the low-magnification phase-contrast images of the biofilms, images were captured using a Nikon TE2000-E inverted epifluorescence microscope.

RESULTS

Identification of RNase J1 and J2 in S. mutans

Almost nothing is known about RNase J1 and J2 mutant phenotypes in streptococci, except for a single study on S. pyogenes which characterized their central roles in global mRNA stability (Bugrysheva & Scott, 2010). In that study, conditional expression mutants were employed for depletion analysis, as knockout mutations of both RNases exhibited lethality. Thus, we were curious as to whether these genes are also essential in S. mutans. We performed a BLASTP search of the S. mutans genome using the B. subtilis RNase J1 and J2 orthologues and identified two candidates (SMU.368c and SMU.1444c) that were annotated as ‘RNA metabolizing β-lactamases’ (Fig. 1a). This is a typical annotation for RNase J orthologues, due to their membership in the widely conserved β-CASP family of metallo-β-lactamases (Dominski et al., 2013). Both putative proteins exhibit extensive homology, but it was possible to distinguish between them using five key residues unique to the B. subtilis RNase J1 (Newman et al., 2011). All five of the definitive RNase J1 residues were conserved in SMU.368c, which is indicative of this protein as the RNase J1 orthologue (Fig. 1b). Interestingly, one of these characteristic RNase J1 residues, Asp78, is also conserved in SMU.1444c, whereas the other four residues differ (Fig. 1b). This indicates that the SMU.1444c protein is likely to be RNase J2, even though the B. subtilis orthologue differs at all five positions. Further evidence for these protein assignments can be found in the operon structures of SMU.368c and SMU.1444c. In B. subtilis, rnjA (RNase J1) is co-transcribed with a small upstream gene ykzG (Jamalli et al., 2014), whereas rnjB (RNase J2) is a single gene operon. The same genomic arrangement is also conserved in S. mutans (Fig. 1a).

Morphology and growth characteristics of RNase J mutant strains

Both rnjA and rnjB were targeted for deletion by allelic replacement. To our surprise, mutations of both genes were readily obtainable, unlike that previously reported for S. pyogenes (Bugrysheva & Scott, 2010). However, transformants did take considerably longer than average (4 days versus 2 days) to appear, indicating likely growth deficiencies. We also tested whether the same was true for a double mutation of both rnjA and rnjB and indeed this was the case. The ΔrnjA/ΔrnjB double mutant transformants were easily obtainable, but only after a much longer incubation period. All three mutant strains exhibited a clumpy appearance in liquid medium, but this phenotype was considerably more...
dramatic in the ΔrneJ mutant (Fig. 2a). Similarly, the ΔrneJ mutant was also the only strain difficult to fully disperse by vortexing. For all three strains, trans expression of the mutated genes fully restored the cultures to their typical appearance. (Fig. 2a). It was also apparent that cellular morphology was strongly impacted as well. Differences in both chain length and cell shape were particularly obvious in cells taken directly from agar plates (Fig. 2b). On plates, the WT rarely formed chains longer than two or three cells, whereas this was typical for the RNase J mutants. Mirroring the results in liquid culture, the ΔrneJ mutant also exhibited the most dramatic defects. It formed exceptionally long chains that frequently contained cells resembling bacterial ghosts (Szostak et al., 1996), which were presumably partially lysed. In contrast, major colony morphological defects were only apparent in the ΔrneA/B double mutant, as it uniquely formed smooth, wet colonies rather than the dry, rough colonies characteristic of the WT (Fig. 2c). A similar, but intermediate smooth colony phenotype occurred in the ΔrneB mutant as well. Surprisingly, the doubling time of the ΔrneA mutant in liquid culture was only slightly longer than the WT and complemented mutant (Fig. 3a). In contrast, the ΔrneA/B double mutant strain grew considerably slower (Fig. 3a). It was not possible to obtain an accurate growth curve measurement for the ΔrneB mutant, due its clumpy phenotype in liquid medium, but it was obviously much

Fig. 2. Morphology phenotypes of RNase J2 mutants. (a) Cultures were grown to stationary phase and then photographed. Strains from left to right are: WT, ΔrneA (J1), complemented ΔrneA (J1c), ΔrneB (J2), complemented ΔrneB (J2c), ΔrneA/B (J1/J2) and complemented ΔrneA/B (J1/J2c). (b) Cells from each of the indicated strains were scraped from agar plates and imaged using phase-contrast microscopy. Black arrows indicate ghost cells. Bars, 1 μM. (c) Colonies were directly photographed from agar plates.
slower to develop colonies on an agar plate (Fig. 3b). It is worth noting that all of the mutant strains exhibited exceptionally long lag phases (data not shown). Therefore, all growth curve measurements were made using dilutions of exponential phase cultures.

**Stress tolerance and viability phenotypes**

Due to the growth and morphology phenotypes of the RNase J mutants, we predicted that they would also exhibit a pronounced sensitivity to stress. However, we were extremely surprised to discover that the mutants only displayed two- to threefold increased sensitivity to oxidation from H₂O₂ or paraquat (data not shown). The same was true for stationary phase survival assays. The proportion of surviving cells was only modestly reduced compared with the WT (data not shown). In contrast, all three RNase J mutants were exquisitely sensitive to acidic pH (Fig. 4a). Survival rates at pH 5 were reduced by four to five orders of magnitude, indicating a near total loss of acid tolerance abilities. We also noticed that each of the RNase J mutants consistently yielded approximately tenfold fewer c.f.u. on the BHI control plates (Fig. 4a). This suggested that a significant portion of the liquid cultures was either dead, even in exponential phase, or that many of the cells were simply viable but non-culturable. To examine this further, we stained exponential phase cultures of the RNase J mutants with a BacLight LIVE/DEAD viability kit. Interestingly, the ∆njjB mutant exhibited a substantial proportion of dead/dying cells, whereas the two other mutants were comparable to the WT (Fig. 4b). Thus, it is conceivable that a large proportion of the ∆njjB mutant cells were dead/dying in actively growing cultures, but apparently this was not the case for the ∆njjA and ∆njjA/B mutants. Given the extreme acid sensitivity of the RNase J mutants, we hypothesized that the apparent reduction in c.f.u. was likely as a result of medium acidification during growth. To test this, each of the mutants was cultured in medium containing sodium bicarbonate and grown to exponential phase before spotting dilutions on agar plates also supplemented with sodium bicarbonate. However, this approach did not appear to increase the total number of mutant c.f.u. recovered, although it did improve their growth (Fig. 4c). Thus, it is unlikely that the disparity between OD₆₀₀ and c.f.u. in exponential phase cultures is attributable to medium acidification.

**Natural competence and biofilm formation**

In addition to stress tolerance, we were also interested to examine the role of RNase J proteins in the two major developmental pathways of S. mutans: natural competence and biofilm formation. All three RNase J mutants were severely deficient in natural competence, as none of the mutant strains ever yielded transformants. This indicated a reduction in transformation efficiency of at least three orders of magnitude for each (Fig. 5). Due to the apparent disparities between OD₆₀₀ and c.f.u. in the RNase J mutants, it is possible that the competence deficiencies are somewhat overestimated, since the standard assay protocol requires growth to the ideal OD₆₀₀ that naturally induces the competence pathway. To at least partially mitigate this issue, transformation reactions were all supplemented with synthetic competence-stimulating peptide to trigger competence independent of cell density. While the competence phenotypes were similar for all three RNase J mutants, biofilm phenotypes varied widely between strains. For the ∆njjA mutant, biofilm structure was only modestly impacted with structural differences apparent only in the low-magnification images (Fig. 6a, b).
In contrast, both the ΔrnjB and ΔrnjA/B mutants grew very sparse biofilms overall, but were interspersed with unusually tall biofilm structures (Fig. 6a, b). By far the most dramatic effects were seen in the ΔrnjB mutants. While a few small microcolony-like clusters were typically scattered between each of the large clusters in the ΔrnjA/B biofilm, almost no intervening biofilm was detectable between the massive ΔrnjB biofilm clusters (Fig. 6a, b). Also, each ΔrnjB biofilm cluster was spaced too far apart to image more than one in a single field of view using magnifications above 10×. The thickness of the ΔrnjB biofilm clusters exceeded 140 μM, whereas the WT and complemented strains generally formed biofilms of about 25 μM under the same growth conditions. Despite the obvious structural differences between the RNase J mutant biofilms, they all similarly exhibited substantial increases in the proportion of dead/dying cells in their biofilms.

**DISCUSSION**

In the current study, we sought to examine the functional roles of RNase J1 and J2 in *S. mutans*. Even though the proteins are widely conserved among bacteria and even
archaea (Dominski et al., 2013; Even et al., 2005), surprisingly little basic information is known about their regulatory roles. Despite the widespread conservation of RNase J orthologues, it is clear from the current study that there are some fundamental differences from B. subtilis in the roles of these enzymes, particularly for RNase J2. While the function of RNase J2 has remained enigmatic and appears nearly dispensable in B. subtilis, it is a pleiotropic regulator in S. mutans. Phenotypically, the ΔrnjB mutation exerted an even greater impact upon cellular physiology than that of ΔrnjA. This was quite surprising and underscores just how little is known about the pathways governing RNA stability in most bacteria.

In the aforementioned phenotypic study of the B. subtilis ΔrnjA mutant, phenotypes related to cell morphology and antibiotic sensitivity suggested a major role for RNase J1 in cell wall metabolism (Figaro et al., 2013). Our results implicate a similar role for both of the S. mutans RNase J enzymes, except that RNase J2 appears to play a wider role. On agar plates we rarely observed cell chains from the WT, whereas the ΔrnjB mutant typically formed chains containing >30 cells under the same conditions (Fig. 2b). In fact, the image shown in Fig. 2(b) is actually one continuous cell chain. By comparison, the ΔrnjA chain length phenotype seemed relatively modest, even though it clearly formed longer chains than the WT as well. Cell shape was also affected and was most evident in the ΔrnjB and ΔrnjA/B mutants. WT S. mutans cells typically form elongated ovals, whereas the RNase J mutants were much more coccoid (Fig. 2b). Perhaps the most suggestive phenotype was the presence of ghost cells in the ΔrnjB mutant, and to a lesser extent, the ΔrnjA/B double mutant. This is highly likely to be a direct consequence of cell wall metabolism defects. We speculate that ghost cells arise due to partial cell lysis, since they were frequently observed in the middle of cell chains. This suggests that the ghost cells were initially viable cells that had successfully divided before lysing. The lack of ghost cells in the ΔrnjA mutant is also consistent with its more modest morphology defects.

While there was considerable heterogeneity among the RNase J morphology and biofilm phenotypes, it was interesting that all three mutants exhibited nearly identical stress tolerance profiles. Contrary to our expectations, the mutants were surprisingly not hypersensitive to stress in general. In fact, they were only modestly sensitive to oxidation and starvation. Yet, all of the mutants were extremely sensitive to low pH. C.f.u. were rarely recovered on pH 5 plates, even when plating undiluted cultures. This suggests a potentially critical, if not essential, role for post-transcriptional regulation of the acid tolerance response. Given the rapid and dramatic acidification that occurs in dental plaque exposed to sugars (Jensen et al., 1982; Jensen & Wefel, 1989), it would not be surprising to discover that many of the principal acid adaptation genetic pathways are regulated post-transcriptionally. Post-transcriptional control mechanisms offer a much faster form of regulatory control compared with transcriptional mechanisms, and also have the distinct advantage of being able to immediately override and reset genetic programmes as conditions change (Gripenland et al., 2010). Presently, most of the S. mutans acid tolerance response has been characterized via transcriptional control mechanisms (Matsui & Cvitkovitch, 2010). However, our results suggest that a vital layer of post-transcriptional control over the acid tolerance response has yet to be discovered. RNases J1 and J2 are likely to be central regulators in these pathways.

Currently, it is still a mystery why the Firmicutes encode multiple RNase J paralogues (Even et al., 2005). A comparative analysis of the B. subtilis RNase J2 suggests that its limited enzymic activity is likely due to the degradation of a critical Zn$^{2+}$-binding catalytic centre (Newman et al., 2011). The strictly conserved consensus motif found in all RNase J1 orthologues is HxHxDH, whereas this sequence in the B. subtilis RNase J2 is HGHEDN. Interestingly, in S. mutans, S. pyogenes and E. faecalis, the sequence is closer to the RNase J1 consensus motif (HGHADA) and RNase J2 appears to play a more significant regulatory role in these organisms as well. Since we also observed identical (HGHADA) sequences in each of the RNase J2 orthologues of many other lactic acid bacteria, it will be very interesting to compare the role of RNase J2 among these species and determine whether this minor sequence difference confers more efficient catalysis. Surprisingly, previous attempts to replace the degenerate B. subtilis RNase J2 motif with that of RNase J1 (HGHEDH) actually resulted in reduced enzymic activity (Mathy et al., 2010).

Despite the vastly different roles of RNase J2 in B. subtilis and S. mutans, the data in the current study suggest a
conserved role for RNase J2 as a regulator of RNase J1 function. This was most evident from the changes in morphology and biofilm phenotypes, which were all severe in the ΔrnjB mutant, intermediate in the ΔrnjA/B double mutant and modest in the ΔrnjA mutant. Based upon these results, we speculate that the more dramatic effect of the

Fig. 6. Biofilm phenotypes of RNase J mutants. Static biofilms were grown to completion in plastic six-well plates. Due to the slower growth of the RNase J mutant biofilms, these strains were grown for 48 h while the others were grown for 24 h. (a) Biofilms were stained with the BacLight LIVE-DEAD viability kit and imaged using confocal microscopy. Z-stack images were processed using Volocity software to generate 3D reconstructions. Red staining is indicative of membrane-compromised dead/dying cells, whereas green staining indicates intact cell membranes. Each unit of the image grid represents 23.4 μm² and arrows represent orientation along the X-Y-Z coordinate axes. (b) The same biofilms were also imaged at 10× magnification using phase-contrast microscopy. Bars, 100 μm.
ΔrnjB mutation can be attributed to the additive effects of a loss of RNase J2 enzymic activity, as well as a gain of function for RNase J1 (i.e. degradation of unnatural substrates). Interestingly, we found no evidence implicating a reciprocal effect of the ΔrnjA mutation on RNase J2 function, as none of the ΔrnjA mutant phenotypes were suppressed in the ΔrnjA/B background.

ACKNOWLEDGEMENTS

We would like to thank the University of Oklahoma Health Sciences Center Laboratory for Molecular Biology and Cytometry Research for their assistance with biofilm imaging. This work was supported by NIH/NIDCR grants DE018893 and DE022083 to J.M., DE019566 to S.K. and DE019940 to F.Q.

REFERENCES


Edited by: W. Crielard