

## Unusual Organization of the rRNA Genes in *Rickettsia prowazekii*

SIV G. E. ANDERSSON,<sup>1\*</sup> ALIREZA ZOMORODIPOUR,<sup>1</sup> HERBERT H. WINKLER,<sup>2</sup>  
AND CHARLES G. KURLAND<sup>1</sup>

*Department of Molecular Biology, Biomedical Center, Uppsala University, Uppsala, S751 24 Sweden,<sup>1</sup>  
and Laboratory of Molecular Biology, Department of Microbiology and Immunology,  
University of South Alabama College of Medicine, Mobile, Alabama 36688<sup>2</sup>*

Received 10 November 1994/Accepted 28 April 1995

**We describe here the organization of the rRNA genes in *Rickettsia prowazekii*. In this organism, the 23S and the 5S rRNA genes are tightly linked to each other, whereas the 16S rRNA gene is separated from this cluster. The 23S-5S unit is preceded by the methionyl-tRNA<sub>f</sub><sup>Met</sup> formyltransferase gene.**

The rRNA genes of eubacteria are typically organized into operons with the general structure 16S-23S-5S (15). In many of these operons, tRNA genes are also found in the spacer between the 16S and the 23S genes (14). It is highly relevant to the present study that unusual arrangements of rRNA genes have so far mainly been associated with organisms having small genome sizes and low copy numbers for rRNA genes. For example, in the 1.0-Mb genome of *Mycoplasma gallisepticum* (10), the 16S rRNA gene is separated from the 23S and the 5S rRNA genes in one of two rRNA configurations (1b). Similarly, in the 950-kb genome of *Borrelia burgdorferi* (6), the single 16S rRNA gene is situated more than 2 kb upstream from the 23S and 5S rRNA genes, and these are duplicated in the order 23S-5S-23S-5S (2, 12). Finally, the 5S rRNA gene is separated from the 16S and 23S rRNA genes by at least 4 kb in the 1.1-Mb genome of *Mycoplasma hyopneumoniae* (10), which only has a single copy of each of these genes (17). Since *Rickettsia prowazekii* has a small genome of 1.2 Mb (5) with only a single copy of its 16S rRNA gene (9), it was of interest to determine how the rRNA genes in *R. prowazekii* are arranged. The data show that the 16S rRNA gene is at least 4 kb distant from the 23S-5S gene cluster and that the flanking sequences of the rRNA genes are not those typical of eubacteria.

**Isolation and cloning of the rRNA genes.** A genomic library was prepared from *R. prowazekii*, which was propagated in the yolk sacs of embryonated hen eggs, harvested, and purified as previously described (19). Random cutting of the rickettsial chromosome was achieved with the aid of *EcoRI*\* activity. This modified enzyme activity was generated by digestion of 2 µg of genomic DNA in a low-ion reaction buffer (2.5 mM MnCl<sub>2</sub>, 2.5 mM MgCl<sub>2</sub>, 10 mM NaCl, 10% glycerol [pH 8.5]) with *EcoRI* (4,000 U) at 37°C (1). The reaction was terminated after 2 to 3.5 min by the addition of 50 mM EDTA. The digested fragments were separated on an 0.8% agarose gel, and fragments ranging from 2.3 to 9.6 kb were excised from the gel. The purified fragments were ligated to *EcoRI*-digested lambda ZAP II vector and packaged with the Gigapack II packaging extract according to the protocol provided with the Stratagene lambda ZAP II cloning kit. The phage titer after one round of amplification was 1.6 × 10<sup>9</sup> PFU/ml. Approximately 400,000

phage plaques were obtained with insert sizes averaging 3.3 kb (20).

In each screening for phage carrying *R. prowazekii* rRNA genes, approximately 2,000 individual phage particles of the *R. prowazekii* library were plated on NZY agar plates and transferred to replica filters (Du Pont, NEN Research products, Boston, Mass.) according to the instructions of the lambda ZAP II cloning system (Stratagene, La Jolla, Calif.). Hybridizations and washings were performed as described previously (11). In vivo excision of the pBluescript vector along with the inserted DNA of each positive clone was done according to the protocol of the supplier of the lambda ZAP II cloning system (Stratagene). In the first screening, we used as a probe an oligomer (or-16S) homologous to positions 796 to 813 in the 16S rRNA gene of *R. prowazekii* (18), which had been labelled at its 5' end with [ $\gamma$ -<sup>32</sup>P]ATP and T4 kinase under standard conditions (11). This probe identified six positive plaques, each of which could be sorted on the basis of restriction fragment analysis into either of two different classes (r2 and r3) as illustrated in Fig. 1A. In the second search, we screened the library with a total RNA mixture that had been labelled with T4 RNA ligase and [ $\gamma$ -<sup>32</sup>P]pCp, as previously described (3, 4). Stable RNA was purified from Renografin-purified *R. prowazekii* obtained from 50 infected hen's yolk sacs resuspended in 1 ml of buffer (0.3 M sucrose plus 10 mM sodium acetate [pH 4.5]). To this suspension, 1 ml of 2% sodium dodecyl sulfate plus 10 mM sodium acetate was added, followed by incubation at 70°C for 5 min and extraction with hot phenol three times. After precipitation, the RNA preparation was resuspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 7.5]). In this screening, more than 100 positive clones were identified. Eleven of the clones were found to contain rRNA genes, and these were selected for further analysis. The clones were sorted into subgroups on the basis of their restriction enzyme digest patterns as well as their hybridization patterns with the oligonucleotide probes or-16S (ATATCGGAGGATTCT CTT), or-23S (AGTACCGTGAGGGAAAGG), and or-5S (ACCGATCCCATCCCGAAC), which were used as markers for each of the rRNA genes. Two subgroups hybridizing to the 23S and the 5S probes are here represented by clones t56 and t63 (Fig. 1B). In the third screening, we used as probes restriction fragments from the 3' and 5' ends of clones r2 and t56, which had been labelled with [ $\alpha$ -<sup>32</sup>P]dCTP and Klenow DNA polymerase by the random priming method (11). In this screening, more than 60 positive clones were identified, one of which is r2w12 (Fig. 1A). Surprisingly, none of the clones isolated hybridized to both the

\* Corresponding author. Phone: 018-174387. Fax: 018-557723. Electronic mail address: siv@bio.embnet.se.

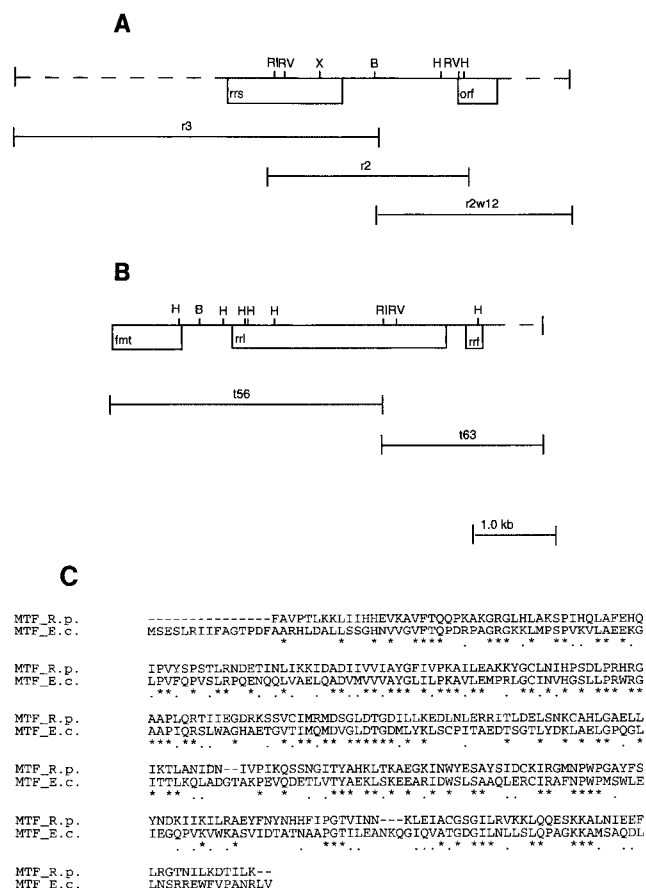


FIG. 1. Schematic representation of the clones containing the 16S (A) and 23S (B) rRNA genes used in sequencing. (C) Alignment of the deduced amino acid sequence of the *fnt* gene product (MTF) of *R. prowazekii* (R.p.) with that of *E. coli* (E.c.) with the CLUSTAL V multiple sequence alignment program (8). A thin line indicates a noncoding region; a box indicates a coding region. Abbreviations used to indicate restriction enzyme sites are as follows: B, *Bgl*II; RI, *Eco*RI; RV, *Eco*RV; H, *Hind*III; X, *Xho*I. Symbols beneath the aligned sequences indicate identical residues (\*) and sites with conservative replacements (·).

16S and the 23S rRNA oligonucleotide-specific probes, nor was simultaneous hybridization obtained when an oligomer specific for the 3' end of the 16S rRNA gene was used in combination with an oligomer specific for the 5' end of the 23S rRNA gene.

**Features of sequences downstream from the 16S rRNA gene.** In order to determine the sequences downstream of the 16S rRNA gene, clones r2 and r2w12 were selected for further analysis. Plasmid DNA was isolated by Qiagen large-scale plasmid preparations (KEBO, Stockholm, Sweden), and the DNA sequences on both strands of the inserts were determined by double-stranded dideoxy sequencing with modified T7 DNA polymerase (Sequenase) and fluorescent dATP (Pharmacia, Uppsala, Sweden). The products of the sequencing reactions were separated and analyzed with the aid of an ALF Sequencer (Pharmacia).

No rRNA sequences could be detected in the 2-kb region immediately downstream of the 16S rRNA gene. Furthermore, both visual inspection of the sequences and analysis with the aid of the tRNA-Search program (13) failed to identify any tRNA genes in this region. The sequence 2 kb downstream from the 3' end of the 16S rRNA gene contains one open reading frame with a length of 483 bp preceded by a potential ribosome-binding site (AGGT) and ending with two termina-

tion codons in a row (TGATAA). That this open reading frame corresponds to a protein-coding gene is consistent with the observation that the putative, synonymous third-codon positions have an average G+C content of 14% (1a). A homology search with different parts of the deduced amino acid sequence failed to identify any homologs in the data banks. We refer to this gene as *orf*(*rrs*-3').

**Sequence features upstream of the 23S rRNA gene.** A sequence analysis of clone t56 failed to identify any rRNA or tRNA genes in the 1.6-kb region upstream of the 23S rRNA gene. Instead, an open reading frame with a length of 879 bp could be identified. That this open reading frame is indeed a protein-coding gene is further supported by the characteristic variations in G+C content in the three codon positions, with the putative synonymous third-codon positions having a G+C content of 17% (1a). A homology search with different parts of the inferred amino acid sequence from this gene matched a gene product from the *E. coli* gene designated *fnt*, which encodes the enzyme catalyzing the formylation of the Met-tRNA<sup>Met</sup> complex (MTF) (7). Translation of the putative *fnt* gene in *R. prowazekii* is expected to produce a protein with a length of at least 293 amino acids. This sequence is incomplete at its 5' end, but probably not by much, since its homolog in *Escherichia coli* is 314 amino acids. A direct comparison of the two proteins yields an overall identity of 39% and suggests that the first 14 amino acids may be missing from the *R. prowazekii* protein (Fig. 1C). The region corresponding to positions 82 to 165 in the *E. coli* MTF sequence is particularly well conserved, with 43 out of 84 residues being identical in the two sequences (Fig. 1C).

**rRNA gene copy number.** It has recently been determined that there is only one copy of the 16S rRNA gene in *R. prowazekii* (9). To test whether there is similarly only one copy of the 23S rRNA gene, a Southern blot analysis was performed. About 1 µg of chromosomal DNA from *R. prowazekii* was digested with 10 to 20 U of *Eco*RI, *Hinc*II, and *Hind*III at 37°C overnight and resolved by electrophoresis through a 0.7% agarose gel, followed by transfer to a nylon membrane (DuPont, NEN Research Products). The 16S-specific probe was made from a 700-bp fragment obtained by digestion of clone r2 with *Xho*I and *Nor*I, covering a region from nucleotides 640 to 1288 in the 16S rRNA gene (Fig. 2A). The 23S-specific probe was generated by PCR with two oligomers (ot56+3 and ot56-5) that amplify a region between nucleotides 435 and 1759 in the 23S rRNA gene (Fig. 2C). To confirm the identities of the probes, clone r2 digested with *Xho*I and *Nor*I and clone t56 digested with *Bgl*II and *Xho*I were included as 16S rRNA and 23S rRNA test samples, respectively (Fig. 2A and C). Indeed, the 16S probe hybridized with the 700-bp fragment obtained in the r2 digest (Fig. 3B), and the 23S probe hybridized with the 5-kb fragment containing the 23S rRNA gene of clone t56 (Fig. 3C).

In the genomic digests, the 16S probe hybridized with only one band in each lane (Fig. 3B), in accordance with the observation that there is only one 16S rRNA gene in *R. prowazekii* (9). Similarly, we found that the 23S probe hybridized to a single band in each of the genomic digests (Fig. 3C). The bands hybridizing to the 23S probe in the *Hind*III and *Eco*RI digests are far too short to accommodate two sets of 23S genes coding for rRNA (2.4 and 3.5 kb, respectively). We note that sequence data of the unit made up of 23S-5S rRNA genes predicts a fragment of 2,534 nucleotides covering the 23S probe and bordered by two *Hind*III sites (Fig. 1B), in good agreement with the value observed. However, since this fragment is located within the 23S-5S unit, it does not provide information about the potential redundancy of the two genes.

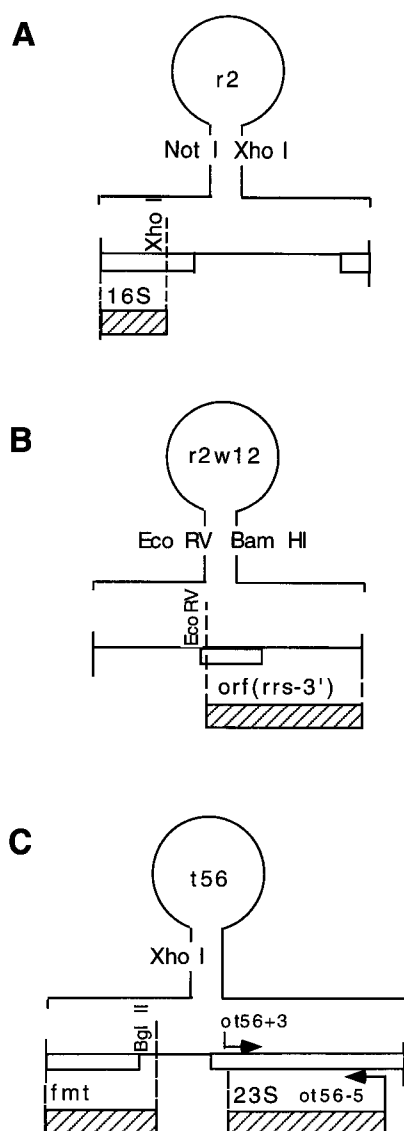


FIG. 2. Plasmids used as positive controls and for the generation of probes in the Southern blot hybridization shown in Fig. 3. (A) Clone r2. (B) Clone r2w12. (C) Clone t56. The hatched boxes under the inserts indicate the lengths and positions of the probes. The arrows in the insert of clone t56 refer to the primers used in PCR to generate the probe for the 23S genes coding for rRNA.

We observed that the insert size of clone t56 (3,485 bp) is similar to the size of the *EcoRI* genomic fragment to which the 23S probe hybridizes, suggesting that these two fragments may in fact be identical. Indeed, the single *EcoRI* site at position 1881 in the sequence of the 23S rRNA gene coincides with one end of the inserted DNA in clone t56 (Fig. 1B). Finally, no *HincII* sites have been observed within the 5 kb of sequence data available in this region, implying that the entire 23S-5S unit as well as the *fmt* gene is contained within the 8.5- to 9.0-kb *HincII* genomic fragment. This suggests that the 23S rRNA gene is present in the genome in only one copy, as is the 16S rRNA gene (9).

**Restriction digest analysis.** To gain evidence for the order of genes observed, the filters were also subject to hybridization with probes specific for *fmt* and *orf(rrs-3')*. The *fmt*-specific probe was derived from a 1.2-kb fragment obtained by digestion of clone t56 with *BglII* and *XhoI* (Fig. 2C). The *orf(rrs-*

*3')*-specific DNA fragment was generated by digestion of clone r2w12 with *EcoRV* and *BamHI* (Fig. 2B). The 1.8-kb fragment harboring the 3' half of clone r2w12 was separated electrophoretically from the 1.2-kb 5' fragment prior to labelling. The identities of the *fmt* and *orf(rrs-3')* probes were confirmed by hybridizations to digested plasmid DNAs. For example, the *fmt* probe can be seen to primarily hybridize to the 1.2-kb fragment corresponding to the 5' end of clone t56, as expected (Fig. 3E). Similarly, the *orf(rrs-3')* probe hybridizes to the 4.8-kb *EcoRV* DNA fragment but not to the 1.2-kb *EcoRV* fragment of clone r2w12 (Fig. 3D). This probe also hybridizes to the 1.8-kb fragment of clone r2, as expected, since this fragment contains the 5' region of *orf(rrs-3')*. Finally, we note that this probe hybridizes to the plasmid-containing bands in all digests, which presumably is due to contamination by a small amount of undigested plasmid DNA that comigrates with this fragment during agarose gel electrophoresis. However, it should be emphasized that *orf(rrs-3')* does not hybridize to the 0.7-kb fragment of clone r2, as does the 16S probe.

The hybridization experiments were designed as follows. After restriction enzyme digestion, each sample was run on two agarose gels and transferred to two filters. In the first round of hybridizations, the 16S probe was hybridized to one of the filters and the 23S probe was hybridized to the other. In the second round of hybridizations, the filter that had first been hybridized with the 16S probe was hybridized with the *fmt* probe and the filter that had first been hybridized with the 23S probe was hybridized with the *orf(rrs-3')* probe.

We found that *fmt* and the 23S rRNA gene are both located within similar size genomic fragments in the *EcoRI* and *HincII* digests (Fig. 3C and E), confirming the sequence data of clone t56. Similarly, we observed that *orf(rrs-3')* and the 16S rRNA gene are both located within fragments of similar sizes in the *EcoRI* and *HincII* digests (Fig. 3B and D), in accordance with the sequence data produced from clones r2 and r2w12. We note that the *EcoRI* fragment, which is more than 5 kb in size (Fig. 3B and D), is inconsistent with an organization in which the 16S rRNA gene is located less than 1 kb upstream of the 23S rRNA gene, because *EcoRI* sites have been found in the 16S rRNA gene (18) as well as in the 23S rRNA gene (16). In contrast, all four probes hybridized to different size bands in the *HindIII* digests. However, this was also expected, since our sequence data demonstrate that the 16S rRNA gene is separated from *orf(rrs-3')* by two *HindIII* sites (Fig. 1A). Similarly, we have observed five *HindIII* sites between the regions covered by the *fmt* and 23S rRNA probes (Fig. 1B). The hybridization to different size bands in the *HindIII* digest also implies that the similarities observed in the *EcoRI* and *HincII* digests cannot be explained by cross-contamination or cross-hybridization. The restriction analysis therefore strongly supports the gene orders observed in the rRNA clones isolated and sequenced.

**Linked PCR amplification.** To finally establish that *fmt* is indeed physically near the 23S rRNA gene, the region between these two genes was amplified by PCR with from 20 to 50 ng of chromosomal DNA in 50- $\mu$ l reaction mixtures with a primer from within the *fmt* gene, ot56+2 (CTAAAGCAGAAGG AAAAATT), together with a primer from within the *rnl* gene, ot56-2 (GCTTCTAGTGCCAAGGCATC). After a hot start, 30 cycles of amplification were performed in a DNA thermal cycler (AMS Biotechnology, Taby, Sweden) with a standard profile of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 to 2 min. This primer pair should, according to sequence analysis, yield a product of 1.0 kb, and this was in fact observed when clone t56 was used to drive the PCR (Fig. 4). When this primer pair was

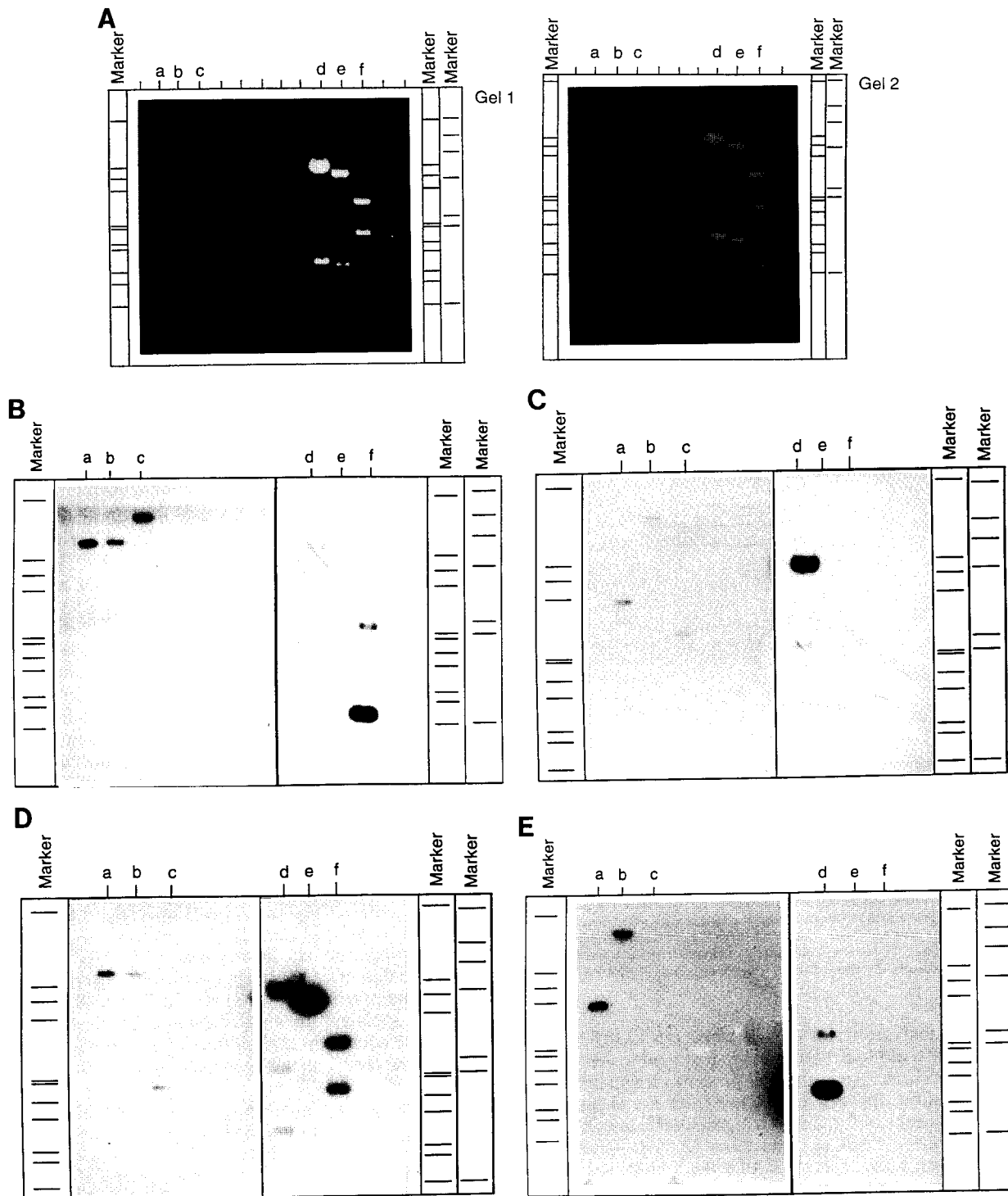


FIG. 3. Restriction enzyme analysis of the region of the *R. prowazekii* chromosome containing the 16S and the 23S rRNA genes. (A) The two agarose gels before blotting. (B to E) Hybridization signals generated with the 16S probe on filter 1 (B), the 23S probe on filter 2 (C), the *orf(rsr-3')* probe on filter 2 (D), and the *fml* probe on filter 1 (E). Lanes: a to c, single digestions of chromosomal DNA with the enzymes *EcoRI*, *HincII*, and *HindIII*, respectively; d, digestion of clone t56 with the enzymes *BglII* and *XhoI*; e, digestion of clone r2w12 with the enzyme *EcoRV*; f, digestion of clone r2 with the enzymes *XhoI* and *NotI*. Molecular markers were based on digests of lambda DNA with the enzymes *HindIII* and *EcoRI* (left lane) and with the single enzyme *HindIII* (right lane). These digests produced the following sizes of bands (in kilobases: left and right lanes, 21.2, 5.1, 5.0, 4.3, 3.5, 2.0, 1.9, 1.6, 1.4, 1.0, 0.8, and 0.6; right lane, 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6. The left halves of panels B to E were subjected to a longer exposure than the right halves.



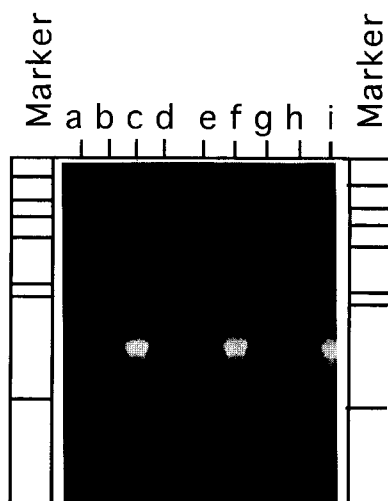


FIG. 4. PCR analysis. Primers ot56-2 and ot56+2 were used in combination with genomic DNA from *R. prowazekii* isolated in H. Winkler's laboratory, Mobile, Ala. (lane c), with genomic DNA from *R. prowazekii* isolated in D. Raoult's laboratory, Marseille, France (lane f), and with plasmid DNA from clone t56 (lane i). Lanes a, b, d, e, g, and h are water controls. Molecular markers were based on digests of lambda DNA with the single enzyme *Hind*III, producing the following sizes of bands (in kilobases): 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6.

used in combination with genomic DNA from *R. prowazekii*, isolated and purified independently from two different sources, a product of exactly the same size was obtained in both cases (Fig. 4).

**Conclusions.** It has been shown previously that there is only one gene for 16S rRNA in *R. prowazekii* (9). Here we suggest that there is only one 23S rRNA gene and that the rRNA genes are not arranged in the typical eubacterial operon. Furthermore, we show that the sequences flanking the 16S and 23S rRNA genes are very different from the spacer sequences normally found between these two genes. Our interpretation is that multiple copies of the rRNA genes serve as sites of intragenomic recombination that leads both to loss of genetic sequences and rearrangement of gene orders. Elsewhere, we will show that the elongation factor EF-Tu- and EF-G-encoding genes are also found in arrangements that are not typical for eubacterial genomes (16a). In particular, there is only one gene encoding EF-Tu in *R. prowazekii*, and the sequences flanking this *tuf* gene suggest that there has been a recombination event between the *tufA* and *tufB* genes in an ancestor of the rickettsiae associated with deletion of one *tuf* gene (16a). All of these observations suggest that the genome of *R. prowazekii* has undergone extensive deletion after intrachromosomal recombination at repeated genes in an ancestor genome that was originally more typical of the bacteria.

**Nucleotide sequence accession number.** The nucleotide sequence data downstream of the 16S rRNA gene and upstream of the 23S rRNA gene reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession numbers Z49076 and Z49077, respectively. The 16S and the 23S-5S rRNA gene sequences are associated

with the accession numbers M21789 (18) and U11018 (16), respectively.

We thank David Wood and Rebecca Waite for the preparation of the Lambda library and Asfar Jamal for library screening with the total RNA probe. We are grateful to Didier Raoult for the generous gift of genomic DNA from *R. prowazekii*.

This work was supported by grants from the Natural Sciences Research Council, Swedish Cancer Society, The Knud and Alice Wallenberg Foundation (C.G.K.), and the National Institute of Allergy and Infectious Diseases through Public Health Service grant AI-15035 (H.H.W.).

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