Ribozyme Stability, Exon Skipping, and a Potential Role for RNA Helicase in Group I Intron Splicing by *Coxiella burnetii*[▽]

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The 23S rRNA gene of *Coxiella burnetii*, the agent of Q fever in humans, contains an unusually high number of conserved, selfish genetic elements, including two group I introns, termed Cbu.L1917 (L1917) and Cbu.L1951 (L1951). To better understand the role that introns play in *Coxiella*'s biology, we determined the intrinsic stability time periods (*in vitro* half-lives) of the encoded ribozymes to be ~15 days for L1917 and ~5 days for L1951, possibly due to differences in their sizes (551 and 1,559 bases, respectively), relative degrees of compactness of the respective RNA structures, and amounts of single-stranded RNA. *In vivo* half-lives for both introns were also determined to be ~11 min by the use of RNase protection assays and an *Escherichia coli* model. Intron RNAs were quantified in synchronous cultures of *C. burnetii* and found to closely parallel those of 16S rRNA; i.e., ribozyme levels significantly increased between days 0 and 3 and then remained stable until 8 days postinfection. Both 16S rRNA and ribozyme levels fell during the stationary and death phases (days 8 to 14). The marked stability of the *Coxiella* intron RNAs is presumably conferred by their association with ribosomes, a stoichiometric relationship that was determined to be one ribozyme, of either type, per 500 ribosomes. Inaccuracies in splicing (exon 2 skipping) were found to increase during the first 5 days in culture, with a rate of approximately one improperly spliced 23S rRNA per 1.3 million copies. The *in vitro* efficiency of L1917 intron splicing was significantly enhanced in the presence of a recombinant *Coxiella* RNA DEAD-box helicase (CBU_0670) relative to that of controls, suggesting that this enzyme may serve as an intron RNA splice facilitator *in vivo*.

Coxiella burnetii is the etiologic agent of Q fever in humans and one of the most infectious pathogens known (17). The bacterium also infects domestic ruminants and has been isolated from a variety of other vertebrates and arthropods (7). *C. burnetii* is an obligate intracellular parasite in nature that replicates within an acidic, parasitophorous vacuole with many features of a mature phagolysosome (31). While the *Coxiella* intracellular niche provides few opportunities for horizontal gene transfer, the bacterium's genome contains an unusually large number of selfish genetic elements that may have been acquired during a free-living past. These include dozens of insertion (IS) elements from eight different families and three unknown transposases (3, 23), an intein within the C-terminal region of replicative DNA helicase (DnaB) (19), an intervening sequence (IVS) with a nested open reading frame (ORF) (CBU_2096) within the single-copy 23S rRNA gene (1), two highly conserved self-splicing group I introns (Cbu.L1917 and Cbu.L1951, here termed L1917 and L1951, respectively) that disrupt the 23S rRNA gene (21), and a homing endonuclease (HE; CBU_0182) encoded within L1951 (21). Remarkably, these selfish genetic elements are highly conserved in all eight genotypes of *C. burnetii* (21), suggesting that they play adaptive roles in the pathogen's biology.

Previous work by our laboratory showed that intron RNAs (ribozymes) encoded by L1917 and L1951, when heterologously expressed, significantly inhibited the growth of *Escherichia coli*. Since ribozymes L1917 and L1951 both reassociate

with the 50S ribosome following splicing and inhibit translation *in vitro* (19), it is conceivable that the introns are involved in regulating growth. In fact, analysis of 14-day synchronized infections of Vero cells showed that the quantities of *Coxiella* genomes were inversely correlated with the amount of ribozyme present. Collectively, the results suggest that ribozymes modulate the *Coxiella* growth rate by interfering with ribosome function and that the inhibition would need to be alleviated to allow maximal replication. The present study was undertaken to better define the potential role of introns in regulating *Coxiella* growth and development by analyzing the stability of ribozymes L1917 and L1951 *in vitro* and *in vivo*. We also present data showing 23S rRNA exon 2 skipping that arises from improper splicing of the two introns and the potential role of RNA helicase (RhIE; CBU_0670) in facilitating L1917 intron splicing from the precursor 23S rRNA.

MATERIALS AND METHODS

Bacterial strains, cell lines, and growth conditions. Nine Mile Phase II *C. burnetii* (strain RSA 439, clone 4) was propagated in African green monkey kidney epithelial (Vero) cells (CCL-81; American Type Culture Collection [ATCC], Manassas, VA), as previously described (19). *C. burnetii* small-cell variants (SCVs) were prepared and purified as previously described (19) and used to inoculate Vero cell monolayers to produce synchronized infections (6). *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C. Kanamycin (50 µg/ml) or ampicillin (100 µg/ml) was added to the medium as required. JM109 and Top10F' *E. coli* strains were used for routine cloning and plasmid production. *E. coli* strain M15 (pREP4) was used as a host to express recombinant RhIE (rRhIE). A list of the strains and cells used in the study is provided in Table 1.

Nucleic acid purification. Chromosomal DNA was purified using a DNeasy blood and tissue kit per the instructions of the manufacturer (Qiagen, Valencia, CA). RNA was purified using a RiboPure Bacteria kit (Ambion, Austin TX) and subsequently cleaned with an RNeasy kit (Qiagen). Plasmid DNA was prepared with a QIAprep Spin Miniprep kit (Qiagen). Nucleic acids were quantified by

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TABLE 1. Bacterial strains, cell lines, plasmids and primers used in the study

Designation	Relevant characteristics	Source or reference
Strains		
<i>C. burnetii</i> RSA 439	Nine Mile Phase II, clone 4	R. A. Heinzen
<i>E. coli</i> JM109	Host strain for cloning	Promega
M15 (pREP4)	Host strain for His ₆ fusion proteins	Qiagen
Top10F ⁺	Host strain for pCR2.1 TOPO constructs	Invitrogen
Cell line		
Vero	Green monkey epithelial line; CCL-81	ATCC
Plasmids		
pQE30	Expression vector for His ₆ fusion proteins	Qiagen
pREP4	<i>lacI</i> ⁺ in <i>trans</i> repressor plasmid	Qiagen
pCR2.1 TOPO	Cloning vector for PCR products	Invitrogen
pUM2	Cbu.L1917 cloned into pCR2.1 TOPO (Invitrogen)	19
pUM3	Cbu.L1951 cloned into pCR2.1 TOPO (Invitrogen)	19
pQrhIE	<i>C. burnetii</i> <i>rhIE</i> cloned into pQE30	This study
PCR primers		
L1917_flank ^a	TAATACGACTCACTATAGGGAGGTGGCTGCGACTGTTTAC	20
L1917_flank_R	GGAATTTTCGCTACCTTAGGACCG	This study
L1951_flank+T7P_F ^a	TAATACGACTCACTATAGGGTCCTAAGGTAGCGAAATTCC	This study
L1951_flank_R	GTGGAGACAGCGTGGCTATCGTTA	This study
L1917_RPA_F	TTTTTTTTTTTTCTTGGCTATATGCTGGAAACTC	This study
L1917_RPA_R ^a	TAATACGACTCACTATAGGTTTTTTTTTTTTCAGGTTCTTTTGAAAACCTCT	This study
L1951_RPA_F	TTTTTTTTTTTTTCGTAACGACTGATCCGAAAG	This study
L1951_RPA_R ^a	TAATACGACTCACTATAGGTTTTTTTTTTTTCCTACGTTTACCTGATGG	This study
<i>Cb_rhIE_F</i>	AAAAAGGATCCTTGTGTGGCCGGTTAGGAGT	This study
<i>Cb_rhIE_R</i>	AAAAAAAAGCTTTACTGAGCACTTTATAAATCGTC	This study
<i>Cb_16S_F</i>	CGATCCGTAGCTGGTCTGAGA	This study
<i>Cb_16S_R</i>	ACTGCTGCCTCCCGTAGGA	This study
Exon_chimera_F	CGGGCGAAGCTCTTGATC	This study
Exon_chimera_R	GGAACCTTATAGTTACG	This study
L1951_internal ^b	TTTAGCAAAGGGCAATCC	19
L1951_internal ^c	TCCATAGTCACTTACTTCTTG	19
L1917_internal ^b	GGACAATCAGCAGGAAAGAC	19
L1917_internal ^c	CGGACTCTATCATCACACTTA	19

^a The T7 promoter sequence is underlined.

^b Forward primer sequence from the indicated reference.

^c Reverse primer sequence from the indicated reference.

spectrophotometry at 260 nm. A list of the plasmids and primers used in the study is provided in Table 1.

In vitro transcription of introns and half-life determination. Introns were amplified by PCR using *C. burnetii* genomic DNA and primers (primer pair L1917_flank and L1917_flank_R or primer pair L1951_flank+T7P_F and L1951_flank_R; Table 1) designed to add a T7 promoter sequence at the 5' end of the amplicons to create templates for the two ribozymes, as previously described (19). *In vitro* transcription was done with a MEGAscript kit (Ambion) following the manufacturer's instructions, and the resulting RNA was purified using 5% (wt/vol) acrylamide and an 8 M urea gel (per Ambion technical bulletin 173). Purified RNA was suspended in phosphate-buffered saline (PBS; pH 7.4) and incubated on days 0 to 9 at 37°C in a capped, RNase-free microcentrifuge tube. RNA samples were removed at 24-h intervals, quenched with loading buffer II (Ambion), and stored at -80°C until analyzed. RNA degradation was assessed by processing RNA samples (250 ng of L1917 and 100 ng of L1951) on ethidium bromide-stained gels (5% [wt/vol] acrylamide, 8 M urea). Densitometry of ribozyme bands was performed using gel images captured by a GelDoc system (Bio-Rad; Hercules, CA). RNA half-lives were calculated as $\ln 2/k$, where k was obtained from the equation for the corresponding exponential curve.

In vivo expression of introns and half-life determinations performed using RPAs. *E. coli* Top10F⁺ strains harboring pUM2 and pUM3 plasmids were previously described (19) and used to express L1917 and L1951 introns, respectively. *E. coli* strains were grown for 16 h in LB broth with ampicillin (LB_{amp}; 100 µg/ml) at 37°C with shaking. A 2-ml aliquot of the culture was then used to inoculate 20 ml of LB_{amp}. Cultures were grown for 2 h at 37°C to an optical

density at 600 nm (OD₆₀₀) of ~0.6, transferred to a 250-ml Erlenmeyer flask, and grown for an additional hour. IPTG (isopropyl-β-D-thiogalactopyranoside) was then added (final concentration, 1 mM), and cultures were allowed to grow for an additional 2 h. Subsequently, rifampin (Sigma; St. Louis, MO) was added (final concentration, 160 µg/ml), samples (2 ml) were removed at 5-min intervals for 30 min and centrifuged (10,000 × *g* for 1 min), and the pellet was suspended in 1 ml of Tri reagent (Ambion). Suspensions were stored at -80°C until used. RNA was purified from the mixture and processed using an RNase protection assay (RPA) III kit (Ambion) per the instructions of the manufacturer. RPA reactions were resolved on denaturing gels (5% [wt/vol] acrylamide, 8 M urea) and then transferred to BrightStar-Plus (Ambion) nylon membranes. Membranes were UV cross-linked using a GS Gene Linker (Bio-Rad) set at 150 mJ. RPA signals were detected using a nonisotopic BrightStart BioDetect kit (Ambion). RPA probes for the introns were prepared using the corresponding primers (primer pair L1917_RPA_F and L1917_RPA_R or primer pair L1951_RPA_F and L1951_RPA_R; Table 1) and a MEGAscript kit as instructed, except biotin-labeled UTP (Bio-16-UTP; Ambion) was added to achieve a final concentration of 0.8 mM. Biotin-labeled RNA probes were subjected to gel purification on acridine orange-stained, 5% (wt/vol) acrylamide-8 M urea gels (per Ambion technical bulletin 173). Final RPA blots were visualized using an LAS-3000 imaging system (Fujifilm, Stamford, CT).

PCR. Quantitative real-time PCR (qRT-PCR) was done as previously described (19). Primer sets were designed using Beacon Designer 7.5 software (Biosoft International, Palo Alto, CA) and were specific to the *C. burnetii* 16S rRNA-encoding gene (primer pair *Cb_16S_F* and *Cb_16S_R*), improperly

spliced 23S rRNA lacking exon 2 (primer pair Exon_chimera_F and Exon_chimera_R), and the two introns (primer set L1917_internal and L1951_internal) (see Table 1 for details). Amplified cDNA was normalized to genome numbers determined by quantitative PCR (Q-PCR) with a primer set specific to the *Coxiella* *rpoS* gene, as described by Coleman et al. (6). Q-PCR data were also used to generate *Coxiella* growth curves, showing genome numbers as a function of time in infected Vero cell cultures. The qRT-PCR product from improperly spliced 23S rRNA (i.e., lacking exon 2) was obtained from 6-day-old and 8-day-old *Coxiella* synchronous cultures. The amplicon was excised from ethidium bromide-stained, 8% (wt/vol) acrylamide gels, cleaned using a QIAquick nucleotide removal kit (Qiagen), and then cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) for sequence analysis per the manufacturer's instructions.

RNA helicase cloning, ATPase, and splicing assays. *C. burnetii* rhlE (Cbu_0670) was amplified from chromosomal DNA by PCR using primers with 5'-terminal BamHI and HindIII sites (*Cb_rhlE*_F and *Cb_rhlE*_R; Table 1) and standard protocols (2). Amplicons were digested using BamHI and HindIII restriction endonucleases and cloned into the corresponding sites of pQE30 (Qiagen) by the use of standard protocols (2). The resulting plasmid, pQrhlE, encodes an in-frame, N-terminal translational fusion associated with the vector's His₆ tag. *E. coli* M15 (pREP4) was transformed with pQrhlE (5). A 2-liter culture of M15 (pREP4 [pQrhlE]) was grown for 2 h in LB_{amp}, induced with IPTG (final concentration, 1 mM), and incubated for another 2 h. The mixture was centrifuged (4,000 × g for 20 min at 4°C), and the resulting pellet was frozen (16 h at -20°C). His₆-tagged rRhlE was purified from the pellet by the use of nickel-nitrilotriacetic acid (Ni-NTA) agarose and native conditions as instructed by the manufacturer (Qiagen), except the following buffers were used: cell lysis buffer (20 mM Na₂HPO₄ [pH 7.4], 500 mM NaCl, 5 mM imidazole); wash buffer (20 mM Na₂HPO₄ [pH 7.4], 500 mM NaCl, 30 mM imidazole); and elution buffer (20 mM Na₂HPO₄ [pH 7.4], 500 mM NaCl, 250 mM imidazole). rRhlE was concentrated using a Slide-A-Lyzer dialysis cassette (Thermo Scientific, Waltham, MA) (3,500 molecular weight cutoff [MWCO]) and TNE buffer (10 mM Tris [pH 8.0], 10 mM NaCl, 1 mM EDTA, 35% [wt/vol] polyethylene glycol [PEG]) (28). rRhlE ATPase activity was assayed using the general methods of Worrall et al. (33) and an EnzChek phosphate assay kit per the instructions of the manufacturer (Molecular Probes, Eugene, OR). *E. coli* tRNA (Sigma) was included in ATPase assays (final concentration, 40 µg/ml). Heat-denatured rRhlE was prepared by boiling for 10 min and cooling to room temperature prior to use.

The ability of rRhlE to facilitate intron splicing was examined by assaying intron splicing rates in the presence of 0 or 0.1 µM rRhlE or 0.1 µM heat-denatured rRhlE, as previously described (20), except 200 µM rATP, 10 mCi of [γ -³²P]GTP (Perkin-Elmer, Waltham, MA), and 100 µM MgCl₂ were used in the reactions. Intron RNAs were prepared as described for the *in vitro* half-life determinations, except L1917 was prepared using alternate primers (i.e., L1917_flank and L1951_internal [reverse]; Table 1).

Automated sequence analysis. DNA was routinely verified by sequencing with an automated DNA sequencer (ABI3130xl) and a BigDye Terminator cycle sequencing ready-reaction kit (ABI, Foster City, CA). Sequence data were analyzed using Accelrys Gene 2.5 software (Accelrys, San Diego, CA).

Statistical analyses and graphics. At least three independent determinations were used to calculate means and standard deviations (S.D.) for numerical data. ImageJ 1.43 software was employed in densitometry. Excel 2010 software (Microsoft, Redmond, WA) was used to generate graphs and statistically analyze ribozyme half-life curves. Instat software (GraphPad Software, La Jolla, CA) was used to conduct paired Student's *t* tests. A *P* value ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

Ribozyme half-lives *in vitro*. Previous work by our laboratory showed that *Coxiella* L1917 and L1951 ribozymes (i) associate with the 50S ribosome, (ii) inhibit translation reactions *in vitro*, and (iii) significantly decrease *E. coli* growth rates when heterologously expressed (19). These results led us to hypothesize that ribozymes play an adaptive role in *Coxiella* biology by slowing the pathogen's growth rate (~11-h generation time) (6), thereby fostering conditions that promote a persistent, chronic infection of the host, as previously described (10, 14, 18, 27, 30). However, because ribozymes can be short-lived, the potential utility of L1917 and L1951 as growth modulators was unclear. To address this issue, we determined the intrinsic

stability of *Coxiella* ribozymes by assaying the RNA half-lives *in vitro*. Transcribed and purified L1917 and L1951 ribozymes were incubated in PBS at 37°C for several days under RNase-free conditions, during which time aliquots of the samples were taken at 24-h intervals and subsequently analyzed on denaturing acrylamide gels. The results of the experiment showed a roughly 3-fold difference in the *in vitro* half-lives of the two ribozymes, with L1917 exhibiting an ~15-day and L1951 an ~5-day half-life (Fig. 1). These results suggest that the L1917 ribozyme (551 bases) is intrinsically more stable than L1951 (1,559 bases), at least under the conditions employed in the assay. It is conceivable that the much smaller size of L1917 allows the formation of a more compact and therefore longer-lived RNA structure, especially considering that L1951 contains a 474-base ORF within its P8 helix that encodes a potential LAGLIDADG HE, CBU_0182 (21; LAGLIDADG refers to a conserved protein motif). A second possibility is that L1951 has less double-stranded RNA than L1917, making it more prone to cleavage by an intramolecular phosphoester transfer mechanism (26).

Ribozyme half-lives in an *E. coli* model. Previous work suggested the possibility that L1917 might be more labile than L1951, since L1917 levels were relatively and consistently lower in *C. burnetii* cells (19). Because those earlier observations contradict what we observed *in vitro* (Fig. 1), we assayed the half-lives of ribozymes L1917 and L1951 *in vivo* by the use of RPAs. Initial assays were done using *C. burnetii* but were consistently problematic due to low RNA yields and the timing of sampling in long-term (14-day) synchronized cultures. We therefore turned to *E. coli* as a model for the experiment. The results of the RPAs showed that both L1917 and L1951 demonstrated *in vivo* half-lives of approximately 11 min in the *E. coli* background (Fig. 2). This value is similar to what was reported for the group I intron of the T4 phage *td* gene, with which half-lives for the excised RNA ranged from 12 to 19 min when expressed in *E. coli* (4). Since the L1917 and L1951 ribozymes display nearly identical half-lives *in vivo*, the results do not help to explain the disparity in ribozyme levels observed in *Coxiella* (19) and suggest that there may be limitations to the model (e.g., RNase activities of *E. coli* may not be comparable to those of *C. burnetii*). RPAs examining the *in vivo* half-life of L1917 gave a single RPA signal of ~288 bases (Fig. 2A), in good agreement with the RNA size predicted *in silico*. However, RPAs used to determine the *in vivo* half-life of L1951 consistently showed three RPA signals of approximately 533 (full-length RNA), 425, and 230 bases (Fig. 2B). Interestingly, the ~425-base and ~230-base bands were also observed in RPAs prepared with *C. burnetii* RNA samples (data not shown) and may represent partial degradation products of the L1951 ribozyme.

Abundance of ribozymes in synchronized cultures of *Coxiella*. In a previous report, we quantified ribozyme levels in *Coxiella* and normalized them to genome numbers determined by Q-PCR (19). However, as a result of using this approach, the relative levels of abundance of ribozymes and rRNAs over time in culture and the stoichiometry of ribosome inhibition *in vivo* could not be determined. To address this lack of information and to help clarify the disparity of ribozyme quantities *in vivo*, as mentioned above, long-term synchronized cultures of *C. burnetii* were prepared and qRT-PCR was used to quantify

FIG. 1. *In vitro* half-lives of L1917 and L1951 ribozymes. (A) Representative example of an *in vitro* L1917 half-life determination. (Left panel) An ethidium bromide-stained, 5% (wt/vol) acrylamide–8 M urea gel showing *in vitro*-transcribed L1917 ribozyme at posttranscription days 0 to 7 (corresponding to column labels). The L1917 band is indicated by an arrowhead; RNA size standards (Ambion) are shown on the left in bases. (Right panel) Densitometric analysis of the L1917 band versus day of sample collection. The equation for the exponential curve and the R^2 value are inset; the equation yields a half-life of 15.0 days. (B) Representative example of an *in vitro* L1951 half-life determination. (Left panel) An ethidium bromide-stained, 5% acrylamide–8 M urea gel showing *in vitro*-transcribed L1951 ribozyme at posttranscription days 0 to 9 (corresponding to column labels). The L1951 band is indicated by an arrowhead; RNA size standards (Ambion) are given to the left in bases. (Right panel) Densitometric analysis of the L1951 band versus time of collection. The equation for the exponential curve and the R^2 value are inset; the equation yields a half-life of 5.3 days.

16S, L1917, and L1951 RNAs. Q-PCR was also done to obtain genome numbers to allow a comparison with our previous work (19). The results of these experiments showed that, on a per-genome basis, 16S rRNA levels were highest at day 3 (~1.4 million copies per cell), or just as *Coxiella* log-phase growth began, and then fell to about 1 million copies per cell at day ≥ 6 postinfection (Fig. 3A). These data are very similar to earlier quantification results for L1917 and L1951 RNAs determined on a per-genome basis and led us to conclude that there was an inverse correlation between levels of ribozyme (per genome) and *Coxiella* genome numbers (19). In comparison to the results shown in Fig. 3A, our previous results more likely reflected rapid ribozyme (intron) splicing from the nascent 23S rRNA precursor, which would be at parity with the 16S rRNA results, since *Coxiella* structural RNAs are encoded in a single operon. When total numbers of 16S rRNA and ribozymes from 1- μ g *Coxiella* RNA samples collected over time in culture were compared, we found that 16S rRNA levels increased significantly over days 0 to 5 ($P < 0.05$ for comparisons of the numbers of RNAs to those determined at the previous collection time) and were stable between day 5 and day 8 (no significant difference; $P > 0.05$). Levels of 16S rRNA decreased significantly over days 8 to 14 ($P < 0.05$ for comparisons of the

numbers of RNAs to those determined at the previous collection time), a time period corresponding to the stationary and death phases. In a fashion similar to the results seen with 16S rRNA, quantities of L1917 and L1951 ribozymes increased significantly ($P < 0.05$) between days 0 and 3, were stable from day 3 to day 8 ($P > 0.05$), and decreased significantly ($P < 0.05$) between days 8 and 14 (Fig. 3B). The long-term stability of both *Coxiella* ribozymes (Fig. 3B) was a surprising result, as our earlier report erroneously concluded that ribozyme levels must fall in order for exponential growth to occur, for the reasons described above (19). Analysis of the RNA levels determined in this study (Fig. 3B) shows that during the *Coxiella* exponential-growth phase (Fig. 3A), there are roughly two ribozymes (one of each kind) for every thousand ribosomes (estimated from 16S rRNA results). Those ribozymes may bind to a ribosome and inhibit translation, as previously demonstrated in *E. coli* and *in vitro*, respectively (19).

A second issue arises from the results presented in Fig. 3B. Although each ribozyme had an *in vivo* half-life of ~11 min (Fig. 2), quantities of both RNAs remained fairly stable at days 3 to 8 in long-term *C. burnetii* cultures. Second, although not always significantly different ($P < 0.05$ for day 0 and day 14 samples only), the amounts of L1917 were consistently less

FIG. 2. *In vivo* half-lives of L1917 and L1951 ribozymes in *E. coli*. (A) Representative example of an *in vivo* L1917 half-life determination by RPA, as described in the text. (Left panel) RPA blot with the L1917 band (288 bases) indicated with an arrowhead. (The blot was prepared using 500 ng of RNA per lane and 50 pg of L1917 probe.) Lanes: 1, biotin-labeled L1917 probe; 2, as described for lane 1 but including pretreatment with RNase A/T1 (Ambion); 3 to 9, RNAs isolated at 5-min intervals from 0 to 30 min following rifampin treatment; 10, *E. coli* RNA. (Right panel) Densitometric analysis of L1917 bands in lanes 3 to 9 versus time of collection following rifampin treatment. The equation for the exponential curve and the R^2 value are inset; the equation yields a half-life of 11.3 min. (B) Representative example of an *in vivo* L1951 half-life determination by RPA, as described in the text. (Left panel) RPA with a full-length L1951 band (533 bases; black arrowhead) plus bands of approximately 425 and 230 bases (indicated by white and gray arrowheads, respectively). (The blot was prepared using 400 ng of RNA per lane and 100 pg of L1951 probe.) Lanes: 1, biotin-labeled L1951 probe; 2, as described for lane 1 but including pretreatment with RNase A/T1 (Ambion); 3 to 9, RNAs isolated at 5-min intervals from 0 to 30 min following rifampin treatment; 10, *E. coli* RNA. (Right panel) Densitometric analysis of the L1951 bands in lanes 3 to 9 versus time of collection following rifampin treatment. The equation for the exponential curve and the R^2 value are inset; the equation yields a half-life of 11.1 min.

than those of L1951 regardless of the collection time (Fig. 3B), as previously observed (19). Long-term stability of the ribozymes may arise from their association with the ribosome after RNA splicing (19). We previously hypothesized that internal guide sequences (IGSs) were being used by the ribozymes to reassociate with 23S rRNA following splicing (19), and work by others has shown that RNA stability increases when a ribozyme binds to a substrate, such as cellular protein (25). We predict that, prior to association with the ribosome, L1917 and L1951 stability is dictated by their *in vivo* half-lives of ~11 min (Fig. 2). Thus, ribozyme levels that were initially on par with the precursor 23S rRNA levels rapidly fell to roughly one copy of each ribozyme type for every thousand ribosomes. Once a ribozyme associates with a ribosome, it becomes very stable until *Coxiella* reaches the stationary and death phases (days 8 to 14; Fig. 3). A lack of parity in ribozyme concentrations (Fig. 3B and reference 19) possibly reflects L1917's greater sensitivity to an RNase(s) present in *Coxiella* but absent in *E. coli*, for which ribozyme half-lives were not found to be significantly different (Fig. 2).

Exon skipping during intron splicing in synchronized cultures of *Coxiella*. Bacteriophage Twort of *Staphylococcus aureus* encodes three group I introns that disrupt a single phage gene (ORF142) in a clustering arrangement that resembles that of the L1917 and L1951 introns of the *C. burnetii* 23S rRNA gene. Previous work showed that the Twort introns exhibited decreased splicing efficiencies during stationary-phase growth of host *S. aureus*, resulting in ribozyme splice variants and exon skipping in the mature RNA (13). We were therefore curious to see whether skipping of the 34-base exon lying between L1917 and L1951 ([21]; exon 2) occurs during *C. burnetii*'s developmental cycle, thereby potentially modulating translation and growth. To that end, qRT-PCR was done using RNA collected over the course of 14-day synchronized culture growth of *C. burnetii* together with a special primer set (Exon_chimera_F and Exon_chimera_R), where the Exon_chimera_R primer targets an exon 1-exon 3 junction that could arise only from improper splicing and omission of exon 2. Results of the experiment showed that quantities of 23S rRNA lacking exon 2 increased over days 0 to 5 postinfection and then re-

FIG. 3. Abundance of L1917 and L1951 ribozymes in 14-day synchronized cultures of *C. burnetii*. (A) Q-PCR and qRT-PCR results showing quantities of *C. burnetii* genomes (white bars) and 16S rRNA per genome (black bars) in millions, respectively, during days 0 to 14 in synchronous cultures. (B) qRT-PCR results showing total numbers of 16S RNAs (black bars), L1917 RNAs (gray bars), and L1951 RNAs (white, speckled bars) in 1- μ g *C. burnetii* RNA samples obtained over days 0 to 14 in synchronous cultures. Data represent the means of the results of six independent determinations $\pm$ S.D.

maintained at a fairly constant level through day 14 (i.e., no significant difference relative to the day 5 sample). The initial increase (days 0 to 5) and plateau (days 5 to 14) in improperly spliced 23S rRNA quantities roughly parallel the 16S rRNA levels in the samples (Fig. 4), albeit at much lower levels. Maximal levels (average, 140.9 ± 38.2 copies) of improperly spliced 23S rRNA were observed on day 5. To ensure that qRT-PCR results were not due to spurious amplification, we cloned PCR products of the Exon_chimera primer set from 6-day-old and 8-day-old cultures into pCR2.1-TOPO (Invitrogen). Plasmids from random clones were subsequently purified and sequenced and were found to contain a 23S rRNA insertion lacking exon 2 (data not shown), verifying the aberrant splicing event. If 16S and 23S rRNA quantities are assumed to be equal (since the two rRNAs are encoded in the same, single-copy operon), these results indicate that improper splicing of 23S rRNA occurs at a rate of about 1 per 1.3 million copies. Although these results reflect only one possible type of 23S splice variant, they suggest that improper 23S rRNA splicing does not play a significant role in regulating ribosome quantities in *C. burnetii*. Interestingly, exon skipping in *Coxiella* 23S rRNA does not increase during the stationary phase, as observed in phage Twort introns (13), but rather is highest during exponential-phase growth, when rRNA transcription is greatest (see Fig. 3A).

Recombinant RNA helicase (rRhIE) facilitates intron splicing. Although most group I introns, including L1917 and L1951, are capable of autonomous self-splicing *in vitro* (21), the efficiency of splicing is often enhanced *in vivo* by proteins that help fold the RNA into a catalytic configuration or disrupt stable, inactive RNA structures that constitute “kinetic traps” (11, 12, 32). *Bona fide* RNA splice facilitators include intron-

FIG. 4. 23S rRNA exon 2 skipping during L1917 and L1951 intron splicing in 14-day synchronized cultures of *C. burnetii*. qRT-PCR results showing total numbers of 16S rRNAs (black bars) and improperly spliced 23S rRNAs (exon 2 omitted; diagonally striped bars) present in 1 μ g of *C. burnetii* RNA obtained over days 0 to 14 in synchronous cultures. Data represent the means of the results of six independent determinations $\pm$ S.D.

encoded HEs (maturases) and host-derived, RNA-dependent helicases. To investigate the potential involvement of a protein facilitator in *Coxiella* intron splicing, we initially focused our efforts on the potential LAGLIDADG HE carried by L1951 (CBU_0182). However, despite numerous attempts and several alternative strategies, we were unable to clone the HE gene, possibly owing to its toxicity in *E. coli* (e.g., a predicted HE restriction site is present in *E. coli* 23S rRNA-encoding genes). We therefore searched the *Coxiella* genome for potential RNA chaperones that could be involved in splicing and identified a single, annotated DEAD-box RNA helicase (RhIE; CBU_0670). DEAD-box RNA helicases are ATP-dependent RNA chaperones in helicase superfamily 2 and are named for a conserved, Walker B DEAD motif involved in ATP binding. Functionally, these enzymes use the energy from ATP hydrolysis to mediate structural rearrangements in substrate RNAs (22, 24, 29). Several reports have demonstrated a role for DEAD-box RNA helicases in facilitating splicing by group I and group II introns (8, 9, 15, 16).

Coxiella recombinant RhIE (rRhIE) was generated and tested for ATPase activity *in vitro* using an EnzChek phosphate assay kit (Molecular Probes). Results of the assay showed that rRhIE has an average ATPase activity of 9.9 ± 2.6 mol of inorganic phosphate (Pi) released per minute per mole of enzyme ($n = 4$). An example of an assay is shown in Fig. 5A. Identical ATPase assays that were conducted in the presence of heat-denatured or 0 μ M RhIE enzyme did not show an increase in Pi levels (data not shown), indicating that the recombinant protein was responsible for the observed ATP hydrolysis (Fig. 5A). Having established that rRhIE is an ATPase, we next wished to determine whether the enzyme was able to enhance the intron RNA *in vitro* splicing rate. Results clearly showed that the L1917 splicing rate was significantly enhanced (greater slope) in the presence of rRhIE compared to the control results ($P < 0.05$ by paired Student's *t* tests versus 0.1 μ M rRhIE or 0.1 μ M heat-denatured rRhIE results; Fig. 5B). In contrast, the difference in the slope values for the two control treatments was not statistically significant ($P > 0.05$). These results suggest that splicing of the L1917 intron may also be enhanced *in vivo* by splice facilitators, such as the RNA helicase enzyme. However, the role that RhIE plays in L1951 intron splicing is still unclear, as splicing

FIG. 5. ATPase activity and enhancement of intron splicing by *Coxiella* rRhIE. (A) *In vitro* ATP hydrolysis by rRhIE. Data are expressed as micromolar concentrations of P_i released per second in 1-ml reaction mixtures containing 1 μM (black circles) or 2 μM (red diamonds) rRhIE after correcting for background (i.e., a reaction mixture containing 0 μM rRhIE). Representative assay curves are shown from four independent determinations at each molarity. (B) Splicing reactions. L1917 RNA was spliced in the presence of 10 μCi of [γ -³²P]GTP in reaction mixtures containing 0 μM (green), 0.1 μM (blue), or 0.1 μM heat-denatured (red) rRhIE as previously described (20). Levels of [γ -³²P]GTP incorporated in the spliced product after 2 and 5 min of incubation at 37°C are shown. Data represent the means $\pm$ S.D. of the results of three independent determinations. Slopes corresponding to the 0.1 mM rRhIE splicing reactions were significantly ($P < 0.05$; paired t tests) steeper than those corresponding to either of the controls, whereas slopes of the controls were not significantly ($P > 0.05$; paired t tests) different from one another.

reactions similar to those performed for L1917 gave inconsistent results (data not shown). It is possible that the HE encoded by L1951 serves as its maturase splice facilitator, but issues surrounding the enzyme's toxicity to *E. coli* need to be overcome or an alternative model needs to be employed to clarify its role in this activity.

The occurrence of five conserved selfish DNA elements in the 23S rRNA-encoding gene of *C. burnetii* is highly unusual in nature, especially for a bacterium that has evolved from a free-living existence to become a specialized, obligate intracellular pathogen. Clarifying the functions and activities of these DNA elements is expected to increase our understanding of their role in *Coxiella* genetics and biology.

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