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Toxic Introns and Parasitic Intein in *Coxiella burnetii*: Legacies of a Promiscuous Past[▽]

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The genome of the obligate intracellular pathogen *Coxiella burnetii* contains a large number of selfish genetic elements, including two group I introns (Cbu.L1917 and Cbu.L1951) and an intervening sequence that interrupts the 23S rRNA gene, an intein (Cbu.DnaB) within *dnaB* and 29 insertion sequences. Here, we describe the ability of the intron-encoded RNAs (ribozymes) to retard bacterial growth rate (toxicity) and examine the functionality and phylogenetic history of Cbu.DnaB. When expressed in *Escherichia coli*, both introns repressed growth, with Cbu.L1917 being more inhibitory. Both ribozymes were found to associate with ribosomes of *Coxiella* and *E. coli*. In addition, ribozymes significantly reduced in vitro luciferase translation, again with Cbu.L1917 being more inhibitory. We analyzed the relative quantities of ribozymes and genomes throughout a 14-day growth cycle of *C. burnetii* and found that they were inversely correlated, suggesting that the ribozymes have a negative effect on *Coxiella*'s growth. We determined possible sites for ribozyme associations with 23S rRNA that could explain the observed toxicities. Further research is needed to determine whether the introns are being positively selected because they promote bacterial persistence or whether they were fixed in the population due to genetic drift. The intein, Cbu.DnaB, is able to self-splice, leaving the host protein intact and presumably functional. Similar inteins have been found in two extremophilic bacteria (*Alkalilimnicola ehrlichei* and *Halorhodospira halophila*) that are distantly related to *Coxiella*, making it difficult to determine whether the intein was acquired by horizontal gene transfer or was vertically inherited from a common ancestor.

Bacterial genomes are in a constant state of flux. Bacteria gain new DNA through horizontal gene transfer (HGT), whereas nucleotide deletion results in loss of DNA (54). While environmental bacteria often have large genomes (e.g., *Pseudomonas aeruginosa*, ~6 Mb) that contain a large number of elements acquired through HGT, host-associated bacteria have relatively smaller genomes (e.g., *Rickettsia rickettsii*, ~1.2 Mb; *Chlamydophila abortus*, ~1.1 Mb) with little or no DNA of foreign origin (18, 72). The advent of whole-genome sequencing has facilitated the detailed comparative analyses of obligate intracellular bacterial genomes, leading to identification of factors such as the prevalence of pseudogenes and insertion sequences that can be used to distinguish early adapters from bacteria that have shifted recently from a free-living to a host-restricted lifestyle (48). A relevant case is the obligate intracellular pathogen *Coxiella burnetii*, whose genome was found to be undergoing reductive evolution and to contain a large number of selfish genetic elements and "young" pseudogenes, suggesting a recent shift to its current niche (66).

C. burnetii, a purple bacterium of the gamma subdivision, is the causative agent of Q fever (12). Most human infections are acquired through inhalation of contaminated aerosols of animal origin and can lead to an acute self-limiting febrile illness and severe, chronic cases of endocarditis or hepatitis (41). This category B select agent has a broad range of susceptible hosts, including arthropods, fish, birds, and wild and domestic mammals, and is distributed almost worldwide (12). *C. burnetii* can survive for months in the extracellular environment, where it is notoriously resistant to heat, UV light, various disinfectants, and desiccation (42, 75). These traits are conferred by a biphasic developmental cycle that alternates between a fragile, metabolically active large cell variant (LCV) and a durable, dormant small cell variant (SCV) (8). Even though *C. burnetii* occupies an acidic parasitophorous vacuole where the opportunity for HGT is likely minimal, its genome contains 29 insertion sequences strewn across the chromosome, two group I introns and an intervening sequence (IVS) within its sole 23S rRNA gene, and a putative intein in the C-terminal region of the replicative DNA helicase (*dnaB*) gene (59, 66).

Group I introns self-splice independently of proteins (ribozyme) and are considered a holdover from a primordial RNA world (77). These genetic elements spread efficiently into an intronless cognate site by a process called homing at the DNA level or by reverse splicing at the RNA level (60). The typical secondary structure of a group I intron consists of about 10 paired (P) elements (59, 74). P1 and P10 are complementary to the 5′ exon and 3′ exon, respectively, and are collectively called the internal guide sequence (IGS), which the intron uses to locate its 5′ and 3′ splice sites (31).

Inteins (internal proteins) are genetic elements similar to introns that have invaded the coding sequence of genes in *Eukarya*, *Bacteria*, and *Archaea*. Inteins are found in proteins with diverse functions; however, enzymes involved in DNA replication and repair predominate (23, 56). Unlike introns, inteins are transcribed and translated together with the host protein. They self-excise, leaving the flanking exteins (external proteins) spliced together and the host protein intact (23).

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TABLE 1. PCR primers and their targets in *C. burnetii*

Target	Designation	Forward sequence	Reverse sequence
Cbu.L1917	L1917_flank	GTGGCTGCGACTGTTTAC	TTCGCTACCTTAGGACCG
	L1917_internal	GGACAATCAGCAGGAAAGAC	CGGACTCTATCATCACACTTA
	L1917+T7P	TAATACGACTCACTATAGGGAAATTA ACCGTTGTAGTTACG	TATTGACGTTATGTTAATCATGGG
Cbu.L1951	L1951_flank	AACGGTCCTAAGGTAGCG	ATTTCCGACCGTGCTGAG
	L1951_internal	TTTAGCAAAGGGCAATCC	TCCATAGTCACTTACTTCTTG
	L1951+T7P	TAATACGACTCACTATAGGGAAAATT GCCCCGCTAAACTG	CACTCTTTCCTATGTTTCCATAGGG
IVS	IVS_flank	CGTGGTGGAAAGGGAAAC	TGTCAGCATTCGCACTTC
	IVS+T7P	ATCAGAAGACAGATGACAGATGACAG	TAATACGACTCACTATAGGGATCA GAAGACAGATGGCAGAAG
23S rRNA	23S	GTGGCTGCGACTGTTTAC	ATTTCCGACCGTGCTGAG
Cbu.DnaB	Intein_flank	TTAAAGGACGACGATTGGC	CCCATTTCGGTGTTTAGC
CBU_1927	ParB	AGAAGTTCCTGCGGTTATCC	CAACACTGGCAATCGGAAG

Four conserved motifs are involved in protein splicing and are related to the C-terminal domain of the Hedgehog protein of eukaryotes (13, 57). Most, but not all, inteins encode a homing endonuclease (HE), which aids in mobility, as observed in group I introns (15, 56). Chemical reactions involved in protein splicing have been extensively studied and are described elsewhere (9, 10, 40).

We recently showed that the 23S rRNA gene of *C. burnetii* contains two group I introns (Cbu.L1917 and Cbu.L1951) and elucidated their secondary structures, splicing properties, and phylogenetic histories (59). Here, we describe the toxicity of both introns as it relates to bacterial growth and consider the possible biological consequences and evolutionary significance. We postulate that *Coxiella*'s past life as a free-living, environmental bacterium with extensive HGT combined with a recent shift to its current host-restricted lifestyle helps explain the occurrence of toxic introns that can potentially influence its biology. In addition, we analyzed an intein (Cbu.DnaB) found in the replicative DNA helicase gene (*dnaB*) and confirmed its functionality. Phylogenetic analyses revealed closely related inteins at the same loci of *Alkalilimnicola ehrlichei*, an anaerobic, haloalkaliphilic, gammaproteobacterium, and *Halorhodospira halophila*, an extremely halophilic gammaproteobacterium (29, 44). Further phylogenetic analyses using 16S and DnaB sequences point toward a possible close evolutionary relationship between *C. burnetii* and these extremophiles, making it difficult to delineate whether *C. burnetii* acquired the intein via HGT or whether the intein was vertically inherited from a common ancestor.

MATERIALS AND METHODS

***E. coli* growth assay.** Both introns were amplified using PCR primers (Table 1; L1917_flank and L1951_flank) and *C. burnetii* genomic DNA, as previously described (59). Amplicons were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). PCR and sequencing were performed to confirm that the inserts were in the proper orientation downstream of the *lac* promoter. *E. coli* (Top10F′) transformed with each plasmid was grown to mid-logarithmic phase at 37°C in Luria-Bertani broth in the presence of 100 μg/ml ampicillin (Sigma-Aldrich, St. Louis, MO) and then used to inoculate 50 ml fresh Luria-Bertani broth with 100 μg/ml ampicillin and 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG; EMD Chemicals, Gibbstown, NJ) to an optical density at 600 nm (OD_{600}) of 0.03 at 0 h. Bacterial growth was assayed spectrophotometrically at 600 nm every 60 min for 5 h (37°C, shaking).

In vitro transcription to synthesize ribozymes and control RNA. Introns were amplified using *C. burnetii* genomic DNA and PCR primers designed to add a T7 promoter sequence at the 5′ end of the amplicons (Table 1; L1917+T7P and L1951+T7P) to generate templates to synthesize the two ribozymes. As a control, the IVS was amplified using *C. burnetii* genomic DNA and a PCR primer set (Table 1; IVS+T7P) that adds a T7 promoter sequence at the 3′ end of the amplicon such that an antisense RNA can be transcribed from it. In vitro transcription was performed using a MEGAscript kit (Ambion, Austin, TX), and resulting RNA was purified using NucAway (Ambion) spin columns and Turbo DNase (Ambion) as per the manufacturers' instructions.

In vitro transcription/translation and luciferase assay. Luciferase was synthesized in vitro using an *E. coli* S30 extract system for circular DNA and pBEST*luc* (Promega, Madison, WI) as per the manufacturer's instructions in the presence of 25 μg of Cbu.L1917, Cbu.L1951, or control antisense RNA. Luciferase activity was measured using a luciferase assay system (Promega) per the manufacturer's instructions and utilizing a luminometer (Perkin-Elmer, Waltham, MA).

Phylogenetic analyses. Intein phylogeny was reconstructed using amino acid sequences. Inteins were identified either by BLAST or from the intein database InBase (55, 71). In addition to the intein from *C. burnetii* (NP819887), other inteins used were from *H. halophila* SL1 (ABM61435), *A. ehrlichei* MLHE-1 (ZP00865879), *Rhodothermus marinus* (AAB66912.1), *Trichodesmium erythraeum* (ABG50752), *Lyngbya* sp. (EAW38924.1), *Microcystis aeruginosa* PCC 7806 (CA090722.1), *Herpetosiphon aurantiacus* ATCC 23779 (ABX06165.1), *Synechocystis* sp. strain PCC 6803 (NP442446.1), *Synechococcus* sp. strain JA-3-3Ab (ABC99714.1), *Guillardia theta* (O78411), and *Pseudomonas fluorescens* Pf-5 (AAY90835.1). Neighbor-joining trees that were bootstrap replicated (1,000 replicates) were built using MEGA4 (64, 69). An optimal tree is shown with branch lengths depicting evolutionary distances computed by the Poisson correction method for the number of amino acid substitutions per site (80). All positions containing gaps were eliminated, resulting in a total of 125 positions being used in the final data set. The 16S nucleotide and DnaB amino acid sequences were obtained from GenBank, including sequences for *C. burnetii* (AE016828), *Rickettsiella grylli* (AAQJ00000000), *Legionella pneumophila* (AE017354), *A. ehrlichei* (CP000453), *H. halophila* (CP000544), *Francisella tularensis* (CP000803), *P. fluorescens* (CP000094), *Pseudomonas syringae* (AE016853), *P. aeruginosa* (NC009656), *Yersinia pestis* (CP000668), *Salmonella enterica* serovar Typhimurium (AE006468), *E. coli* K-12 (CP000948), *Neisseria meningitidis* (AL157959), *Neisseria gonorrhoeae* (AE004969), *Bartonella bacilliformis* (CP000524), *Bartonella quintana* (BX897700), *Rickettsia bellii* (CP000849), *Rickettsia prowazekii* (NC000963), *Chlamydophila abortus* (CR848038), and *Chlamydia pneumoniae* (BA000008). Neighbor-joining trees were built with 1,000 bootstrap replicates using MEGA4 (69). Evolutionary distances in 16S trees were computed using the maximum composite likelihood method in units of base substitutions per site, whereas evolutionary distances for the DnaB trees were computed similar to the intein tree (70). Positions containing gaps were removed, with 1,351 positions in the final 16S tree and 423

positions in the final DnaB tree. Maximum parsimony trees were also built using the close neighbor interchange algorithm to confirm the evolutionary history inferred from the neighbor-joining trees (16, 50).

RNA and DNA preparations. DNA was isolated using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) and RNA was isolated using a RiboPure-Bacteria kit (Ambion) as per the manufacturers' instructions.

***C. burnetii* culture.** *C. burnetii* Nine Mile phase II (RSA 439; clone 4) was used in this study and was propagated in African green monkey kidney (Vero) fibroblasts (CCL-81; American Type Culture Collection, Manassas, VA). Cultures were grown in RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Bacteria were purified from infected cells by Renografin (Bracco Diagnostics, Princeton, NJ) gradient centrifugation as previously described (76). To generate SCVs, Vero cell monolayers were infected with *C. burnetii* and incubated for 4 weeks without replenishing the medium: the first week at 37°C in 5% CO_2 followed by 3 weeks at room temperature with the lids tightened, as previously described (8).

Sequence analyses. Sequence data were obtained with an automated DNA sequencer (ABI3130x1) and a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). Sequence analysis was accomplished using the BLAST 2 sequences tool at the NCBI website (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) (71).

Ribosome isolation and intron detection. *E. coli* containing both Cbu.L1917 and Cbu.L1951 cloned in pCR2.1TOPO (pUM1) was grown to an OD_{600} of 0.5 in the presence of 100 μg/ml ampicillin and 1 mM IPTG. The cells were centrifuged at 10,000 × *g* for 10 min at 4°C and lysed in buffer A (20 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$) containing 10 mg/ml lysozyme (Sigma) and 200 U/ml RNaseOUT (Invitrogen) by a freeze-thaw procedure (62). Cell debris was removed by centrifugation at 16,000 × *g* for 10 min at 4°C. Lysates were loaded on a linear 5 to 20% sucrose gradient made in buffer A containing 2 mM 2-mercaptoethanol and centrifuged for 15 h at 43,000 × *g* (SW28 rotor; Beckman Coulter, Fullerton, CA) at 4°C. Fractions were collected from the gradient and assayed at 260 nm, and ribosomes were recovered by ethanol precipitation (67). *C. burnetii* ribosomes were isolated using a similar procedure except that bacteria were purified from Vero cells 72 h postinfection (~2×10^8 genomes) and lysed twice with a French pressure cell (10,000 lb/in^2) (67). RNA was isolated from ribosome fractions using a RiboPure-Bacteria kit (Ambion), and cDNAs were generated using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). 23S rRNA was detected by PCR (Table 1; 23S). The presence of intron RNA was detected using primer sets specific for each intron (Table 1; L1917_internal and L1951_internal).

qRT-PCR. To prepare synchronized cocultures, Vero cells were infected with SCVs prepared as described above. RNA and DNA were isolated from the same flask on days 0, 2, 4, 6, 8, 10, 12, and 14 postinfection using TRI reagent (Ambion) as per a protocol recommended by the manufacturer (http://www.ambion.com/techlib/tn/123/13.html). RNA was treated with Turbo DNase (Ambion) and quantified using spectrophotometry. One μg of RNA from each sample was converted to cDNA using an iScript cDNA synthesis kit (Bio-Rad). Quantitative RT-PCR (qRT-PCR) and qPCR were performed (Table 1; L1917_internal and L1951_internal) on cDNA and genomic DNA, respectively, using iQ Sybr green supermix (Bio-Rad) on a MyiQ single-color real-time PCR detection system (Bio-Rad). Cycling parameters were 95°C for 5 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. To control for DNA contamination of RNA samples, PCR was also performed on RNA that had not been reverse transcribed. Differences were calculated by comparing each value (2 to 14 days) to that of day 0 (taken as 1.0). Amplified cDNA was normalized to genomic equivalents and plotted as the fold difference.

Intein analyses. Cbu.DnaB, along with proximal flanking sequences, was amplified using the PCR primer set Intein_flank (Table 1) and *C. burnetii* genomic DNA. The amplicon was cloned in frame into pQE-31 (Invitrogen) and cloned out of frame into pQE-30 (Invitrogen) utilizing BamH1 and Pst1 to produce pUM6 and pUM12, respectively. The whole *dnaB* was not cloned into the expression vector, to avoid toxicity in *E. coli*, as reported previously (78). *E. coli* [M15(pREP4)] transformed with pUM6, pUM12, or pQE-31 was grown to logarithmic phase (OD_{600}, 0.5), and 1 mM IPTG was added to induce expression for 4 h at 37°C. Resulting cell pellets were solubilized in Laemmli sample buffer and used in gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10 to 15% acrylamide [wt/vol]). Proteins were visualized by staining with Coomassie brilliant blue, and appropriate bands were excised from the gel and submitted to Alphalyse, Inc. (Palo Alto, CA) for matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) peptide mass fingerprinting and MALDI-TOF/TOF peptide sequencing, after trypsin digestion.

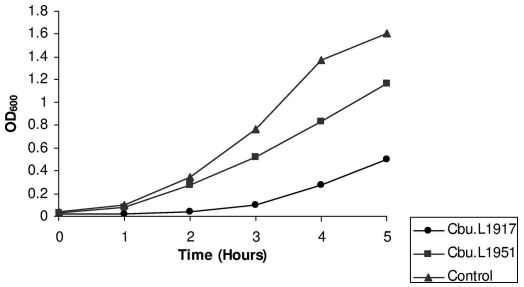

FIG. 1. Effect of *Coxiella* ribozymes on *E. coli* growth. *E. coli* cells expressing cloned Cbu.L1917, Cbu.L1951, or an irrelevant control RNA (pUM2, pUM3, and pUM4, respectively) were induced with IPTG (1 mM) and assayed spectrophotometrically for growth at 37°C over 5 h. A representative growth curve is shown.

Statistical analyses and graphics. SigmaPlot 8.0 (Systat Software Inc., San Jose, CA) was used for statistical analyses. PowerPoint 2003 and Excel 2003 (Microsoft, Redmond, WA) were used to generate graphs and figures.

RESULTS

Introns retard the growth rate of *E. coli*. A previous article showed that *E. coli* expressing the 26S rRNA intron from *Tetrahymena thermophila* displayed a significantly decreased growth rate relative to controls, prompting us to check whether the same is true for the 23S rRNA introns of *C. burnetii* (51). Both Cbu.L1917 and Cbu.L1951 were cloned individually into pCR 2.1-TOPO (Invitrogen) to produce pUM2 and pUM3, respectively. As a control, the IVS located upstream of the introns in the 23S rRNA gene was similarly cloned but in the opposite orientation to the vector's *lac* promoter to generate pUM4 and to transcribe a nonsense RNA of intermediate length (445 bases) relative to the introns (Cbu.L1917, 288 bases; Cbu.L1951, 720 bases). Growth rates of *E. coli* strains transformed with pUM2, pUM3, or pUM4 were monitored spectrophotometrically for 5 h. As shown in Fig. 1, *E. coli* expressing either intron exhibited a significantly retarded growth rate when compared to the control, with Cbu.L1917 being more inhibitory than Cbu.L1951. Similar growth inhibition was observed when purified intron RNAs (25 μg) were electroporated directly into *E. coli* (not shown). Also, when Cbu.L1917 antisense RNA was electroporated into *E. coli* transformed with pUM2, normal growth was restored (not shown).

Ribozymes associate with both *E. coli* and *C. burnetii* ribosomes. In the same earlier report, *Tetrahymena* intron RNA (ribozyme) was found associated with *E. coli* ribosomes (51). We therefore analyzed ribosomes from both *C. burnetii* and from *E. coli*(pUM1) for ribosome-ribozyme complexes. Ribosomes were harvested from *C. burnetii* and *E. coli*(pUM1). RNA was isolated from ribosomal pellets, converted to cDNA, and analyzed by PCR (Table 1; L1917_internal and L1951_internal) for the presence of ribozymes. Ribozymes were found associated with ribosomes of both *E. coli*(pUM1) and *C. burnetii* (Fig. 2A, lanes 1 and 5, and 2B, lanes 1 and 5, respectively). Further, PCR done with primers (Table 1; 23S) designed to give a large (1,559-bp) amplicon if introns are unspliced and a small (551-bp) product if introns are spliced out, confirmed that the mature 23S rRNA does not contain unspliced introns (not shown). To verify these results, PCR analysis using a primer set (Table 1; ParB) for *Coxiella parB*

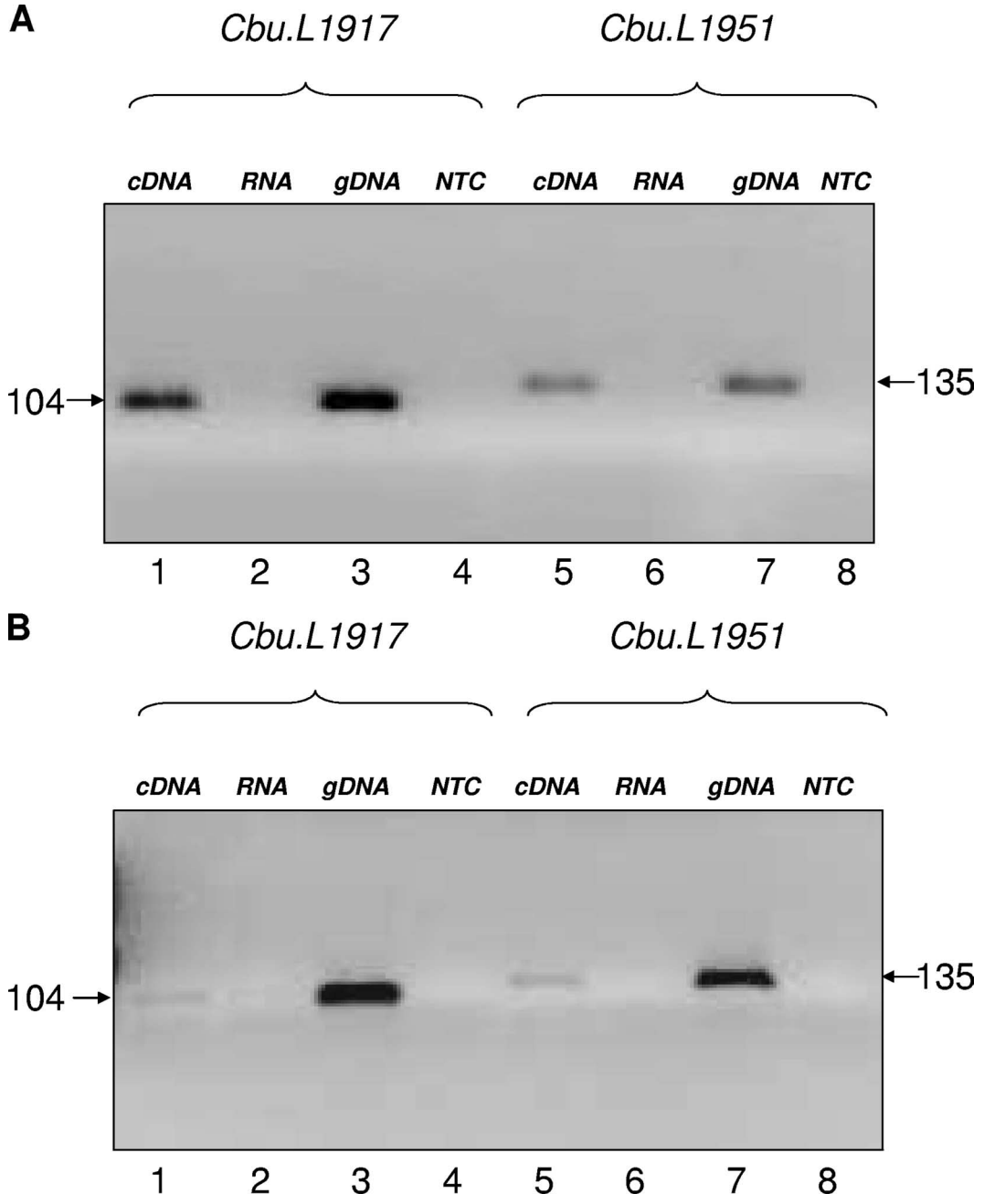

FIG. 2. Ribozyme-ribosome association in *E. coli* (A) or *C. burnetii* (B). PCR was done using L1917_internal or L1951_internal primer sets on RNA isolated from *E. coli* or *C. burnetii* ribosomes, cDNA made from RNA, *C. burnetii*-genomic DNA (gDNA), or a no-template control (NTC). An ethidium bromide-stained agarose gel (2% agarose [wt/vol]) is shown. Amplicon sizes were determined from standards and are given on either side in base pairs.

(CBU_1927, encoding a chromosomal partitioning protein) was also performed. *parB* has been previously shown to be strongly expressed alongside rRNA genes of *Chlamydia trachomatis* (another obligate intracellular bacterium) during an entire 7-day infection period in vitro (20, 30). Results showed that while *parB* mRNA is abundant in 0- to 8-day *Coxiella* cocultures, it was not detectable in *Coxiella* ribosomal fractions, suggesting that intron-specific cDNA PCR products from ribosomes (Fig. 2B) did not arise from contaminating RNA (not shown).

Introns inhibit in vitro transcription/translation. To directly analyze the hypothesized impact of intron RNA on protein synthesis, we used an *E. coli* S30 in vitro transcription/translation system in conjunction with pBEST*luc* (encoding luciferase) in the presence of ribozymes or control RNA followed by a luciferase assay (Promega). Following a 60-min reaction, a luciferase substrate was added and luminescence immediately quantified by luminometry. Results showed that both Cbu.L1917 and Cbu.L1951 RNAs significantly decreased

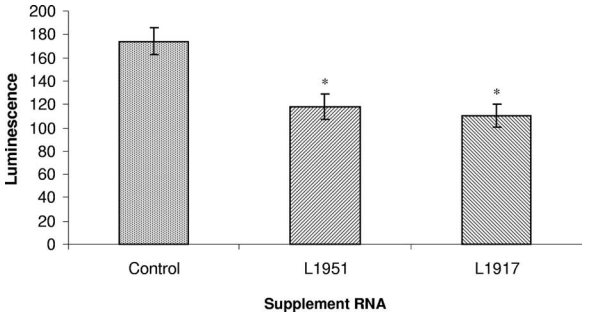

FIG. 3. Ribozymes inhibit in vitro translation of luciferase. In vitro translation of luciferase from pBEST*luc* in the presence of 25 μg of Cbu.L1917, Cbu.L1951, or control RNA was done for 60 min, and luminescence was immediately measured thereafter. Data represent the means of three independent experiments ± standard deviations. *, $P < 0.05$ by Student's *t* test.

luminescence (>30%; $P < 0.05$) compared to the control RNA (Fig. 3).

Inverse correlation between *Coxiella* genome and intron RNA quantities. To build upon our observation that slow growth results from ribozyme-mediated inhibition of translation, we hypothesized that if the introns affect the growth rate of *Coxiella*, then their RNA quantities should inversely correlate to the rate of growth. Since *C. burnetii* is an obligate intracellular bacterium that does not grow in axenic medium, toxicity could only be measured indirectly. To this end, we compared *C. burnetii* genome content and ribozyme quantities measured over time in cocultures. Total RNA and genomic DNA were isolated every 48 h from 0 to 14 days postinfection. *C. burnetii* genome and ribozyme quantities were estimated by qPCR and qRT-PCR, respectively, using primer sets specific for each intron (Table 1; L1917_internal and L1951_internal). Resulting data showed a clear, inverse correlation between the quantities of *C. burnetii* genomes and either ribozyme, suggesting that the amount of intron RNA influences the rate of growth (Fig. 4). After an initial rapid decline when *C. burnetii* transitions from SCV to LCV (2 to 4 days postinfection) (8), intron RNA quantities stabilized at 6 to 14 days at a nominal level. *Coxiella* genome amounts increased slowly from 0 to 2 days (lag phase), then rapidly from days 2 to 8 (log phase), and changed little between days 10 and 12 (stationary phase); however, it was observed to decrease between days 12 and 14.

Intein splicing from DnaB. The putative intein (Cbu.DnaB) detected in the replicative DNA helicase gene (CBU_0868) was analyzed to determine its splicing properties (55, 66). PCR was performed on *C. burnetii* genomic DNA using the Intein_flank primer set (Table 1) to amplify the intein and proximal flanking sequences. The resulting amplicon was cloned into pQE31 (Invitrogen) to produce pUM6, which encodes a translational fusion protein. *E. coli*(pUM6) was used to study the intein's splicing activity at the protein level. As a control, the same insert was cloned out of frame into pQE30 (Invitrogen) to form pUM12. Expression was induced using IPTG, and the protein products were analyzed using SDS-PAGE (Fig. 5). The intein (I, ~16.8 kDa) was observed to splice out of the precursor protein (P, ~35.6 kDa), leaving the two exteins (E, ~23.6 kDa) spliced together (Fig. 5). The molecular masses of I and P determined from the gel correspond well with their in

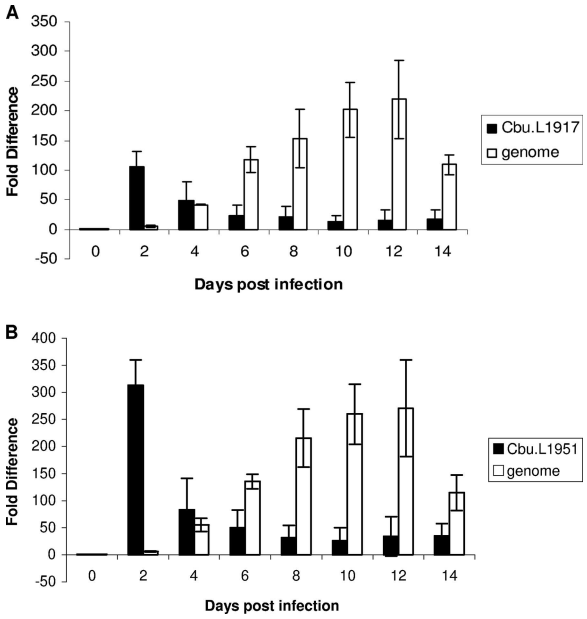

FIG. 4. Inverse correlation between quantities of *Coxiella* genome and ribozymes during growth. q-PCR and qRT-PCR data show relative *C. burnetii* genome and Cbu.L1917 (A) and Cbu.L1951 (B) ribozyme quantities as a function of time. Data represent the means of three experiments ± standard deviations.

silico-predicted values of 16.3 kDa and 34.7 kDa, respectively. The E band seems to have run slightly slower than its predicted molecular mass of 18.3 kDa. The identities of P, E, and I bands were confirmed using MALDI-TOF peptide fingerprinting and MALDI-TOF/TOF peptide sequencing (data not shown). The P, E, and I bands were not visible in the vector-only or pUM12 lanes, showing that the bands are insert specific and are produced only when the insert is in frame. The large precursor band suggests that intein splicing progresses slowly, unlike group I introns, which splice rapidly (59).

Phylogenetic analyses. Neighbor-joining trees were built using MEGA4 to reconstruct the plausible evolutionary history of the intein (69). Phylogeny reconstruction showed that Cbu.DnaB clustered with two very similar inteins inserted at the same position in *dnaB* genes (alleles) of *A. ehrlichei* (Aeh.DnaB2) and *H. halophila* (Hha.DnaB2), with the exclusion of inteins inserted in other sites (nonalleles) (Fig. 6A). These extremophilic bacteria have not been previously shown to be related to *C. burnetii*, suggesting HGT was used by the intein to invade *C. burnetii*'s genome. To get a clearer picture, phylogenetic trees were constructed using 16S rRNA sequences and the amino acid sequences of the *dnaB* gene. DnaB was chosen to verify the 16S tree not only because it is the site of intein insertion but also due to the high degree of replicative DNA helicase conservation between bacterial species. Results show that although *L. pneumophila* and *R. gryllii* are the closest known relatives to *C. burnetii*, as previously reported (11, 63), *A. ehrlichei* and *H. halophila* are also related to *C. burnetii*, albeit with weak bootstrap support (Fig. 6B and C). Similar results were obtained when phylogenetic trees were built using the maximum parsimony method (not shown) (16).

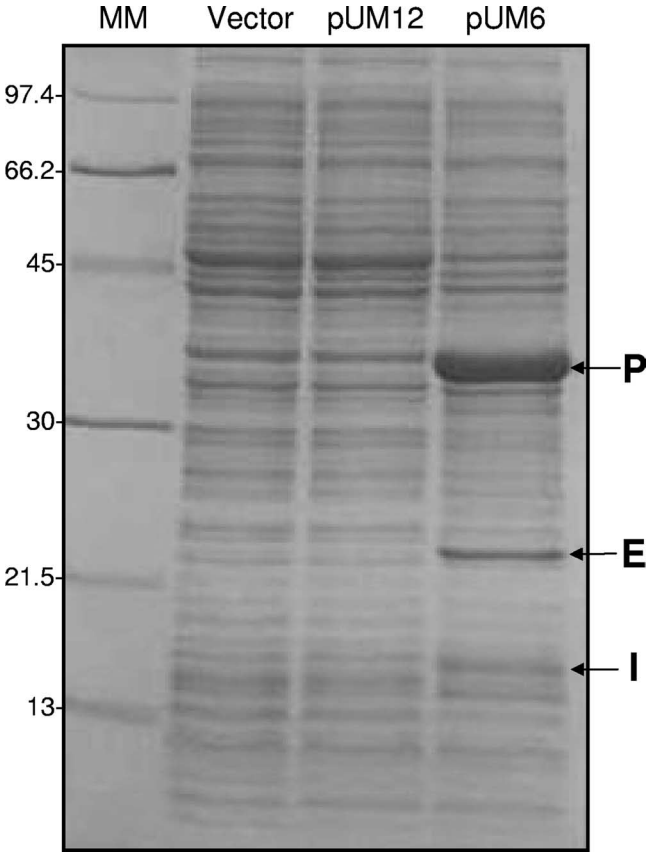

FIG. 5. Intein splicing. *E. coli* (pQE-31 ["vector"], pUM6, or pUM12) was induced with IPTG for 4 h, and protein profiles were analyzed using SDS-PAGE. P, precursor fusion protein (~35.6 kDa); E, spliced exteins (~23.6 kDa); I, intein (~16.8 kDa). Molecular masses (MM) were determined from standards and are given to the left in kilodaltons.

DISCUSSION

All genes can be considered selfish, but in most instances a gene increases its chance of maintenance by increasing the fitness of the host organism (14). However, many genetic elements like introns and inteins reside and replicate within the genome at the host's expense, hence it is perhaps more appropriate to describe them as parasitic (1, 23). Group I introns and inteins invade highly conserved host genes involved in vital functions utilizing an encoded HE. Curiously, HEs themselves are selfish/parasitic elements that have invaded introns and inteins. Based on the presence of conserved amino acid motifs, HEs are divided into four families: LAGLIDADG, GIY-YIG, HNH, and His-Cys box, with LAGLIDADG being the most common (25). While the route of entry for both group I introns and inteins might be the same, i.e., HE-mediated target site cleavage and host-mediated double-stranded DNA repair, their exit strategies are dramatically different: introns splice out as RNA, whereas inteins splice out of proteins. In this study, we describe the "toxic" (i.e., growth-inhibitory) property exhibited by two self-splicing group I introns, Cbu.L1917 and Cbu.L1951, that interrupt the 23S rRNA and the functionality of Cbu.DnaB, the intein found in the C-terminal portion of the replicative DNA helicase (DnaB) of *C. burnetii*.

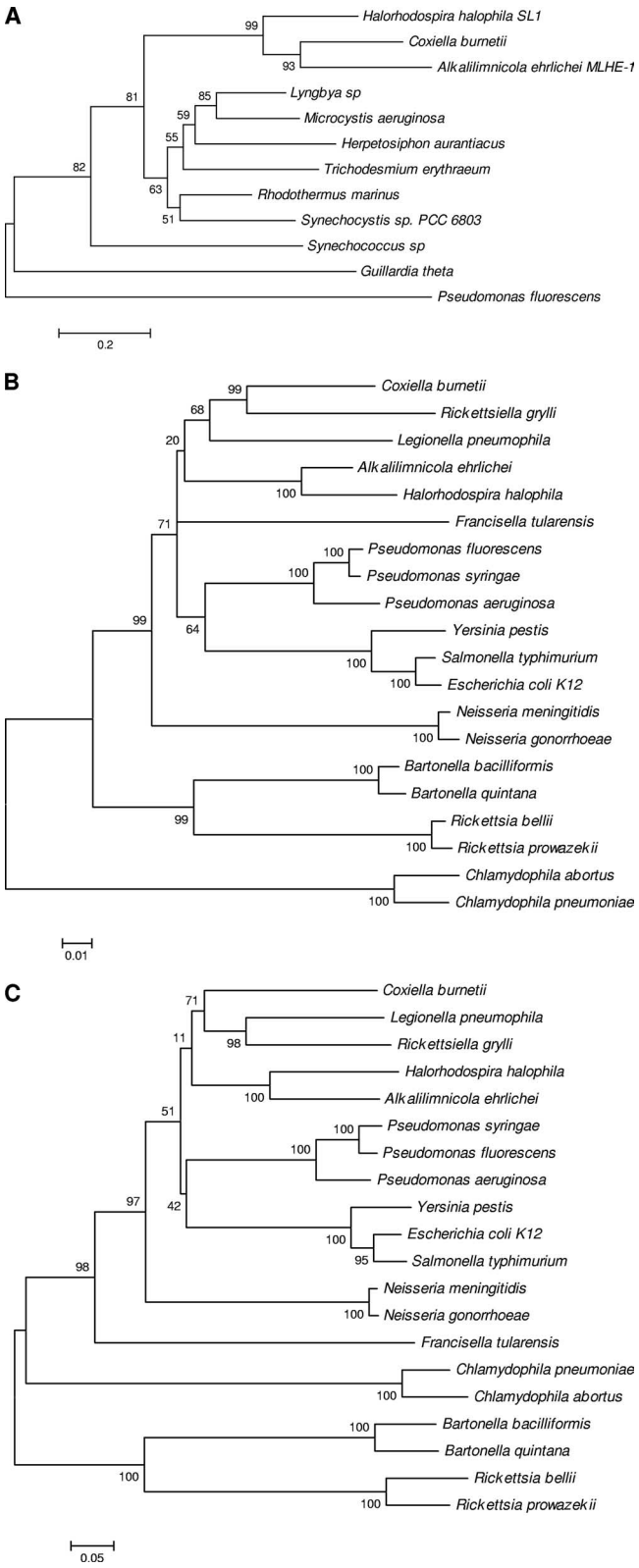

FIG. 6. Phylogenetic analyses. Neighbor-joining trees are shown for inteins (A), 16S (B), and DnaB (C). Intein and DnaB phylogenies were computed using amino acid sequences (125 and 434 positions, respectively) and the Poisson correction method. The 16S tree was built using DNA sequence (1,351 positions) and the maximum composite likelihood method. Bootstrap values (1,000 replicates) are indicated at the nodes.

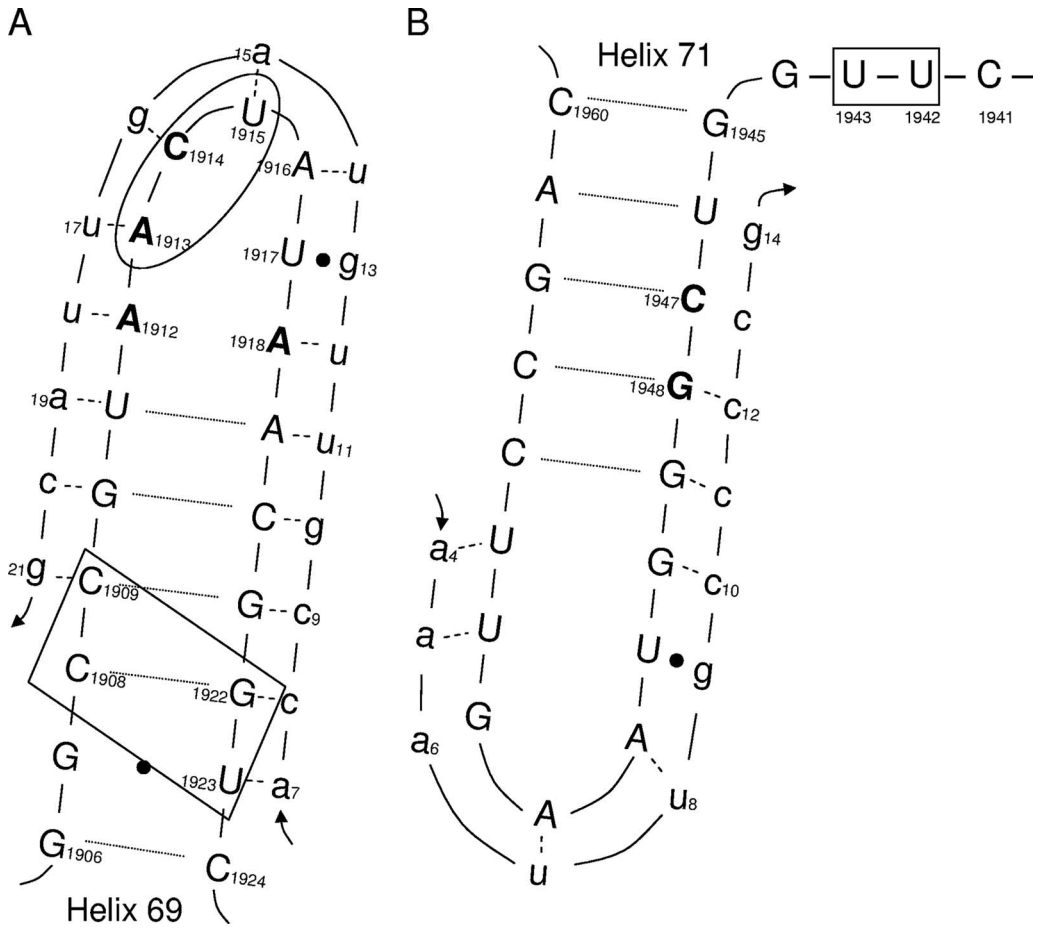

FIG. 7. Potential ribozyme-ribosome interactions. (A) Masking of 23S rRNA helix 69 (uppercase letters) by Cbu.L1917's IGS (lowercase letters). Nucleotides at positions 1912, 1913, 1914, and 1918 (in bold) are involved in forming the intersubunit bridge B2a. Positions 1913, 1914, and 1915 (within oval) interact with A-site tRNA. Positions 1908, 1909, 1922, and 1923 (within parallelogram) interact with P-site tRNA. Arrows, flanking portions of Cbu.L1917. (B) Binding of Cbu.L1951's IGS (lowercase letters) with 23S rRNA helix 71 (uppercase letters). Nucleotides 1947 and 1948 (in bold) are involved in forming the intersubunit bridge B3. Positions 1942 and 1943 (within rectangle) interact with P-site tRNA. Arrows, flanking portions of Cbu.L1951.

Due to the lack of genetic systems for *C. burnetii*, we resorted to *E. coli* models for most of our experiments. However, both in vivo *E. coli* experiments and the in vitro S30 system are appropriate models for studying *Coxiella* introns because of the high degree of conservation between the 23S rRNA of *C. burnetii* and *E. coli*. In fact, exon sequences surrounding and separating the two introns in *C. burnetii* are 100% identical between the two bacteria. The observed association of ribozymes with the ribosomes of both bacteria supports the utility of the model. Since the IGSs of both ribozymes are complementary to their respective splice junctions, it is very likely that intron RNAs associate with the 23S rRNA at these regions (Fig. 7). Binding of Cbu.L1917 RNA to helix 69 and Cbu.L1951 RNA to helix 71 of 23S rRNA would likely interfere with the formation of vital intersubunit (30S-50S) bridges B2a and B3, respectively (79). This interference could make the ribosome unstable, thereby affecting its ability to translate efficiently, leading to a retarded growth rate (37). Cbu.L1917 elicited greater inhibition of *E. coli* growth than Cbu.L1951, consistent with the in vitro observations where inhibition of luciferase translation by Cbu.L1917 RNA was slightly but consistently greater than by Cbu.L1951 RNA. Interestingly, predicted ribozyme-ribosome associations (Fig. 7) showed more complementary interactions between Cbu.L1917's IGS and helix 69 than between Cbu.L1951's IGS and helix 71 (15 and 9 complementary bases, respectively). Moreover, if Cbu.L1917 RNA were to bind to helix 69 as predicted (Fig. 7A), it would mask contact sites with P-site tRNA (bases 1908, 1909, 1922, and 1923) and with A-site tRNA (bases 1913, 1914, and 1915). It would also interfere with the formation of intersubunit bridge B2a (bases 1912, 1913, 1914, and 1918). On the other hand, binding of Cbu.L1951 RNA to helix 71 would partially interrupt bridge B3 (base 1948) and might interfere with the contact sites with P-site tRNA (bases 1942 and 1943) (28, 36).

A potential disparity in target affinity and structural hindrance could explain observed differences in growth inhibition caused by the two ribozymes. During the growth cycle of *C. burnetii*, Cbu.L1917 RNA was found consistently at lower amounts than Cbu.L1951, especially on day 2 of our experiment, when the bacterial growth rate was poised to accelerate (Fig. 4), suggesting that RNA from Cbu.L1917 is more toxic than that of Cbu.L1951. Since equal amounts of both intron

RNAs will be synthesized at any given time, the observed difference in concentration is likely due to differences in intron RNA half-lives, with Cbu.L1917 being more labile than Cbu.L1951, again supporting the possibility that Cbu.L1917 is more toxic than Cbu.L1951. In contrast to the relatively stable 16S rRNA levels observed during the growth cycle of *C. burnetii* (8), a rapid fall in the levels of both intron RNAs is observed as the bacteria transit from lag to log phase. This suggests a reduced half-life for the excised intron RNAs during log phase compared to lag phase. This possibility fits well with earlier observations showing that the half-life of *Tetrahymena* intron RNA is much lower (5 s) during rapid growth than during slow growth (30 s) (5) and that the half-life of total *E. coli* mRNA is longer during lag and stationary phases compared to log phase (33). Also, a study by Chan et al. demonstrated that the half-life of an excised group I intron RNA is 8 to 22 times longer than that of a typical *E. coli* mRNA (6). Consequently, in *C. burnetii* it is possible that when the relatively fast-growing LCVs transition to metabolically quiescent SCVs, potentially toxic intron RNAs become more stable, causing further growth retardation. However, this negative effect may not be significant, since transcription and translation are minimal in SCVs.

Although group I introns are abundant in structural RNA genes of mitochondria and chloroplasts of lower eukaryotes, they are extremely rare in bacterial counterparts (19, 21). We recently described the unusual occurrence of two self-splicing group I introns in the 23S rRNA gene of *C. burnetii* (59). Given the predilection toward genome reduction in obligate intracellular bacteria, the presence of multiple introns in a single gene was intriguing by itself, but the additional observation that these introns can reduce bacterial growth rates raises some interesting questions. Why would a bacterium contain multiple introns in its vital and sole 23S rRNA gene when introns are so rare in other bacteria? Are the introns toxic but still tolerated by the bacterium? Is the growth rate reduction caused by the introns beneficial to the bacterium or is it neutral? We believe that by examining the current lifestyle/biology of *C. burnetii* in light of its evolutionary history, we can elucidate this apparent paradox.

Bacterial genomes are constantly evolving under two opposing forces: gene loss and gene gain (3, 54). The mutational process, which is strongly biased toward deletion in bacteria, is responsible for most of the gene loss that results in condensed genomes with high densities of functional genes (45, 52). Bacteria acquire new genes via HGT, a well-established avenue for bacterial diversification and innovation, with acquisition of pathogenicity islands and antibiotic resistance being two of the most notorious examples (49, 53). Usually these two opposing processes are more or less in equilibrium; however, when a bacterium shifts from a free-living to an intracellular lifestyle, the balance between these two forces tips in favor of gene loss, resulting in shrunken genomes, as observed in obligate intracellular pathogens and endosymbionts (4, 48). Evolutionary forces acting on an obligate intracellular bacterium like *C. burnetii* are quite different from those acting on an environmental bacterium. Constant availability of nutrients, low effective population size, and bottlenecks during transmission render obligate intracellular bacteria susceptible to low purifying selection and genetic drift. This leads to the stochastic loss of several beneficial genes and accumulation of some slightly deleterious mutations (Muller's ratchet) (46, 47). In the obligate endosymbiotic bacterium *Buchnera aphidicola*, the aforementioned forces have not only reduced the genome but also have caused the loss of beneficial DNA repair genes and accumulation of slightly deleterious mutations in vital genes such as *groEL* and 16S rRNA (27, 34). A similar picture can be envisioned in *C. burnetii*. Available evidence suggests that an ancestor of *C. burnetii* recently moved from the environment into the intracellular niche, with concomitant loss of some beneficial genes, like *recBCD* (66). The starter population would possibly have suffered severe bottlenecks before the extant bacterium, which is fully adapted to living in acidic parasitophorous vacuoles, arose. It has been shown that nutrient-laden vesicles of endocytic and/or autophagic pathway(s) fuse with *Coxiella*'s parasitic vacuoles providing a constant supply of nutrients (26, 61). Living in a privileged niche with adequate nutrients and low effective population size conceivably resulted in fixation of genes with low fitness, like the toxic introns. In bacteria like *E. coli*, which lives in unpredictable environments and with large effective populations, these low-fitness genes would be rapidly removed by selection, hence the observed paucity of group I introns (17). Given the likelihood for reductive evolution and limited opportunities for HGT in *C. burnetii*, it is likely that these introns will be lost at some future time unless the elements serve an adaptive role and are hence retained by positive selection. It is important to point out that while it is assumed that the intracellular niche occupied by *C. burnetii* precludes HGT, it is possible that some of the parasitic genetic elements originated from other coinfecting bacteria or from host cell organelles, such as mitochondria and chloroplasts (intracellular arena hypothesis [4]).

The possibility that the introns are beneficial to *C. burnetii* is strengthened by the observation that intron sequences are highly conserved among all eight genomic groups of the pathogen, isolated from disparate hosts and geographic regions (59). It is conceivable that the growth rate reduction caused by the introns fosters chronic infection and persistence in the host. Slow growth rate is a trait shared by most bacteria that cause chronic infections (32, 38, 58, 68, 73). While acute Q fever is self-limiting, the immune system is often unable to clear all bacteria, leading to persistence in the host. A study by Marmion et al. showed that over 88% of infected persons were PCR positive for *C. burnetii* 12 years after acute primary infection (39). By promoting slow growth, introns might in turn be promoting host survival and their own positive selection. Another possibility is that the introns are evolutionarily neutral, with minimal additional negative impact on the growth rate of *C. burnetii*, which already is extremely slow. In this scenario, the introns would eventually be lost from the population and would not reappear due to a lack of HGT, thus resulting in a more compact genome (22).

Like group I introns, inteins invade a new host via HE-mediated targeting (15). Once inteins are fixed in a population they tend to lose the HE gene (HEG) and accumulate mutations in regions not essential for splicing. Subsequently, the intein itself is lost from the population by accurate deletion, and only through another HGT event can the intein regain access to the population (56). Cbu.DnaB has apparently lost its HEG, suggesting that it is in the second phase of its cycle and has been present for some

time in *C. burnetii*. Interestingly, the G+C ratio and codon bias of the intein is similar to that of the *C. burnetii* genome (data not shown), again suggesting that intein invasion occurred a long time ago, providing the bacterium with adequate time to ameliorate possible nucleotide differences (35). Given enough time, the intein will likely be lost and due to the paucity of HGT may not be able to reinvade *C. burnetii*. Phylogenetic and BLAST analyses using the intein sequence identified inteins inserted at the same loci of *A. ehrlichei* and *H. halophila* as Cbu.DnaB's closest neighbors (71). To date, these are the only inteins found at this specific site in *dnaB* (alleles), and it has been previously shown that intein alleles are more closely related to each other than to nonalleles (inteins at different insertion sites) (55). The presence of homologous inteins in two extremophiles suggests that HGT is the most parsimonious explanation for their source. However, additional phylogenetic and BLAST analyses using 16S and DnaB sequences suggest that *C. burnetii*, *A. ehrlichei* (a haloalkaliphile found in soda lakes), and *H. halophila* (one of the most halophilic bacteria known) share a closer evolutionary relationship than previously suspected. Thus, even though HGT is the most probable route of intein acquisition, it is possible that the element was inherited vertically from a common ancestor. Since a nonfunctional intein could convert a functional gene into a pseudogene (23), and given the pseudogenization going on in *C. burnetii* (66), we were curious to assess the intein's functionality. Our analyses demonstrate that the intein is indeed functional (Fig. 5), hence the mature DnaB of *C. burnetii* is undoubtedly functional. In a recent analyses of the *C. burnetii* proteome, the spot identified as DnaB (no. 16) is clearly smaller (~50 kDa) than the molecular mass predicted from GenBank (accession no. NC002971; ~67 kDa), suggesting that the intein is removed to form the mature DnaB protein (65). These observations are not surprising since DnaB is essential (2).

A fascinating possibility is that the presence of the intein in an essential protein is beneficial to *C. burnetii*. Intein splicing appears to be a slow process (Fig. 5), and before the intein is excised, the DnaB precursor is probably nonfunctional, thereby reducing the pool of mature DnaB (24). Since the amount of DnaB in each bacterial cell is thought to be very low (43), intein-mediated scarcity of functional DNA helicase might create a lag in replication, thereby fostering slow growth. Again, similar to the argument made for introns, *Coxiella*'s slow growth rate may obviate the urgent need for mature DNA helicase proteins during replication, thus rendering the intein functionally neutral (molecular atavism). In any case, the availability of an obligate posttranslational processing step provides opportunity for regulation. Although a regulatory role for inteins has not been demonstrated to date, it is possible that the intein excises in response to a signal, thus making available mature, functional proteins at the appropriate time. Indeed, inteins have been engineered with single amino acid substitutions to respond to pH and temperature (7). In conclusion, the parasitic genetic elements acquired by *C. burnetii* in the past, and retained in its present obligatory intracellular niche, undoubtedly influence its biology. Further research is needed to delineate precise roles played by these intriguing elements in *C. burnetii*.

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