NOTE

Conservation of the unique rickettsial rRNA gene arrangement in *Anaplasma*

¹ Program in Vector Borne Diseases, Department of Veterinary Microbiology and Pathology, Pullman, WA 99164-7040, USA

² Animal Disease Research Unit, Agricultural Research Services, US Department of Agriculture, Pullman, WA 99164-6630, USA

Fred R. Rurangirwa,¹ Kelly A. Brayton,¹ Travis C. McGuire,¹ Donald P. Knowles^{1,2} and Guy H. Palmer¹

Author for correspondence: Fred R. Rurangirwa. Tel: +1 509 335 6023. Fax: +1 509 335 8529. e-mail: ruvuna@vetmed.wsu.edu

The organization of the rRNA genes of *Anaplasma marginale*, the type species of the genus *Anaplasma*, was identified to determine if the atypical rRNA gene arrangement identified in rickettsiae preceded divergence of the order *Rickettsiales* into the families *Anaplasmataceae* and *Rickettsiaceae*. The rRNA genes are encoded by two unlinked units, each present in a single copy per *A. marginale* genome. The 16S rRNA gene is separated from the linked 23S and 5S rRNA genes by a minimum of 100 kb. Similar to species belonging to the genus *Rickettsia*, the typical bacterial 16S–23S spacer region containing tRNA genes has been lost in *A. marginale*. In contrast, the *fmt* gene located upstream of the 23S rRNA gene in most *Rickettsia* spp. is not maintained in *A. marginale*, consistent with the *fmt* arrangement being a relatively late event in the evolution of rickettsial species.

Keywords: *Rickettsiales*, *Rickettsiaceae*, *Anaplasmataceae*, *Anaplasma*, *Rickettsia*

Bacterial rRNA genes are typically organized in an operon, with multiple complete operons per bacterial genome (Srivastava & Schlessinger, 1990, 1991; Blattner *et al.*, 1997). The most common structure of the operons is 16S (*rrs*), 23S (*rrl*) and 5S (*rrf*), with tRNA genes located within the 16S–23S spacer. Deviations from the typical 16S–23S–5S operon structure are rare, with the exception of obligate intracellular bacteria with small (< 1.5 Mb) genomes. Alternative rRNA gene arrangements are characterized by very low copy numbers of the rRNA genes with loss of the classical operon structure (Pang & Winkler, 1993; Andersson *et al.*, 1995). These atypical structures are hypothesized to have arisen from a progenitor with the classical multiple operon structure by mutation and then differential deletion of individual 16S, 23S or 5S rRNA genes (Andersson *et al.*, 1995; Kurland & Andersson, 2000). Consistent with this hypothesis, atypical rRNA gene arrangements are conserved among closely related bacterial species. Among species belonging to the genus *Rickettsia*, the 16S rRNA gene is widely separated from the linked 23S and 5S rRNA genes, and the typical 16S–23S spacer sequences are not retained 3' to the 16S or 5' to the 23S rRNA genes (Andersson *et al.*,

1995, 1998). This unique structure is highly conserved in at least 13 species of *Rickettsia* representing both the spotted fever group and the typhus group, indicating that the rRNA gene rearrangement preceded speciation as well as divergence into these two predominant biotypes (Andersson *et al.*, 1999).

If loss of the typical rRNA operon structure occurred as a consequence of obligate intracellular parasitism by a rickettsial progenitor, the unique rRNA gene arrangement of *Rickettsia* species, or a closely related atypical structure, would be predicted for other bacteria believed to have descended from this common progenitor. Bacteria in the order *Rickettsiales* are obligate intracellular parasites and are, with few exceptions, transmitted by arthropod vectors (Weiss & Moulder, 1984). The order has recently been emended to two families, *Rickettsiaceae*, which includes the genus *Rickettsia*, and *Anaplasmataceae*, which includes and is named for the genus *Anaplasma* (Dumler *et al.*, 2001). In this study, we used the type species of the genus *Anaplasma*, *Anaplasma marginale*, to determine if the unique organization of the rRNA genes is maintained in both families of the order *Rickettsiales*.

The 16S rRNA gene of the St Maries strain of *A. marginale* (Eriks *et al.*, 1994) was sequenced (GenBank accession no. AY048816) and was shown to be identical to that previously reported for other *A.*

Abbreviation: BAC, bacterial artificial chromosome.

The GenBank accession numbers for the 23S–5S sequence and the 16S rRNA gene sequence of *Anaplasma marginale* St Maries are AY048815 and AY048816, respectively.

marginale strains (Dame *et al.*, 1992; Dumler *et al.*, 2001). A bacterial artificial chromosome (BAC) library derived from the St Maries strain, constructed as described previously (Brayton *et al.*, 2001), was screened for clones containing the 16S rRNA gene. A 540 bp digoxigenin-labelled 16S rRNA probe (nucleotides 382–921) was generated by PCR using the forward primer 5'-CGTGAGTGAGGAAGGCCTTAGGGT-3' and the reverse primer 5'-GTTGCATCGAATTA-AACC-3'. Two overlapping BAC clones, H1 (97 kb) and B7 (100 kb), containing the 16S rRNA gene were identified by probing with the digoxigenin-labelled 16S probe. The sequences of the 16S rRNA gene and its flanking regions were identical in each BAC clone. Although the 23S rRNA gene of *A. marginale* has not been reported previously, a partial (600 bp) sequence of the 23S rRNA gene had been identified and was used to generate a specific 543 bp probe using the forward primer 5'-GTGACAGCGTACCTTTTGCA-T-3' and the reverse primer 5'-GCTTTCTTTAAAG-GATGGCTG-3'. Two overlapping BAC clones, D3 (50 kb) and G7 (48 kb), containing the 23S rRNA gene were identified. Each BAC clone contained a complete 23S rRNA gene, an intergenic spacer of 80 bp and a 5S rRNA gene. The sequences from each BAC clone were identical and are reported as GenBank accession number AY048815.

Analysis of the copy numbers and linkage of the 16S and 23S–5S rRNA genes was done using four *A. marginale* strains (St Maries, South Idaho, Florida and Virginia), one *Anaplasma centrale* strain (Israel) and one *Anaplasma ovis* strain (Idaho). The strains were maintained as liquid nitrogen-cryopreserved stabilates of infected bovine or ovine erythrocytes (Love, 1972). The source and characterization of the *A. marginale* and *A. ovis* strains has been described previously by McGuire *et al.* (1984) and Ndung'u *et al.* (1995), respectively. Genomic DNA was isolated from blood collected from infected animals during acute rickettsemia using the Puregene DNA Extraction Kit (Gentra Systems), following the manufacturer's instructions. *A. centrale* genomic DNA was a gift from Dr Varda Shkap (Kimron Veterinary Institute).

Digestion of *Anaplasma* chromosomal DNA using restriction enzymes that do not have cleavage sites within the 16S rRNA gene or within the linked 23S–5S rRNA genes and hybridization of the same blot with either the 16S probe (Fig. 1a) or, after stripping, the 23S probe (Fig. 2a) revealed only single chromosomal fragments. Furthermore, the 16S and 23S probes did not bind to the same DNA fragment in any of the enzyme digests. As predicted from the sequence data, a 5S probe bound the same chromosomal fragments as the 23S-specific probe (data not shown). To confirm the separation of the 16S and 23S–5S rRNA genes, the 100 kb BAC clone B7, containing the 16S rRNA gene, and the 50 kb BAC clone D3, containing the 23S–5S rRNA genes, were digested using restriction enzymes that did not cleave within the rRNA gene sequences. The cleavage products were hybridized using the 16S

Fig. 1. Southern-blot analysis of the 16S rRNA gene in genomic DNA and in the BAC clones. (a) Genomic DNA from the St Maries strain of *A. marginale* or (b) DNA from BAC clones B7 (16S clone) and D3 (23S–5S clone) was digested with endonucleases that do not have cleavage sites within the 16S gene or within the 23S–5S rRNA genes. The fragments were separated by agarose gel electrophoresis, blotted onto a nylon membrane, prehybridized at 50 °C in Dig EasyHyb buffer (Roche Molecular Biochemicals) for 2–4 h and then hybridized in the same buffer with the 16S rRNA gene probe overnight. Generation and digoxigenin labelling of the probe, hybridization, detection and stripping were done as recommended by the manufacturer of the PCR Labelling Kit (Roche Molecular Biochemicals). Washing conditions were as follows: two washes of 5 min each in 2 × SSC (1 × SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) containing 0.1% (w/v) SDS at room temperature, and two final washes of 15 min each at 68 °C in 0.5 × SSC containing 0.1% SDS. Molecular size markers are shown at the left of the images.

probe (Fig. 1b); the membranes were then stripped and re-probed using the 23S probe (Fig. 2b). Neither clone contained both the 16S and 23S genes. Separate single DNA fragments were bound by the 16S probe in BAC clone B7 (Fig. 1b) and the 23S probe in BAC clone D3 (Fig. 2b). The pattern of hybridizing fragments was identical for the genomic DNA of the four *A. marginale* strains (St Maries, South Idaho, Florida and Virginia) and the BAC clones, indicating that these clones are representative of the genomic arrangement of the genes. The chromosomal and BAC clone hybridization data and the sequence data derived from the BAC clones indicate that *A. marginale* does not retain the classical bacterial 16S–23S–5S rRNA operon structure and that the 16S rRNA gene is separated from the linked 23S–5S rRNA genes. Analysis of genomic DNA from *A. ovis* and *A. centrale* by the same strategy, digestion using restriction enzymes that do not cleave within the genes, probing using the 16S rRNA gene probe, and then stripping and re-probing using the 23S rRNA gene probe, also revealed only single fragments (Figs 3 and 4). Similar to the results with *A. marginale*, the 16S and 23S probes did not bind the same DNA fragment in the restriction enzyme digests of *A. centrale* (Fig. 3) or *A. ovis* (Fig. 4). It is noteworthy that the enzyme restriction fragment length polymorphisms of *A. centrale* and *A. ovis* are

Fig. 2. Southern-blot analysis of the 23S rRNA gene in genomic DNA and in the BAC clones. (a) Genomic DNA from the St Maries strain of *A. marginale* or (b) DNA from BAC clones B7 (16S clone) and D3 (23S-5S clone) was digested with endonucleases that do not have cleavage sites within the 16S gene or within the 23S-5S rRNA genes. After detection of the 16S rRNA genes (Fig. 1), the same membranes as used in Fig. 1 were stripped by washing them briefly in distilled water, then washing them for 15 min (twice) in 0.2 mM NaOH containing 0.1% SDS, with a final rinse in $2\times$ SSC. Southern blotting, prehybridization and hybridization conditions, and generation of the 23S rRNA probe were done as described for Fig. 1. Molecular size markers are shown at the sides of the images.

Fig. 3. Southern-blot analysis of the 23S and 16S rRNA genes in the *A. centrale* genome. Genomic DNA from the Israel vaccine strain of *A. centrale* was digested with endonucleases that do not have cleavage sites within the 16S gene or within the 23S-5S rRNA genes. Following hybridization with the 16S rRNA probe, the membranes were stripped and re-hybridized using the 23S rRNA probe. Prehybridization and hybridization conditions, stripping and generation of the probes were done as described for Figs 1 and 2. Molecular size markers are shown at the left of the images.

different from each other (Figs 3 and 4), as well as being different from *A. marginale* (Figs 1-4).

Are the 16S rRNA gene and, separately, the 23S-5S rRNA genes present as tandem repeats within the *A. marginale* genome? Digestion with *EcoRI* or *PstI*,

Fig. 4. Southern-blot analysis of the 23S and 16S rRNA genes in the *A. ovis* genome. Genomic DNA from the Idaho strain of *A. ovis* was digested with endonucleases that do not have cleavage sites within the 16S gene or within the 23S-5S rRNA genes. Following hybridization with the 16S rRNA probe, the membranes were stripped and re-hybridized using the 23S rRNA probe. Prehybridization and hybridization conditions, stripping and generation of the probes were done as described for Figs 1 and 2. Molecular size markers are shown at the left of the images.

which have single cleavage sites in the 16S rRNA gene, generated the predicted two fragments that hybridized with the 16S probe (Fig. 5a). The sequence conservation between individual rRNA genes when multiple copies are present in the bacterial genome allows the detection of tandem genes using a combination of enzymes that do and do not cleave within the gene sequence (Pang & Winkler, 1993). Digestion using

Fig. 5. Double digestion and Southern-blot analysis of the (a) 16S and (b) 23S BAC clones. BAC clones B7 (16S clone) and D3 (23S clone) were initially digested using enzymes that had a single cleavage site within the rRNA genes (*EcoRI* and *PstI* for 16S; *NcoI* and *HindIII* for 23S). They were then digested a second time using enzymes that did not cut within the rRNA genes (*BamHI*, *NotI*, *EcoRV* and *NdeI* for 16S; *EcoRI*, *BamHI*, *NotI*, *EcoRV* and *NdeI* for 23S). Prehybridization and hybridization with either the 16S rRNA or the 23S rRNA probes were done as described for Figs 1 and 2. Molecular size markers are shown at the left of the images.

EcoRI or *PstI* followed by a second digestion with enzymes that do not cleave within the 16S rRNA gene generated only two fragments that hybridized using the 16S probe (Fig. 5a). Digestion using *NcoI* or *HindIII* cleaves once in the 23S rRNA gene sequence and generated the predicted two fragments bound by the 23S probe (Fig. 5b). Double digestion using *NcoI* or *HindIII* in combination with enzymes that do not cleave within the 23S–5S rRNA genes also generated only two fragments (Fig. 5b). This strongly suggests that there are no tandem repeats of the 16S or 23S–5S rRNA genes. This conclusion was corroborated by sequencing 1 kb of sequence flanking the 16S and 23S–5S rRNA genes in the BAC clones, which did not reveal any related sequences.

Overall, the organization of the *Anaplasma* rRNA genes, with the loss of the typical operon structure and the presence of a single 16S gene that is separated from the linked 23S–5S genes, is similar to that reported for the genus *Rickettsia* (Andersson *et al.*, 1995, 1998, 1999). The distance separating the 16S and the 23S–5S genes in *A. marginale* has not been determined, but it is at least 100 kb. This minimum separation is based on the position of the 16S gene, in the sense orientation, at the end of the BAC clone B7 (data not shown) followed by 100 kb of downstream sequence that does not include the 23S gene (Figs 1b and 2b). In addition,

there were no tRNA genes present in the 1 kb immediately 3' of the 16S rRNA gene. This loss of the normal 16S–23S spacer region sequences containing tRNA genes also reflects the pattern seen with *Rickettsia* spp. (Andersson *et al.*, 1995, 1998, 1999). In contrast, in *A. marginale*, the *fmt* gene encoding methionyl-tRNA^{Met} formyltransferase was not identified immediately upstream of the 23S rRNA gene as occurs in 13 species of *Rickettsia* (Andersson *et al.*, 1999). This inability to detect the *fmt* gene in *A. marginale* may be due to sequence divergence such that the gene is no longer recognizable or because the *fmt* gene is not located within 1 kb upstream of the 23S rRNA. Interestingly, two species in the family *Rickettsiaceae*, *Rickettsia bellii* and *Orientia tsutsugamushi*, also lack a recognizable *fmt* gene in this position (Andersson *et al.*, 1999). This is consistent with *O. tsutsugamushi* representing a separate genus (Tamura *et al.*, 1995) and with the suggestion, based on analysis of 16S and 23S rRNA genes (Stothard *et al.*, 1994; Stothard & Fuerst, 1995) and the citrate synthase gene (Roux *et al.*, 1997), that *R. bellii* diverged relatively early in the speciation of the genus *Rickettsia*.

In conclusion, the unusual arrangement of the rRNA genes in bacteria belonging to the order *Rickettsiales* appears to have been generated prior to the divergence of the families *Rickettsiaceae* and *Anaplasmataceae*.

Whereas the present study examined *A. marginale*, *A. ovis* and *A. centrale*, with the detailed study of BAC clones limited to *A. marginale*, Massung *et al.* (2001) recently reported that the same arrangement of rRNA genes occurs in *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*. *Ehrlichia* represents a second examined genus within the family *Anaplasmataceae* (Dumler *et al.*, 2001). Interestingly, bacteria in both families share the requirement of transition from obligate intracellular parasitism in a vertebrate host cell to an invertebrate host cell. In *A. marginale* this involves replication within the bovine erythrocyte with transition to replication within the tick midgut and then the salivary gland epithelium (Kocan, 1986). The occurrence of multiple operons with the typical 16S–23S–5S structure has been hypothesized to be important in the ability of bacteria to respond to changing growth conditions (Klappenbach *et al.*, 2001). Clearly, rickettsiae manage the transition between the different environments of mammalian and arthropod hosts using a single set of separately arranged 16S and 23S–5S rRNA genes. Their ability to adapt to their different hosts most likely reflects a strategy of specific invasion of target cells and relatively slow intracellular replication in a protected site rather than them depending on rapid growth to survive amidst other microbial competitors.

Acknowledgements

This research was supported by a grant from the National Institute of Allergy and Infectious Diseases (AI44005), USDA-ARS-CRIS 5348-32000-012-00D and USDA-ARS-SCA 58-5348-8-044. We thank Dr Varda Shkap (Kimron Veterinary Institute) for providing *A. centrale* genomic DNA.

References

- Andersson, S. G. E., Zomorodipour, A., Winkler, H. H. & Kurland, C. G. (1995). Unusual organization of the rRNA genes in *Rickettsia prowazekii*. *J Bacteriol* **177**, 4171–4175.
- Andersson, S. G. E., Zomorodipour, A., Andersson, J. O. & 7 other authors. (1998). The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* **396**, 133–140.
- Andersson, S. G. E., Stothard, D. R., Fuerst, P. & Kurland, C. G. (1999). Molecular phylogeny and rearrangement of rRNA genes in *Rickettsia* species. *Mol Biol Evol* **16**, 987–995.
- Blattner, F. R., Plunkett, G. III, Bloch, C. A. & 14 other authors. (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453–1474.
- Brayton, K. A., Knowles, D. P., McGuire, T. C. & Palmer, G. H. (2001). Efficient use of a small genome to generate antigenic diversity in tick-borne ehrlichial pathogens. *Proc Natl Acad Sci USA* **98**, 4130–4135.
- Dame, J. B., Mahan, S. M. & Yowell, C. A. (1992). Phylogenetic relationship of *Cowdria ruminantium*, agent of heartwater, to *Anaplasma marginale* and other members of the order *Rickettsiales* determined on the basis of 16S rRNA sequence. *Int J Syst Bacteriol* **42**, 270–274.
- Dumler, J. S., Barbet, A. F., Bekker, C. P. J., Dasch, G. A., Palmer, G. H., Ray, S. C., Rikihisa, Y. & Rurangirwa, F. R. (2001). Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol* **51**, 2145–2165.
- Eriks, I. S., Stiller, D., Goff, W. L., Panton, M., Parish, S. M., McElwain, T. F. & Palmer, G. H. (1994). Molecular and biological characterization of a newly isolated *Anaplasma marginale* strain. *J Vet Diagn Invest* **6**, 435–441.
- Klappenbach, J. A., Dunbar, J. M. & Schmidt, T. M. (2001). rRNA operon copy number reflects ecological strategies of bacteria. *Appl Environ Microbiol* **66**, 1328–1333.
- Kocan, K. M. (1986). Development of *Anaplasma marginale* in ixodid ticks: coordinated development of a rickettsial organism and its tick host. In *Morphology, Physiology and Behavioral Ecology of Ticks*, pp. 472–505. Edited by J. R. Sauer & J. A. Hair. Chichester, UK: Horwood Inc.
- Kurland, C. G. & Andersson, S. G. E. (2000). Origin and evolution of the mitochondrial proteome. *Microbiol Mol Biol Rev* **64**, 786–820.
- Love, J. N. (1972). Cryogenic preservation of *Anaplasma marginale* with dimethyl sulfoxide. *Am J Vet Res* **32**, 2557–2560.
- Massung, R. F., Lee, L., Mael, M. & Gusa, A. (2001). Characterization of the rRNA genes of *Ehrlichia chaffeensis* and *Anaplasma phagocytophila*. In *Abstracts of the American Society for Rickettsiology – Bartonella as an Emerging Pathogen Group 2001 Joint Conference, Big Sky, Montana*, abstract 80, p. 58. American Society for Rickettsiology.
- McGuire, T. C., Palmer, G. H., Goff, W. L., Johnson, M. I. & Davis, W. C. (1984). Common and isolate-restricted antigens of *Anaplasma marginale* detected with monoclonal antibodies. *Infect Immun* **45**, 697–700.
- Ndung'u, L. W., Aguirre, C., Rurangirwa, F. R., McElwain, T. F., McGuire, T. C., Knowles, D. P. & Palmer, G. H. (1995). Detection of *Anaplasma ovis* infection in goats by major surface protein 5 competitive inhibition enzyme-linked immunosorbent assay. *J Clin Microbiol* **33**, 675–679.
- Pang, H. & Winkler, H. H. (1993). Copy number of the 16S rRNA gene in *Rickettsia prowazekii*. *J Bacteriol* **175**, 3893–3896.
- Roux, V., Rydkina, E., Ereemeeva, M. & Raoult, D. (1997). Citrate synthase gene comparison, a new tool for phylogenetic analysis, and its application for the rickettsiae. *Int J Syst Bacteriol* **47**, 252–261.
- Srivastava, A. K. & Schlessinger, D. (1990). Mechanism and regulation of bacterial ribosomal RNA processing. *Annu Rev Microbiol* **44**, 105–129.
- Srivastava, A. K. & Schlessinger, D. (1991). Structure and organization of ribosomal DNA. *Biochimie* **73**, 631–638.
- Stothard, D. R. & Fuerst, P. A. (1995). Evolutionary analysis of the spotted fever and typhus groups of *Rickettsia* using 16S rRNA gene sequences. *Syst Appl Bacteriol* **18**, 52–61.
- Stothard, D. R., Clark, J. B. & Fuerst, P. A. (1994). Ancestral divergence of *Rickettsia bellii* from the spotted fever and typhus groups of *Rickettsia* and antiquity of the genus *Rickettsia*. *Int J Syst Bacteriol* **44**, 798–804.
- Tamura, A., Ohashi, N., Urakami, H. & Miyamura, S. (1995). Classification of *Rickettsia tsutsugamushi* in a new genus, *Orientia* gen. nov., as *Orientia tsutsugamushi* comb. nov. *Int J Syst Bacteriol* **45**, 589–591.
- Weiss, E. & Moulder, J. M. (1984). Order I. Rickettsiales Gieszkiewicz 1939. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 687–729. Edited by N. R. Kreig & J. G. Holt. Baltimore: Williams & Wilkins.