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Evolution of Mycoplasmas and Genome Losses

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Streptococci and acholeplasmas have a close evolutionary relationship. We examined their genomes to determine what chromosomal losses occurred to produce the smaller acholeplasma genomes and found by RNA-DNA hybridization that *Streptococcus cremoris* and *S. lactis* possess at least five or at least six ribosomal RNA gene sets, respectively, while acholeplasmas have only two rRNA gene sets. Other important deficiencies identified in mycoplasmas are associated with envelope or RNA genes, and analysis of these chromosomal deletions may lead to an understanding of how mycoplasmas evolved from walled bacteria.

INTRODUCTION

Previously we have shown that mycoplasmas evolved from various bacteria and particularly that the sterol non-requiring mycoplasmas (acholeplasmas) are phylogenetically related to streptococci and are descended from streptococci. Consequently, the mycoplasmas do not constitute a true phylogenetic class, but rather are an assemblage of diverse wall-less bacteria [1,2]. Our conclusion that mycoplasmas evolved from bacteria and are not a true phylogenetic class is supported by the work of Woese and co-workers [3].

On the basis of physiological properties [1], lipid composition [4,5] and 16S rRNA sequences [3], several other mycoplasmas as well would appear to be descendants of gram-positive bacteria. However, whether all mycoplasmas descended from gram-positive bacteria and consequently only gram-positive bacteria had the capacity to give rise to mycoplasmas remains to be determined, because these characters have not been examined in non-fermentative mycoplasmas.

Although acholeplasmas are closely related to streptococci and have retained many of their characteristics, it is evident that acholeplasmas are radically altered from streptococci. Because of the close phylogenetic relationship demonstrated between streptococci and acholeplasmas, it becomes possible now to analyze this progenitor-descendant relationship by examining the differences between the acholeplasmas and their specific bacterial antecedents—the streptococci. It also becomes possible now to formulate questions concerning the evolutionary mechanisms by which mycoplasmas evolved from their progenitor bacteria.

We will focus our discussion here on chromosomal changes in mycoplasmas. Comparison of the chromosome sizes of acholeplasmas (1×10^9 daltons) and the known values for streptococci which range from 1.2×10^9 to 1.4×10^9 daltons [6,7] indicates that acholeplasma genomes are approximately 20 to 30 percent smaller than streptococcal genomes and consequently large genome losses must have occurred in

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the transition from streptococci to acholeplasmas. Some important questions are: *(1)* What genome losses have occurred to produce the smaller genome mycoplasmas? *(2)* Were the losses scattered around the chromosome or were losses mainly in certain chromosome segments? *(3)* What mechanism(s) brought these changes about? *(4)* Why do all known mycoplasma genome sizes cluster closely near the two values 5×10^8 and 1×10^9 daltons rather than forming a range of genome sizes and is the separation in the size of these values by a factor of two significant? These questions can be examined directly by using gene probes to examine and compare the chromosome organization of mycoplasmas and their bacterial progenitors.

MATERIALS AND METHODS

Mycoplasma and streptococcal strains, cultivation conditions and immunological methods [2], and enzyme assays [8] were described previously. DNA was isolated as described [9]; ribosomal RNA was extracted from ribosomes, purified by electrophoresis, partially hydrolyzed, and 5'-labeled with (γ-^{32}P) ATP with T4 polynucleotide kinase [10]. Total chromosome DNA was cleaved with restriction endonucleases according to the conditions described by the supplier (New England Biolabs, Beverly, MA, or BRL, Gaithersberg, MD). The resulting DNA fragments and lamda phage DNA molecular weight standards were separated in horizontal 0.7 percent agarose gels in 0.04 M Tris-0.02 M Na acetate buffer-2 mM Na_2EDTA and transferred to nitrocellulose paper by the method of Southern [11]. Hybridizations described here were carried out with *S. cremoris* ^{32}P-5'-labeled 5S, 16S, or 23S rRNA probes at 37°C in 4 × SSC-50 percent formamide-0.4 percent SDS for 16 hours. Filters were washed at 22°C in 2 × SSC, treated with 10 μg heated pancreatic RNAse per ml, washed in 0.4 percent SDS-2 × SSC and autoradiographed.

RESULTS

The rRNA genes in acholeplasmas and streptococci were examined and compared by hybridizing ^{32}P-5'-labeled *S. cremoris* 5S, 16S, and 23S rRNA to southern blots of restriction fragments obtained from whole chromosomes digested with various restriction endonucleases. For purposes of comparing acholeplasma rRNA genes, the most useful digests were obtained with the endonucleases Eco RI, Hind III, and Bgl II, all of which recognize AT rich sequences. Mycoplasmas generally have relatively low GC contents [9], and it would be expected that restriction endonucleases that recognize GC rich DNA sequences would cleave relatively few sites in mycoplasma genomes. Nevertheless, acholeplasma DNAs were treated with several such enzymes, with the view that there could be fortuitous sequences in the chromosome that could provide fragments useful for the analysis of the acholeplasma chromosomes. The enzymes BamH I, Xho I, and Sma I indeed did produce mainly a smear of very large fragments which were not useful for rRNA gene analysis. However, certain acholeplasma DNAs in addition to the smear of large fragments produced a small number of bands which formed a pattern characteristic for that species. For example, *A. hippikon* DNA produced six well-separated, characteristic bands in BamH I digests and such distinct digest fragments may prove useful in genome analysis. Interestingly, some of these bands matched in size the bands from *S. lactis* BamH I digests.

The number of rRNA gene sets was determined by counting the fragments which hybridized to 5S, 16S, and 23S rRNA. Five *Acholeplasma* species have been examined and, in each DNA digest, two DNA restriction fragments reacted with all three rRNA probes. Results are shown for three species, digested with each of two

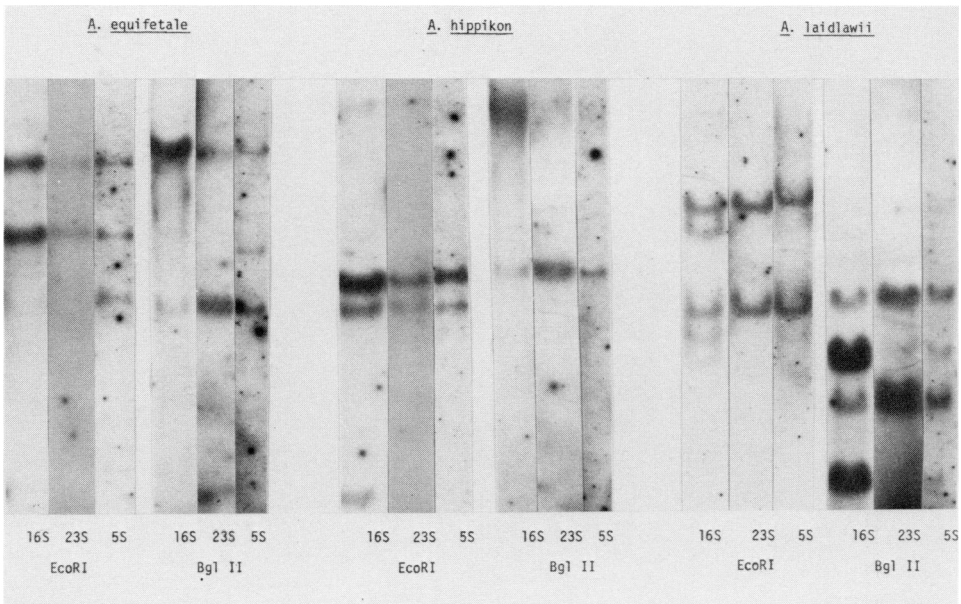

FIG. 1. Autoradiograph of *A. equifetale, A. hippikon,* and *A. laidlawii* DNAs digested with the restriction enzymes EcoRI and Bgl II and hybridized with ^{32}P-5′-labeled 5S, 16S, and 23S rRNAs.

enzymes (Fig. 1). Similar results were obtained for *A. granularum* and *A. axanthum* (not shown). We conclude that *Acholeplasma* species chromosomes each contain only two rRNA gene sets. It is apparent also that the individual acholeplasma rRNA genes are physically linked together on the chromosome, and, from the fact that the 16S genes are associated with the 23S genes and the 5S genes are associated with the 23S genes, the order probably is in the usual 16S-23S-5S eubacterial arrangement.

Results of hybridizations with EcoR1 digests of DNA from two group N streptococci, *S. lactis* and *S. cremoris,* are shown in Fig. 2. We estimate that there are at least five rRNA gene sets in *S. cremoris* and at least six rRNA gene sets in *S. lactis* (assuming that the darker, lower band in the *S. lactis* 23S and 5S rRNA autoradiographs are composed of two co-migrating rRNA gene-bearing fragments). Analysis of hybridizations with digests obtained with Bgl II and Hind III support these results.

Interestingly, the closely related group N streptococci, *S. cremoris* and *S. lactis,* shared two identically sized rDNA fragments bearing 5S, 16S, and 23S rDNA gene sequences. It seems probable that these shared restriction fragments will contain identical or nearly identical sequences. Some of the acholeplasmas also shared apparently identically sized rDNA fragments bearing both 16S and 23S rDNA sequences; for example, in Bgl II digests, *A. equifetale* shared one of its two rRNA gene fragments with one *A. laidlawii* fragment. These fragments too could share sequences. Especially interesting was the fact that some of the acholeplasma and streptococci shared apparently equal-sized cut rDNA fragments with one another. For example, *A. laidlawii* and *S. lactis* shared a 16S-23S rRNA gene fragment, and this could be coincidental or could result from the sharing of large nucleotide tracts. In addition, in both streptococcal and acholeplasma digests, one or a small number of fragments were detected that hybridized only to 16S rRNA but not to 23S rRNA,

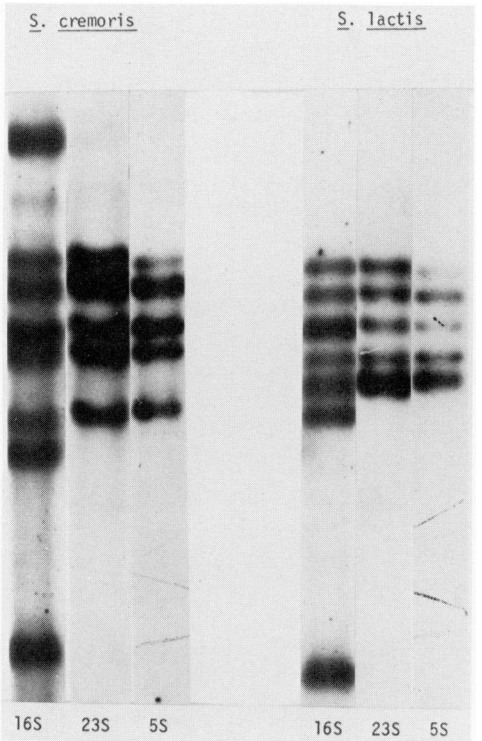

FIG. 2. Autoradiograph of hybridization analysis of streptococcal rRNA gene sets. Each streptococcus DNA was cut with EcoRI. Hybridization probes were as described in Fig. 1.

and these must reflect restriction sites within the 16S rRNA genes or adjacent to the 5′ ends of the genes.

DISCUSSION

We have shown here that the reduction in genome size of acholeplasmas relative to streptococci is accompanied by remarkable losses of entire rRNA gene sets. Thus, the rRNA genes have not been uniformly conserved and consequently certain of the rRNA genes must not be required absolutely. (However, the number of rRNA gene copies in bacteria does not necessarily parallel genome size because *Halobacterium halobium* has only one rRNA gene set even though its genome size is 2.6×10^9 daltons [12].) It seems likely that a parallel situation involving rRNA gene losses will be found to hold for other mycoplasmas. Information is available for the number of rRNA genes in one mycoplasma species, *M. capricolum,* and it has only two rRNA gene copies [13,14].

It will be valuable to learn whether the rRNA genes remaining in acholeplasmas contain spacer tRNAs. In *Escherichia coli* all seven rRNA gene operons contain tRNA genes located in a spacer region between the 16S and 23S rRNA; three of these rRNA gene sets each contain the same two tRNA genes (tRNA $^{Ile}_{1}$ and tRNA $^{Ala}_{1B}$) while the other four operons each contain a single tRNA $^{Glu}_{2}$ gene [15]. By contrast, in the gram-positive *Bacillus subtilis,* only two of the nine or ten rRNA genes contain spacer tRNAs, and they are the same two tRNA genes [16] that are located together in *E. coli* and also in chloroplast rRNA genes. It seems probable that spacer tRNA genes will be found in streptococci and it will be important to know whether they are conserved in the rRNA genes retained by mycoplasmas.

In addition to the complete absence of several rRNA genes, the remaining rRNA genes appear to lack some nucleotides or nucleotide sequences. Reff et al. [17] reported that the 16S rRNA (but not 23S rRNA) of all mycoplasmas examined appeared to be smaller in size than bacterial 16S rRNAs by about 12,000 daltons (this value would indicate the absence of about 37 nucleotides). Also, Woese and coworkers [3] reported that a number of sequences highly conserved in bacteria were not present in mycoplasma 16S rRNAs. In addition, the length of the 5S rRNAs from *M. capricolum* [18] and *Spiroplasma sp.* BC3 [Walker, this symposium] is only 107 nucleotides, the shortest known for any bacteria, suggesting that mycoplasma 5S rRNAs may also be missing some nucleotides (5S rRNAs from gram-positive bacteria are 116 or 117 nucleotides long). We have compiled the specific deficiencies or genome losses that have been identified in acholeplasmas (most of these also occur in other mycoplasmas), and all occur either in rRNA genes or in cell envelope-related functions (Table 1). Thus, losses of rRNA gene sets and certain rRNA gene sequences together with losses of genes coding for several cell envelope-related functions comprise the most prominent chromosomal losses universally missing from mycoplasma genomes. Any explanation for the genesis of mycoplasmas will have to account for these massive chromosomal losses. The apparent universality of these two classes of genome losses among mycoplasmas suggests that they could have been primary events in the formation of mycoplasmas.

The mechanism(s) by which large reductions in genome size occurred to produce the mycoplasmas is not known, and we have already speculated on how large or small chromosome losses could have occurred [2]. Because of our finding that acholeplasmas have only two rRNA gene copies compared to at least five or six copies for streptococci, it seems appropriate to focus our attention on chromosome alteration mechanisms involving rRNA genes. In *E. coli* there are seven rRNA operons distributed around the chromosome [15] and in *B. subtilis* there are nine or ten gene copies [16]. Because rRNA operons are largely homologous there is the potential for unequal recombination between these genes, and such recombination could lead to major rearrangements of the chromosome. These rearrangements might take the form of tandem duplications, inversions, translocations, or, of

TABLE 1
Comparison of Streptococcal and Acholeplasma Genomes and Envelopes

	Streptococci	Acholeplasmas
Genome		
Size	1.2–1.47 × 10^9	1 × 10^9
G + C content	34–42%	29–35%
RNA gene sets	At least 5 or 6 sets	Two sets
rRNA		
G + C content		Lower range
16S rRNA		12,000 daltons smaller Sequences missing
5S rRNA		Sequences missing?
Envelope		
Penicillin binding proteins	Present	Absent
PEP sugar transferase	Present	Absent
NADH oxidase	Membrane soluble	Membrane only

greatest interest here, deletions. The type of rearrangement would depend on the configuration on the chromosome of the interacting genes. The work of Hill and co-workers [19] shows that in fact at least some of these alterations can occur readily in *E. coli*. Thus, a conceivable mechanism for the loss of rRNA genes (and, possibly, genes in the intervening chromosome segments) is that a chromosome segment bounded by homologous rRNA genes could be deleted instead of being transposed. It seems probable that similar events could occur in gram-positive bacteria. Trowsdale and Anagnostopoulos [20] showed that in *B. subtilis* very large genome segments—as large as one-third of the chromosome—can undergo translocation and inversion or duplication, and recently Loughney et al. [16] identified a *B. subtilis* strain in which a single rRNA gene set was deleted.

Deletions of cell wall-related genes and rRNA genes are the classes of deficiencies found universally in mycoplasmas. It is interesting to note that the rRNA (and tRNA) genes are located on the chromosome near the origin of replication [21] and that the replication origin is associated with the cell membrane [22,23]; in *B. subtilis,* inhibition of cell-wall synthesis inhibits or prevents initiation of chromosome DNA replication [24]. Whether perturbation of the interaction of these elements could have had some role in mycoplasma formation is being explored.

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