Common Deoxyribonucleic Acid Sequences in Mycoplasma genitalium and Mycoplasma pneumoniae Genomes

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The new methodology of molecular genetics has provided useful tools for establishing genetic relatedness among bacteria, thus influencing bacterial taxonomy and phylogeny in a most profound way. Methods based on comparison of bacteria, based on detection of common genomic DNA sequences, as revealed by Southern blot hybridization of digested DNA with ribosomal ribonucleic acid (rRNA) gene probes, were recently reported as a way to reveal common sequences as a preliminary step for their cloning and expression. The small size of the mycoplasma genome (700 to 1,400 kilobase pairs [kbp]) led us to the idea of using an entire mycoplasma genome as a probe in Southern blots (22) to detect common nucleotide sequences or genes among mycoplasma species. The data presented in the present report show that it is possible in this way to detect and identify the hybridization bands of rRNA genes, revealed by specific rRNA gene probes. rRNA genes are highly conserved and exhibit a high degree of sequence homology among members of the class Mollicutes and other prokaryotes (1, 17). In addition, the hybridization blots may reveal other homologous nucleotide sequences, apparently representing genes shared by different species of mycoplasmas.

MATERIALS AND METHODS

Organisms and growth conditions. M. genitalium M30 was obtained from J. G. Tully (National Institutes of Health, Frederick, Md.), and Spiroplasma citri (Maroc, R8A2) was provided by R. F. Whitcomb (U.S. Department of Agriculture, Beltsville, Md.). M. pneumoniae (FH), M. pulmonis (6510), and M. gallisepticum A5969 were from our collection.

M. genitalium was grown in 100-ml quantities of modified SP-4 medium distributed in 500-ml tissue culture flasks (Nunc, Roskilde, Denmark). The medium contained 20% (vol/vol) inactivated horse serum in place of fetal bovine serum, and the CMRL component of the original SP-4 medium (26) was omitted. After 4 to 5 days of incubation at 37°C, the layer of cells sticking to the plastic was scraped off into cold 0.25 M NaCl containing 0.1 M ethylene-diaminetetraacetate (EDTA), pH 7.0. The cells were sedimented by centrifugation at 12,000 x g for 30 min at 4°C, and the pellet was washed once in the same solution and kept at −20°C for DNA extraction. Cultivation of M. pneumoniae was carried out in the same medium and under the same conditions as prescribed for M. genitalium, but the cell sheet was usually collected after 3 to 4 days of incubation. M.

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**M. pneumoniae** and *M. gallisepticum* were grown in modified Edward medium (19) at 37°C. *S. citri* was grown in SM medium (28) at 30°C. The organisms were harvested after 1 to 3 days of incubation and were washed as described above for *M. genitalium*.

**DNA preparation and cleavage.** DNA was extracted and purified by the method of Marmur (15). The DNA was digested by restriction enzymes (New England Biolabs, Inc., Beverly, Mass.) for 2 h at 37°C with buffer mixtures recommended by the manufacturer. The digested DNA was electrophoresed at 30 V for 18 h in 0.8% agarose (Seakem; FMC Corp., Marine Colloids Div., Rockland, Maine) slab gels in buffer containing 0.04 M tris(hydroxymethyl)aminomethane, 0.005 M sodium acetate (pH 8.0), and 0.001 M EDTA.

**Southern blot analysis.** DNA fragments from gels were transferred to nitrocellulose sheets by the method of Southern (22) and were hybridized with nick-translated probes. The probes employed included plasmid pMC5 carrying the rRNA operon of *M. capricolum* (23) and parts of the 16S, 23S, and part of the 16S gene of one of the two rRNA operons of *M. capricolum* (1, 2). Plasmid pKK3535, containing the entire rRNA operon rrnB of *Escherichia coli* (6), was digested by EcoRI (1) to give three fragments covering the 5' end, the central portion, and the distal portion of the rrnB operon, respectively (Fig. 1). Total DNAs of *M. genitalium*, *M. pneumoniae*, *M. gallisepticum*, *M. pneumonias*, and *S. citri* were also used as probes. All probes were nick translated (27) with deoxycytosinetriphosphate (3P) phosphate (New England Nuclear Corp., Boston, Mass.) to a level of 2 × 10^8 to 4 × 10^8 cpm/μg of DNA, and hybridization with the DNA fragments on the nitrocellulose sheets was done by a modification of the procedures described by Weinstock et al. (27) and Pollack et al. (16). To prevent nonspecific binding of DNA during hybridization, the sheets were soaked in 6 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.2]) containing 0.2% (wt/vol) each of polyvinylpyrrolidone (Sigma Chemical Co., St. Louis, Mo., PVP-360), bovine serum albumin (Sigma; fraction V), Ficoll (Sigma; type 400), and 100 μg of denatured salmon sperm DNA per ml (Sigma; type III) for 6 h at 56°C. Hybridization was carried out in the same buffer solution containing the labeled probe for 18 h at 56°C. The sheets were then washed at 56°C by soaking for 30 min with shaking in two changes of 2 × SSC plus 0.1% sodium dodecyl sulfate (SDS) followed by three changes in 0.5 × SSC plus 0.1% SDS and three additional changes in 0.2 × SSC plus 0.1% SDS. After air drying, the nitrocellulose sheets were exposed at −70°C to Agfa Gevaert X-ray film with an intensifying screen.

**DNA-DNA hybridization in solution.** DNAs used as probes for these tests were prepared by nick translation as described above and were denatured by heating in boiling water for 10 min. To remove residual double-stranded DNA, the denatured probe solution was passed through a hydroxyapatite column at 56°C, and single-stranded DNA was eluted with 0.12 M phosphate buffer (pH 6.8) and stored at 4°C (23). The unlabeled DNAs tested for hybridization were sheared by sonication (model W350; Heat Systems Ultrasonics, Inc., Plainview, N.Y.) to a fragment size of approximately 400 to 600 nucleotides, as determined by their electrophoretic migration in 0.8% agarose gel, with an HaeIII digest of ΦX174 DNA as a size marker. The sheared DNAs were kept at 4°C until used in the hybridization test.

The hybridization mixture contained 120 μg of unlabeled sheared DNA and 125,000 cpm of the 32P-labeled DNA probe per ml in a final volume of 100 μl of 0.48 M phosphate buffer (pH 6.8) supplemented with 1 mM EDTA and 0.4% SDS. The reaction mixture was heated for 10 min in boiling water to denature the DNAs and then allowed to anneal overnight at 56°C. The reaction was stopped by chilling and by 10-fold dilution of the reaction mixture with 0.012 M phosphate buffer. The sample was then loaded onto a hydroxyapatite column (0.5-ml bed volume), equilibrated at 56°C with 0.12 M phosphate buffer containing 0.2% SDS. Radioactive material not adsorbing under these conditions was considered unhybridized single-stranded DNA. Hybrid DNA was then eluted with at least 5 ml of 0.48 M phosphate buffer.
buffer containing 0.2% SDS. Radioactivity was measured by liquid scintillation spectrometry.

RESULTS

RNA genes in M. genitalium and M. pneumoniae. Analysis of Southern blot hybridizations of M. genitalium and M. pneumoniae DNAs digested by several restriction enzymes and combinations thereof with the various rRNA gene probes enabled the construction of physical maps of the rRNA genes of the two organisms, by using the conventional methodology used in previous structural analyses of the rRNA genes in other mycoplasmas (1, 9). Both mycoplasmas contained one copy of each of the three rRNA genes clustered together in a chromosomal segment of about 5 kbp and forming a single operon organized in the classical procaryotic fashion 16S-23S-5S, yet the two mycoplasmas differ in restriction sites within the operons and in the flanking sequences, therefore yielding different hybridization patterns with the various rRNA gene probes (Fig. 1).

Total chromosomal DNAs as probes. Use of nick-translated total mycoplasmal DNAs as probes in Southern hybridization tests with EcoRI-digested DNAs of the tested mycoplasmas revealed in all cases the rRNA gene bands reacting with the specific rRNA gene probe pMC5 (Fig. 2-4). However, the use of the M. genitalium, M. gallisepticum, and M. pneumoniae DNAs as probes revealed, apart from the rRNA gene bands, additional bands with digested DNAs of these three mycoplasmas (Fig. 2 and 3). The presence of bands additional to rRNA gene bands was most pronounced with the M. genitalium-M. pneumoniae pair. Hybridization with M. pulmonis and S. citri total DNAs as probes revealed, however, only the RNA gene bands in the digested DNAs of the other mycoplasmas (Fig. 4).

To achieve some assessment of the degree of nucleotide sequence homology of the common hybridization bands, we raised the temperature of both hybridization and washings from 56 to 66°C. Despite the consequent increase in stringency conditions, the hybridization patterns obtained with pMC5 and with the total genomic DNA probes were essentially the same as those obtained at 56°C (Fig. 2-4). It should also be taken into account that our washing procedure, including washes in low SSC concentrations, would by itself impose rather high stringency conditions. Furthermore, the low guanine plus cytosine content of the mycoplasmal DNAs was another factor reducing nonspecific hybridization.

DNA-DNA hybridization in solution. DNA-DNA hybridization in solution with nick-translated M. genitalium and M. pneumoniae DNAs as probes (Table 1) indicated a small, but significant, degree of DNA homology between these two mycoplasmas, a lower degree of homology with M. gallisepticum, and essentially no homology with S. citri.

DISCUSSION

Our hybridization data with M. genitalium-M. pneumoniae provide genetic support for previous reports of serological relatedness of these two mycoplasmas of humans, revealed in a variety of conventional serological tests (13, 14, 24) and in Western immunoblots (5, 7, 10, 12). Our Southern blots revealed that in addition to the homologous rRNA genes, the genomes of these two mycoplasmas possess other common nucleotide sequences, probably constituting genes for shared proteins, responsible for the serological cross-
reactions. It is also possible that some of the nucleotide sequences common to \textit{M. genitalium} and \textit{M. pneumoniae} DNAs represent conserved transfer RNA gene sequences. However, analysis of the hybridization data with \textit{M. pulmonis} and \textit{S. citri} DNAs as probes (Fig. 4) appears not to support the above possibility, by identifying the hybridization bands obtained with DNAs of \textit{M. genitalium} and \textit{M. pneumoniae} with fragments carrying rRNA gene sequences.

The hybridization blots do not allow an estimation of the number of common genes. DNA-DNA hybridization in solution (Table 1) showed 6 to 8% homology between \textit{M. genitalium} and \textit{M. pneumoniae}. The present study also shows that \textit{M. genitalium} possesses one rRNA operon, as does \textit{M. pneumoniae} (1, 9). The single rRNA operon occupies a 5-kbp segment of the chromosome, that is, less than 1% of the 700-kbp genome of \textit{M. pneumoniae} (3). The size of the \textit{M. genitalium} genome is not known, but in all probability it resembles that of \textit{M. pneumoniae} and the other sterol-requiring animal mycoplasmas. Data for DNA-DNA hybridization in solution are known to be liable to marked variations, depending on the technique and hybridization conditions employed (8). Thus, our homology values of 6 to 8% are considerably higher than the 1.8% homology value reported for the \textit{M. genitalium-M. pneumoniae} pair by Lind et al. (14). However, visual assessment of band density in the Southern blots of digested \textit{M. genitalium} DNA with total \textit{M. pneumoniae} DNA as a probe (Fig. 2, lane 2) would support a homology value higher than 1.8%, considering that the three bands representing the rRNA operon of \textit{M. genitalium} constitute about 1% of the genome. In any case, the hybridization data would not indicate a large number of genes shared by both organisms; however, it is reasonable to speculate that these common genes, though few, are responsible for the similar properties of the two mycoplasmas.

Our data for DNA-DNA hybridization in solution appear to be in line with the data for Southern blots. Thus, \textit{M. gallisepticum} DNA, the blots of which exhibited some common nucleotide sequences with \textit{M. pneumoniae} and \textit{M. genitalium}, apart from the rRNA genes, also yielded higher percent homology values than did \textit{S. citri} DNA which, apart from the rRNA gene sequences, failed to exhibit any hybridization bands with the \textit{M. pneumoniae} or \textit{M. genitalium} DNA probes. It is of interest that immunoblot revealed some antigens common to \textit{M. gallisepticum} and \textit{M. pneumoniae} (5, 7) but not to \textit{M. pulmonis} (7, 10). Nevertheless, \textit{M. gallisepticum} differs from \textit{M. pneumoniae} and \textit{M. genitalium} in possessing two rRNA operons (Razin and Yogev, in press). Although \textit{M. genitalium} and \textit{M. pneumoniae} resemble each other in having a single rRNA operon, this operon differs in restriction sites (Fig. 1). However, the weight that can be attributed to this finding appears limited, in light of the observation that various strains of \textit{M. gallisepticum}, \textit{Ureaplasma urealyticum} (Razin and Yogev, in press), and \textit{M. hominis} (S. Razin, D. Halachmi, and D. Yogev, unpublished data) exhibited some intraspecies heterogeneity with respect to their hybridization patterns with rRNA gene probes. It should also be mentioned that the physical map of the rRNA operon of \textit{M. pneumoniae} reported by Gobel et al. (9) differs in some detail from ours, a result which could probably be attributed to strain differences.

Among the possible applications of the new hybridization approach is its apparent usefulness as a new taxonomic tool in providing visual evidence for genetic relatedness among mycoplasmas and other procaryotes. The total genomic probes reveal also the characteristic patterns of rRNA gene sequences found useful in mycoplasma identification in contaminated cell cultures (18). There is no need in this instance for specific rRNA gene probes, though initial identification of the rRNA gene bands requires a specific rRNA gene probe, such as pMC5. The new approach also facilitates the identification and cloning of DNA segments of genes common to two mycoplasmas. Thus, use of \textit{HindIII}-digested \textit{M. genitalium} DNA extracted from the gel section above the rRNA gene bands (Fig. 2, lane 2) enabled the easy cloning in \textit{pUC13} of DNA segments containing nucleotide sequences common to \textit{M. genitalium} and \textit{M. pneumoniae} that are not rRNA genes (D. Yogev and S. Razin, unpublished data). Preliminary analysis of these clones revealed the possibility that the shared genomic sequences may carry genes associated with components of the tip or bleb structures characterizing these mycoplasmas. Expression of these clones in \textit{E.}

TABLE 1. Extent of hybridization in solution of DNAs from various mycoplasmas with $^{32}$P-labeled DNA of \textit{M. genitalium} and \textit{M. pneumoniae}.

<table>
<thead>
<tr>
<th>Source of unlabeled DNA</th>
<th>% Hybridization with $^{32}$P DNA probe of:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>\textit{M. genitalium}</td>
</tr>
<tr>
<td>\textit{M. genitalium} M-30</td>
<td>100.0$^\circ$ (77.4)$^a$</td>
</tr>
<tr>
<td>\textit{M. pneumoniae} FH</td>
<td>81.6 (6.2)</td>
</tr>
<tr>
<td>\textit{M. gallisepticum} A969</td>
<td>4.0 (3.0)</td>
</tr>
<tr>
<td>\textit{S. citri}</td>
<td>0.9 (0.7)</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>0 (0.8)</td>
</tr>
</tbody>
</table>

$^a$ Normalized values obtained after subtraction of calf thymus DNA values, considered as background.

$^b$ Actual hybridization values are given in parentheses.
coli may also be expected to throw light on the controversial issue of whether the P1 attachment protein of *M. pneumoniae*, or epitopes of it, are present in *M. genitalium* and *M. gallisepticum* (5, 7).

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**LITERATURE CITED**


