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Novel Arrangement of rRNA Genes in *Mycoplasma gallisepticum*: Separation of the 16S Gene of One Set from the 23S and 5S Genes

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Large restriction fragments from the DNA of *Mycoplasma gallisepticum* S6 and PG31, which were prepared by digestion with *Bgl*I, *Bss*HII, *Sma*I, or *Xho*I and which were separated by pulsed-field electrophoresis, were hybridized with probes containing most, or different parts, of an rRNA operon of *Mycoplasma capricolum*. The results showed that the genomes contained three widely separated rRNA loci. One locus contained genes for all three rRNA species and another contained 23S and probably 5S rRNA genes, whereas the third appeared to have only a 16S rRNA gene.

Members of the class *Mollicutes*, which have relatively small genomes, have been shown to have only 1 or 2 copies of rRNA genes compared with 5 to 10 copies in other procaryotes (8). As noted by Razin and Yogev (9), previous data indicated that the rRNA genes in all the *Mollicutes* tested are linked in the classic order found in procaryotes, i.e., 5′-16S-23S-5S-3′. More recently, an exception to this indicated arrangement has been noted for the single-copy rRNA genes of *Mycoplasma hyopneumoniae* (12), in which the 16S and 23S rRNA genes are contiguous, but the 5S rRNA gene is separated from them by at least 4 kilobase pairs (kb).

This documentation of a separation of one of the rRNA genes from the other two of a set suggests a reinterpretation of observations (1, 9) on the reaction of *Eco*RI and *Bgl*II digests of *Mycoplasma gallisepticum* DNA when pMC5 is used as a probe. The recombinant plasmid pMC5 has an insert of *Mycoplasma capricolum* DNA containing the entire 23S and 5S rRNA genes plus most of the 16S rRNA gene (2). It reacted with three fragments of 8.0, 8.2, and 9.0 kb in an *Eco*RI digest of DNA from *M. gallisepticum* A5969 (1). Probing of the *Eco*RI digests with cloned DNA corresponding to different regions of the *Escherichia coli rrnB* operon indicated that one of the bands contained 16S, 23S, and 5S genes; another contained the 16S genes; and the third contained 23S and 5S genes. The simplest interpretation (1) of the data was that the genome of *M. gallisepticum* contains two operons which differ somewhat in their nucleotide sequence so that one contains an *Eco*RI site, presumably at the 16S region. An alternative explanation is that the 16S rRNA gene from one set is separated from the 23S and 5S genes, whereas all three genes are closely linked in the other set. We examined this possibility by using pMC5 (kindly made available by S. Razin) as a probe to locate rRNA genes on large restriction fragments that were prepared by digesting *M. gallisepticum* DNA with infrequently cutting restriction endonucleases and that were separated by pulsed-field electrophoresis.

By using previously reported procedures from this laboratory (5–7), restriction fragments of *M. gallisepticum* DNA were prepared and digested in low-melting-point agarose and were separated by field-inversion gel electrophoresis (3) that was controlled with a pulse programmer (FIGET; Acronym Pty. Ltd., Boronia, Victoria, Australia). Strains S6 and PG31 (kindly supplied by K. Whithear, Department of Veterinary Paraclinical Sciences, University of Melbourne, Parkville, Victoria, Australia) were used. Restriction endonucleases giving a suitably small number of fragments included *Bgl*I, *Sma*I, and *Xho*I (supplied by Boehringer-Mannheim, Sydney, New South Wales, Australia) and *Bss*HII (supplied by New England BioLabs, Inc., Beverly, Mass.) and used according to the instructions of the manufacturers. The gels were stained with ethidium bromide and were photographed on a UV transilluminator to locate the fragment bands. To detect the fragments containing rRNA genes, the fragments were fixed to hybridization transfer membranes [GeneScreen Plus; Du Pont (Australia), Sydney, New South Wales, Australia] by alkaline Southern transfer (11), and the membranes were probed with ^{32}P-labeled pMC5 as described previously (7, 10). Figure 1A shows the patterns of fragment bands on the gels. The *Bgl*I digests, which were on another gel with a closely similar movement of the marker DNA bands, are not shown. Estimates of fragment sizes to the nearest 5 kb gave values of 375, 290, 140, 130, 55 (two fragments), and 35 kb for *Sma*I digestions of S6 DNA and 405, 250, 105 (two fragments), 75 and 40 (two fragments) kb for those digestions of PG31 DNA. A 10-kb fragment was also detected in *Sma*I digests of S6 DNA separated by ordinary agarose gel electrophoresis. The totals of 1,090 kb for S6 and 1,020 kb for PG31 fragments suggest that the genome sizes were close to that of 1,050 kb reported previously (5) for PG31 from estimates of the size of the genome as a single fragment obtained by comparison of its mobility with those of yeast chromosomal DNA markers by pulsed-field gel electrophoresis. Reliable estimates of the genome size from the other digestions was made difficult by the occurrence of many bands and of overlapping bands.

Figure 1B shows the results that were obtained after autoradiography of the probed membranes. For S6, each of the four digests showed three bands that hybridized with pMC5. For PG31, the *Bgl*I and *Sma*I digests showed three reacting bands in Fig. 1B, whereas the *Bss*HII and *Xho*I digests showed only two bands each. However, for both of the latter two digests, the smaller of the two reacting bands appeared to be a doublet from the intensity of its staining with ethidium bromide (data not shown) and from the intensity of its reaction with pMC5 in Fig. 1B. Confirmation that this band was a doublet in the *Bss*HII digest came from the demonstration that it was split into two reacting bands under different electrophoresis conditions. Further evidence

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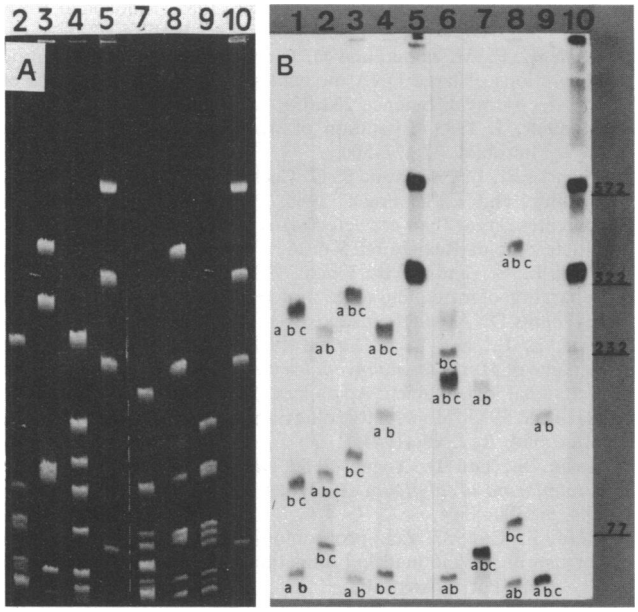

FIG. 1. Hybridization of restriction fragments of *M. gallisepticum* S6 and PG31 with probes containing sequences for various rRNAs. Fragments in digests of intact genomic DNA prepared in agarose blocks were separated by field-inversion gel electrophoresis and then bound to nylon membranes by alkaline Southern transfer. The membranes were autoradiographed after they were probed with ^{32}P-labeled DNA of plasmid pMC5 containing coding sequences for 16S, 23S, and 5S rRNAs. They were then stripped of the probe and the probings were successively repeated with subfragments of the plasmid containing part or complete sequences for 16S only (a), 16S plus some 23S (b), and 23S plus 5S (c). (A) Gel stained with ethidium bromide (the *Bgl*I digests were on a separate gel [data not shown]). (B) Autoradiographs from reaction of the membranes with pMC5. The letters a, b, and c are used to indicate those subfragments from pMC5 that hybridized to the different reactive bands from S6 DNA. Lanes 1 to 4, DNA of S6 digested with *Bgl*I (lane 1), *Bss*HII (lane 2), *Sma*I (lane 3), and *Xho*I (lane 4); lanes 5 and 10, DNA of *M. mycoides* subsp. *mycoides* Y digested with *Xho*I; lanes 6 to 9, DNA of PG31 digested with *Bgl*I (lane 6), *Bss*HII (lane 7), *Sma*I (lane 8), and *Xho*I (lane 9). The positions and sizes (in kilobase pairs) of the fragments in the *M. mycoides* digest (7) are marked on the right margin of panel B.

that these strongly reacting bands in both the *Bss*HII and the *Xho*I digests contained two restriction fragments came from the observation of three reacting bands after digestions (double digests) with an additional restriction endonuclease. For both strains, probing of double digests from various combinations of the four endonucleases never showed more than three reacting bands. This observation, together with the pattern of change in the sizes of the reacting fragments between single and double digestions (shown for strain S6 in Table 1), excludes the possibility that any of the restriction enzymes had cleavage sites within the regions that hybridized with pMC5. Thus, the occurrence of three, rather than two, reacting fragments in a digest did not result from the cleavage of an rRNA operon by any of the enzymes. The data thus indicate that there are three loci for rRNA in the *M. gallisepticum* genome. The data from double digestions of S6 shown in Table 1 require that no pair of loci have a separation of less than 80 kb.

In their detection of three bands that hybridized with pMC5 in *Eco*RI digests of DNA from *M. gallisepticum* A5969, Amikam et al. (1) observed that the middle-sized band gave the weakest response. This was the band that responded only to the 16S rRNA gene sequences. The weakness of its response to pMC5 is consistent with the absence of some of the 16S sequence from the insert in this plasmid. The smallest band hybridized with all three rRNA gene sequences as probes, and the largest band hybridized with 23S and 5S, but not 16S, rRNA sequences. For *M. gallisepticum* S6 DNA, Amikam et al. (1) reported that only one *Eco*RI fragment hybridized with pMC5 and the other rRNA gene probes that were used. However, with our strain of S6 we found the pattern in the response of *Eco*RI fragments to by very similar to that reported for A5969 (1). Three bands (corresponding to sizes of 8.2, 8.4, and 8.8 kb) responded, with the middle-sized band again giving the weakest response and the smallest band giving the strongest response. Differences in the intensities of the responses of the three bands in a digest were also generally observed with the large restriction fragments separated by pulsed-field electrophoresis (Fig. 1B) for both S6 and PG31 DNAs. The faint band with the lowest mobility appearing in the *Bgl*I digest of PG31 DNA (Fig. 1B, lane 6) was a partial digestion product.

TABLE 1. Restriction fragments of *M. gallisepticum* S6 DNA reacting with probes for rRNA genes

Restriction endonuclease(s)	Fragment size (kb)[a]		
	A	B	C
*Bgl*I	270	40	115
*Bss*HII	120	250	55
*Sma*I	290	35	140
*Xho*I	250	165	40
*Bgl*I + *Bss*HII	55	40	55
*Bgl*I + *Sma*I	220	20	70
*Bgl*I + *Xho*I	220	5	40
*Bss*HII + *Sma*I	115	35	55
*Sma*I + *Xho*I	245	25	40

[a] Fragments sizes are estimated to the nearest 5 kb. Single digest fragments listed under A, B, and C correspond, respectively, to those labeled abc, ab, and bc for the S6 digests shown in Fig. 1B. The double-digest fragments derived from DNA within the appropriate single-digest fragments are listed in the same column.

To explore the basis of these differences in intensities between bands, we used the three *Pst*I restriction fragments (2) from pMC5 as probes. We designated these as a, b, and c; and they contained, respectively, sequences corresponding to parts, or all of, 16S rRNA, 16S and 23S rRNAs, and 23S and 5S rRNAs. The S6 DNA bands that were shown to respond to pMC5 DNA are marked in Fig. 1B with these designations to indicate those pMC5 fragments that hybridized to them. The fragment bands fell into three classes: those that reacted with a and b; those that reacted with a, b, and c; and those that reacted with b and c. These correspond, respectively, to the classes which slowed a weak, strong, or intermediate reaction with pMC5. The PG31 digestion fragments which reacted with pMC5 fell into the same three classes, except that the class that reacted with b and c was not observable in the *Bss*HII and *Xho*I digests. Evidence indicates that it is included in the doublet bands along with the fragment that reacted with a, b, and c. From these data and their analogy with the data of Amikam et al. (1) for A5969 DNA, it appears that the restriction fragments in the three classes contain loci coding for 16S rRNA; 16S, 23S, and 5S rRNAs; and 23S and 5S rRNAs, respectively. The data on the hybridization of pMC5 to restriction fragments of DNA from other *M. gallisepticum* strains (1, 9) are

also consistent with the possession by those strains of a similar arrangement of rRNA loci.

From data on rRNA sequences, mycoplasmas have been postulated to have arisen by degenerative evolution from more complex gram-positive eubacterial forms (4). This evolution would require genomic rearrangements or deletion of much of the eubacterial genome, including the loss of several loci coding for rRNA. From the examples of *M. hyopneumoniae* (10) and now *M. gallisepticum*, these rearrangements can also include disruption of the coding pattern for all three rRNA species within the same operon. The operon is transcribed with the usual pattern from promoters preceding the 16S gene. It is not known whether the changed arrangement for one set of rRNA genes in *M. gallisepticum*, with 23S and 5S genes far removed from the 16S rRNA, still permits the full expression and function of the set. It would be of interest to know this and whether the dispersal of the loci can offer some selective advantage.

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