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Characterization of the Mycoplasma Genome

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Recent advances on the properties of the mycoplasma genome, including size, base composition, replication, extrachromosomal DNA, and transfer of genetic material are briefly reviewed, with emphasis on their phylogenetic implications. The use of cleavage patterns of the mycoplasma genome by restriction endonucleases as "finger-prints" indicating genetic relatedness among strains is discussed. The data support the notion that strains of mycoplasma species of strict host and tissue specificity exhibit marked genetic homogeneity, suggesting a clonal origin for some species. The regions of the mycoplasma genome carrying the ribosomal RNA (rRNA) genes have been studied using restriction endonucleases, cloning, and hybridization procedures. The mycoplasmal rRNA cistrons cross-hybridized among themselves, and with the seven rRNA cistrons of *Escherichia coli,* demonstrating the marked conservation of structure during evolution of this part of the procaryotic genome. In most of the mollicutes tested so far the number of rRNA cistrons is two, but a few species appear to carry only one rRNA cistron in their genome.

INTRODUCTION

The genome of *Mollicutes* is distinguished by its small size and low guanine + cytosine (G + C) content. According to genome size all the mollicutes examined thus far fall into two clusters: one comprising *Mycoplasma* and *Ureaplasma* species with a genome of about 5×10^8 daltons, and the other of *Acholeplasma* and *Spiroplasma* species, with a genome about twice as large (Table 1). The wall-less *Thermoplasma acidophilum* resembles the *Acholeplasma-Spiroplasma* cluster in genome size. However, the *Thermoplasma* gene differs in having a significantly higher G + C content and a histone-like protein, in line with the recent inclusion of this organism in *Archaebacteria* [17]. Based on the gap in genome size between the two clusters, Morowitz and Wallace [4] proposed that the existing *Mycoplasma* and *Ureaplasma* species represent the descendants of the organisms ("protocaryotes") which preceded the procaryotic-eucaryotic cell split, and that evolution occurred through DNA doubling, leading to the 1×10^9 daltons of the *Acholeplasma-Spiroplasma* genome. Accordingly, the acholeplasmas and spiroplasmas may be regarded as intermediates in the evolution from protocaryotes to wall-covered bacteria. Although this hypothesis has been challenged [18], new data on genome

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TABLE 1
Genome Size of Mollicutes and Several Representative Procaryotes

Organisms	Genome Size ($\times 10^8$ daltons)	Method of Determination	References
MYCOPLASMA			
M. agalactiae PG2	4.7 ± 0.3	DNA renaturation	[1]
M. arginini G230	4.0 ± 0.3	DNA renaturation	[1]
M. arthritidis (formerly *M. hominis*) H39	5.1	Electron microscopy	[2]
PG6	4.4	DNA renaturation	[3]
M. bovigenitalium PG11	4.0 ± 0.3	DNA renaturation	[1]
M. bovirhinis PG43	4.4 ± 0.2	DNA renaturation	[1]
M. bovis (Donetta)	4.4 ± 0.1	DNA renaturation	[1]
(Donetta)	5.8	Electron microscopy	[4]
M. capricolum kid	6.8	Electron microscopy	[5]
M. dispar 462/2	5.3 ± 0.4	DNA renaturation	[1]
M. fermentans PG18	4.8	DNA renaturation	[3]
M. gallisepticum PG31	4.9	DNA renaturation	[3]
M. hominis PG21	4.5	DNA renaturation	[3]
M. hyorhinis ATCC 25021	5.4	Electron microscopy and restriction fragments	[6]
M. hyorhinis	4.4	Electron microscopy	[7]
M. meleagridis 529	4.2 ± 0.5	Electron microscopy and DNA renaturation	[8]
M. mycoides subsp. *capri* PG3	5.0 ± 0.3	DNA renaturation	[1]
M. mycoides subsp. *mycoides* PG1	5.0 ± 0.1	DNA renaturation	[1]
PG1	5.7(5.2–6.0)	DNA renaturation	[9]
M. orale Patt	4.7	DNA renaturation	[3]
M. pneumoniae Mac	4.8	DNA renaturation	[3]
M. salivarium PG20	4.7	DNA renaturation	[3]
VV	4.2	DNA renaturation	[10]
Group L (Al Aubaidi) B144P	4.9 ± 0.4	DNA renaturation	[1]
Group 7 (Leach) PG50	5.6 ± 0.2	DNA renaturation	[1]
M. alkalescens PG51	4.9 ± 0.3	DNA renaturation	[1]
M. gatae (formerly Group H, Al Aubaidi B139P)	4.4 ± 0.4	DNA renaturation	[1]
M. gallinarum (formerly Group I, Al Aubaidi B142P)	4.8 ± 0.1	DNA renaturation	[1]
UREAPLASMA			
U. urealyticum (T-strain no. 27, Ford)	4.7	DNA renaturation	[3]
(T-strain, no. 58, Ford)	4.4	DNA renaturation	[3]
ACHOLEPLASMA			
A. axanthum (Bovine K, B107PA)	10.7(8.5–12.7)	DNA renaturation	[11]
B107PA	9.9 ± 0.8	DNA renaturation	[1]
A. granularum (Friend)	9.5	DNA renaturation	[3]
A. laidlawii A (F1,PG8)	11.0	DNA renaturation	[3]
PG8	10.9(9.1–13.3)	DNA renaturation	[9]
B,F8	10.0	DNA renaturation	[3]
A	11.1(9.7–12.4)	DNA renaturation	[11]
A. modicum PG49	9.9 ± 1.3	DNA renaturation	[1]

TABLE 1—*Continued*

Organisms	Genome Size ($\times 10^8$ daltons)	Method of Determination	References
SPIROPLASMA			
S. citri Maroc R8A2	10.0(8.0–11.6)	DNA renaturation	[11]
California	10.1(8.7–13.2)	DNA renaturation	[11]
	9.4(8.0–12.1)	DNA renaturation	[9]
S. citri group IB(AS576 honeybee)	12.0	DNA renaturation	[12]
S. citri group IC(I-747 corn stunt)	9.0	DNA renaturation	[12]
S. floricola 23-6	17.0	DNA renaturation	[12]
	10.9(9.5–13.0)	DNA renaturation	[9]
Flower spiroplasma SR3	14.0	DNA renaturation	[12]
Flower spiroplasma serogroup IV (PPS1)	10.1(8.6–13.0)	DNA renaturation	[9]
Thermoplasma acidophilum 122-1B2(ATCC 25905) 122-1B3	9.4–10.0	DNA renaturation	[13]
OTHER PROCARYOTES			
Pseudomonas aeruginosa (NCTC 7244)	69.6 ± 12.0	DNA renaturation	[14]
Escherichia coli B (MMCA 56)	28.4 ± 1.7	DNA renaturation	[14]
Haemophilus influenzae (MMCA 29)	10.1 ± 1.8	DNA renaturation	[14]
Rickettsia quintana Fuller	10.1 ± 0.03	DNA renaturation	[15]
Chlamydia trachomatis	6.6	Electron microscopy	[16]

size in mollicutes still support the existence of the gap by failing to reveal mollicutes with genome sizes within the range of 5×10^8 to 1×10^9 daltons (Table 1). Nevertheless, it must be stressed that genome size data are available only for a minority of the established species, so that the possibility of finding mollicutes with intermediate genome sizes should not be ruled out.

The generalization that the genome of mollicutes is poor in G + C is based on data available for almost all of the established species [19] and determination of the DNA G + C content has been included among the obligatory tests in the definition of new species [20]. The highest G + C content, found in *M. pneumoniae* and in some anaeroplasmas, is about 40 mol%, while in all other mollicutes the values range between 25 and 35 mol%. As in other procaryotes, some of the adenine residues in the mycoplasmal DNA are methylated, though the extent of methylation in the *M. capricolum* DNA is definitely lower than in other bacteria [21]. The different extent of adenine methylation noted among the mycoplasmas tested and the finding of both 6-methyl adenine and 5-methyl cytosine in *M. hyorhinis* can be added to the many findings indicating the wide phylogenetic diversity in mollicutes. Since eucaryotic DNA does not contain 6-methyl adenine, the detection of this methylated base in cell cultures may serve as an indicator for contamination by mycoplasmas [21].

The replication of the mycoplasma genome resembles that of other procaryotes in

being semiconservative. The need for DNA polymerases in this process is self-evident, and some new data have recently been reported on the properties of DNA polymerases of *S. citri* [22] and *M. orale* [23], supplementing the earlier study of Mills et al. [24] on the DNA polymerases of *M. hyorhinis* and *M. orale.* The absence of 3′→5′ exonuclease activities in the purified enzyme preparations from the mycoplasmas distinguishes them from the DNA polymerases of other procaryotes. In this respect the mycoplasma enzymes resemble eucaryotic DNA polymerases.

Although there are strong indications for the presence of extrachromosomal DNA in mollicutes [25,26], little progress has been made in this field. The most significant findings were achieved with spiroplasmas, in which the presence of plasmids is apparently rather common [25,27]. The number and size of the plasmids detected varied according to the spiroplasma examined. Plasmid size ranged between 1.5 to 40 kilobase (kb) pairs. All spiroplasma plasmids studied to date are cryptic, and no reports are available on transfer of plasmids between spiroplasma strains. Only one report is available on plasmid curing by continuous subculture of the spiroplasma, but with no loss of any known phenotypic characteristic [27]. Obviously, characterization of mycoplasmal plasmids may be most rewarding, with prospects of developing them into specific and efficient cloning vehicles in mycoplasmas. The first stride towards this aim was recently accomplished by Archer et al. [27] who mapped, by restriction endonucleases, a spiroplasma plasmid pIJ2000, with a circular DNA of approximately 7.8 kb.

The lack of cell walls in mollicutes would be expected to facilitate genetic transformation by direct transfer of chromosomal DNA from one mycoplasma to another. However, early trials met with failure [26]. Two recent reports [28,29] on successful transformation of tetracycline-sensitive *M. hominis* and *M. salivarium* strains to resistance by DNA extracted from a tetracycline-resistant *M. hominis* are therefore of considerable interest, particularly as these authors argue against the involvement of plasmids in this transfer. Nevertheless, the conditions for rendering the cells competent and the method of DNA purification are factors which, according to Cerone-McLernon and Furness [28], should be checked very carefully, indicating the difficulties inherent in this approach.

The recent application of the powerful tools of genetic engineering to studies of the mycoplasma genome has already produced some interesting data. The rest of this contribution will deal with these findings, focusing on two topics: *(1)* the contribution of cleavage patterns of mycoplasmal DNA by restriction endonucleases to elucidation of the genome structure, and determining inter- and intra-species genetic relatedness, and *(2)* determination of the number and nature of ribosomal RNA cistrons in the mycoplasma genome, and the contribution of these findings to our understanding of mycoplasma phylogeny.

GENOME CLEAVAGE BY RESTRICTION ENDONUCLEASES

The idea of employing cleavage patterns of the mycoplasma genome as a taxonomic aid was first suggested by Bove and Saillard [30]. However, exploitation of this new approach lagged, probably due to the difficulties encountered in its initial application to spiroplasma strains. Many spiroplasmas harbor extrachromosomal DNA, a fact which complicates the interpretation of cleavage data [31]. Recent reports by Robberson et al. [32] and by Darai et al. [6,33] gave a boost to the restriction enzyme approach by demonstrating its usefulness in identifying mycoplasmal DNA in contaminated cell cultures.

Our studies [34–36] have been directed to evaluate critically the potential of restriction endonucleases as tools in determining genetic relatedness among strains of various *Mycoplasma, Ureaplasma,* and *Acholeplasma* species. The generally low C + G content of mycoplasmal DNA proved an advantage, as restriction endonucleases with recognition sites rich in G + C cleave the mycoplasma genome at relatively few sites (Table 2). As a result, the electrophoretic patterns of the cleavage products exhibit a limited number of bands and are easier to compare.

Strains of each of the established pathogens *M. pneumoniae* and *M. gallisepticum,* and of the newly discovered and possibly pathogenic human genital mycoplasma, *M. genitalium* [37,38] exhibited remarkably similar species-specific cleavage patterns, indicating genetic homogeneity of strains within each of these three species. Thus, the cleavage patterns of the DNA from strains G-37 and M-30 of *M. genitalium* isolated from two different patients under different cultural conditions [37] showed identical patterns. Moreover, an isolate of *M. genitalium* from the urethra of a chimpanzee artificially infected with *M. genitalium* G-37 exhibited the same pattern as the original strain (Fig. 1). Hence, the *M. genitalium* strains fit well the concept of a clone, a property of great importance in the epidemiology, taxonomy, and evolution of pathogenic bacteria [39]. Despite the fact that the *M. pneumoniae* strains tested by us were isolated by different laboratories, and differed greatly in the number of *in vitro* passages, in their virulence to hamsters, and in their adherence ability, their DNA cleavage patterns proved to be remarkably similar [34,35]. These results indicate considerable genetic relatedness among strains of this pathogen, a conclusion supported by the findings that the same strains were essentially indistinguishable by standard serological techniques and by electrophoretic patterns of their cell proteins [34]. Similar data were obtained on analysis of the DNA cleavage patterns of *M. gallisepticum* strains, pointing to the conclusion that this pathogen also represents a genetically homogenous species [36].

Somewhat different results were obtained with the human genital *U. urealyticum* strains [35]. In this case, the cleavage patterns showed that the *U. urealyticum* serovars fall into two definite clusters, one consisting of serovars 1, 3, and 6 and the

TABLE 2
Effect of G + C Content of Mycoplasmal DNA on Its Susceptibility to Cleavage by Restriction Endonucleases

		Number of Cleavage Bands Observed			
Enzyme	Recognition Sequence	*U. urealyticum* (G + C = 28 mol%)	*M. genitalium* (G + C = 32 mol%)	*M. gallisepticum* (G + C about 33 mol%)	*M. pneumoniae* (G + C = 40 mol%)
*Sma*I	CCC/GGG	0	NT	0–1	35
*Kpn*I	GGTAC/C	0–2	18	9	>50
*Xho*I	C/TCGAG	0–1	NT	1–6	ca.9
*Bam*HI	G/GATCC	8	12	19	ca.30
*Pst*I	CTGCA/G	6–16	>40	>40	ca.45
*Eco*RI	G/AATTC	>50	NT	NT	>50
*Hind*III	A/AGCTT	>50	NT	NT	>50
*Hpa*I	GTT/AAC	>50	>50	>50	>50
*Xba*I	T/CTAGA	ca.30	NT	NT	ca.35

NT = Not tested

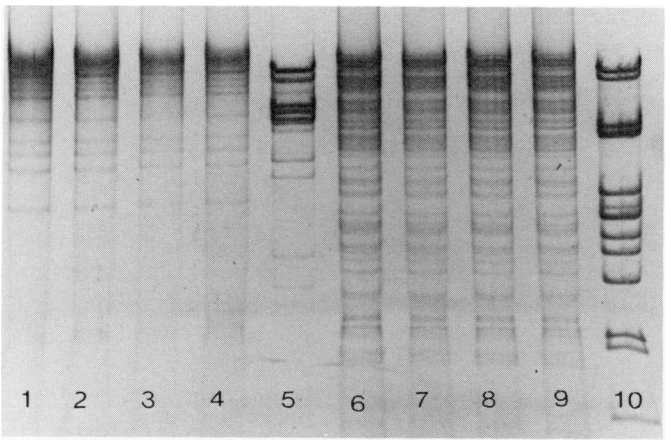

FIG. 1. Cleavage patterns of the DNA of *M. genitalium* strains and of phage lambda DNA by *Bam*HI (lanes 1–5) and by *Pst*I (lanes 6–10). The strains tested: G-37, passage 17 (lanes **1** and **6**); G-37, passage 8 (lanes **2** and **7**); G-37 isolated from the urethra of an experimentally infected chimpanzee (lanes **3** and **8**); M-30, passage 4 (lanes **4** and **9**); lambda phage DNA (lanes **5** and **10**).

other of 2, 4, 5, 7, 8, and 9, in agreement with results obtained by electrophoresis of cell proteins [40] and DNA hybridization [41]. The questions of whether the two *U. urealyticum* clusters represent two different species or subspecies of the same species requires consideration of all available serological and biochemical data, in view of the ill-defined species concept on procaryotes. It would be helpful to find out whether or not the two clusters differ in pathogenicity, as this property may considerably influence the decision on this question.

As against the pronounced genetic homogeneity of the *Mycoplasma* species tested by us, the DNA cleavage patterns of nine *A. axanthum* strains, isolated from a variety of hosts and habitats, differed markedly from each other, indicating their considerable genotypic heterogeneity [36]. This finding is consistent with recent DNA-DNA hybridization data showing DNA homology values ranging from 48 to 100 percent among *A. axanthum* strains [42]. Heterogeneity was less marked among the *A. oculi* tested by the restriction endonuclease technique [36]. All the above data support the notion that genetic heterogeneity among strains of the same species is associated with their recovery from diverse habitats [42]. Accordingly, adaptation of an organism to a new host may have pressured it to change genotypically in order to survive.

TABLE 3
Merits and Deficiencies of the Restriction Enzyme Approach

Merits	Deficiencies
1. A few μg of DNA suffice for test.	1. DNA has to be pure and undegraded by endogenous nucleases.
2. No need for labeling the DNA	2. Presence of extrachromosomal DNA (plasmids, viruses) complicates reading of results.
3. No interference by culture medium components	3. Although cleavage patterns are adequate for determining identity, close similarity, or non-identity of strains, it is difficult to determine degree of relatedness as cleavage data are not expressed by numbers, unlike hybridization data.
4. Easy reading of results visually, or on densitometer tracings of photographs	
5. Excellent means of determining clonality of strains	
6. Means of determining mycoplasmal DNA in contaminated cell cultures	
7. Means of estimating G + C content, genome size, and possibly constructing a physical map of the genome	

As with any other method, the use of restriction endonucleases for genome analysis and mycoplasma classification has its merits and deficiencies. These are summarized in Table 3.

RIBOSOMAL RNA GENES

As discussed above, the DNA cleavage patterns as well as the DNA hybridization data provide valuable information on the overall nucleotide sequence in the mycoplasma genome, and thus serve as effective tools in determining genetic relatedness among strains. The new cloning techniques open the way for investigating specific regions of the mycoplasma genome. The first region to be studied is that coding for ribosomal RNAs (rRNAs). The reasons for this choice are obvious: rRNAs are major and ubiquitous cell products, and the genes responsible for their synthesis as well as the factors regulating their expression have been major topics for research. In addition, rRNAs are highly conserved molecules, so that variations in their nucleotide sequences may have phylogenetic implications [18].

We have recently shown [43] that ribosomal RNA synthesis in *M. capricolum* responds to amino acid starvation in a similar fashion to the intensively studied *E. coli* system; that is, cessation of stable RNA synthesis and accumulation of 5′-triphosphoguanosine-3′-diphosphate (pppGpp). Hence, the stringent control mechanism appears to operate in mycoplasmas. Obviously, knowledge of the mycoplasmal rRNA genes may facilitate the use of these relatively simple organisms as models in studying the genetic aspects of ribosomal RNA synthesis and its control.

Realization of the fact that rRNA cistrons are highly conserved led Ryan and Morowitz [5] to try and isolate these cistrons from *M. capricolum,* assuming that the region of the genome containing these cistrons has a much higher G + C content than the 25 mol% G + C characterizing the *M. capricolum* genome as a whole. Their assumption proved to be correct, as sonication of the DNA followed by hydroxyapatite chromatography separated a 46 mol% G + C fragment which hybridized with 16S and 23S rRNA and with tRNA. Based on the percentage of this high G + C DNA of the total *M. capricolum* DNA, Ryan and Morowitz [5] concluded that this organism contains only one rRNA cistron.

We have recently reinvestigated this problem by employing cloning techniques [44]. Using Southern blotting analysis with ^{32}P-labeled ribosomal RNA of *M. capricolum* as a probe, two fragments (1kb and 5kb) were detected in an *Eco*RI digest of the DNA of this mycoplasma. This analysis revealed that the 5kb fragment carries both 16S rRNA sequences and the entire 23S rRNA gene of this mycoplasma. The 1kb fragment contains 16S rRNA sequences only. As shown in Fig. 2 the 5kb *Eco*RI fragment was cloned in the pBR325 plasmid. The *E. coli* colonies carrying the chimera plasmids were screened by the Hogness screening technique [45], using ^{32}P-labeled *M. capricolum* rRNA. A plasmid designated pMC5 was found to carry a 5kb mycoplasma DNA insert which hybridized both with 16S and 23S rRNA. This clone was then used as a probe for analysis of other mycoplasma genomes. Hybridization of this plasmid to *Eco*RI digests of *M. mycoides* subsp. *capri* and *A. laidlawii* DNAs supported the presence of two rRNA cistrons in these mycoplasma. In our earlier experiments we failed, apparently for technical reasons, to observe the 20kb hybridization band with *M. capricolum* DNA (Fig. 2) leading to the erroneous conclusion that *M. capricolum* carries only one rRNA cistron [44]. Our new hybridization data [Amikam D, Glaser G, Razin S: to be published] support the conclusion of Sawada et al. [46] that this mycoplasma also has two rRNA cistrons.

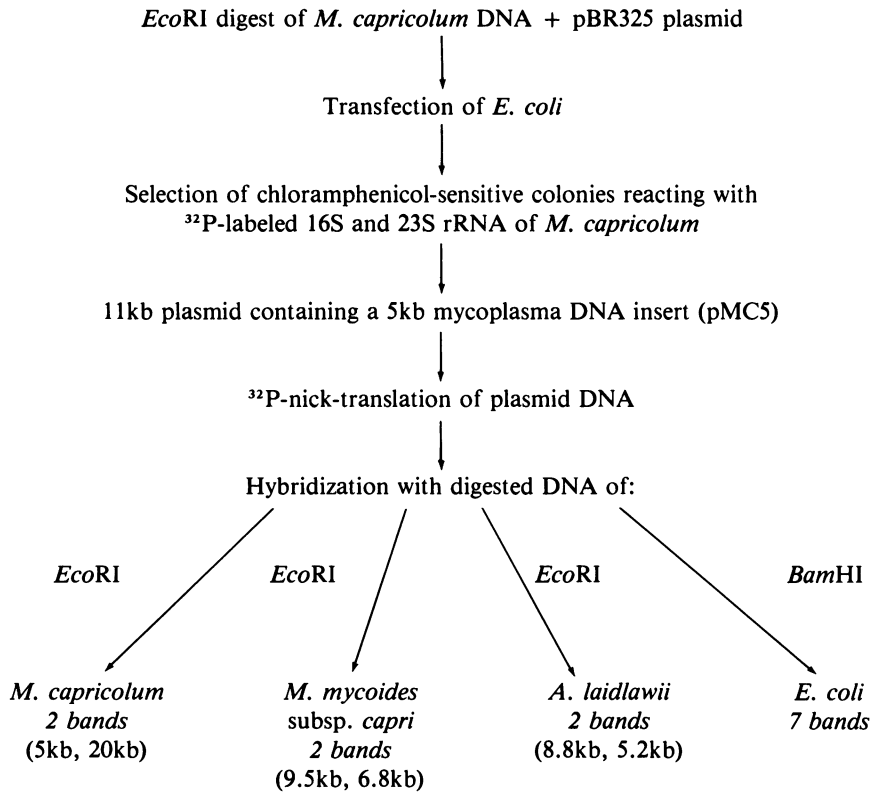

FIG. 2. Flow sheet of cloning experiments designed to determine the number of rRNA cistrons in mycoplasmas (for details see [44]).

Nevertheless, extension of our studies to a representative series of *Mycoplasma, Acholeplasma, Ureaplasma,* and *Spiroplasma* species revealed that, while most species carry two rRNA cistrons, some such as *M. orale* and several spiroplasmas contain one rRNA cistron only [Amikam D, Glaser G, Razin S: to be published]. The probe pMC5 when hybridized with *Bam*HI-digested DNA of *E. coli* produced seven bands (Fig. 2), in line with the established number of rRNA cistrons in this bacterium [46].

Additional support for the above findings comes from experiments using as a probe the plasmid pGG1 which carries the *rrn*B ribosomal promoter region and part of the 16S ribosomal gene of *E. coli*. This plasmid hybridized weakly with the 5kb *Eco*RI fragment of *M. capricolum* DNA and strongly with the 1kb fragment [44]. This suggests that the 5kb fragment, though containing most of the rRNA cistron, does not contain the promoter found apparently in the 1kb fragment together with part of the cistron coding for 16S rRNA.

The evolutionary implications of the above findings are obvious. They show that the number of rRNA cistrons in mycoplasmas is very limited, in accordance with the small genome size. The hybridization experiments reveal the structural similarity of the rRNA genes among the mycoplasma themselves and, more important, their relatedness to *E. coli* rRNA genes. These findings demonstrate the pronounced con-

servation of structure during evolution of this part of the procaryotic genome. On the other hand, the number of rRNA cistrons appears to differ in the few mycoplasmas tested so far.

It can be speculated that the number of rRNA cistrons in procaryotes is grossly proportional to the size of the genome and is influenced by their habitat. Thus, bacteria capable of growing in versatile habitats may benefit from the presence of a significant number of rRNA cistrons, as these amplify RNA synthesis, enabling fast growth whenever possible. On the other hand, the needs of the slow-growing parasitic mycoplasmas, well adapted to constant and secure ecological niches, are satisfied by only one or two rRNA cistrons.

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