Physical Mapping of the *Mycoplasma gallisepticum* S6 Genome with Localization of Selected Genes

TIMOTHY S. GORTON, M. STEPHANIE GOH, AND STEVEN J. GEARY*

Department of Pathobiology, University of Connecticut, Storrs, Connecticut 06269-3089

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We report the construction of a physical map of the *Mycoplasma gallisepticum* S6 genome by field-inversion gel electrophoresis of DNA fragments generated by digestion of genomic DNA with rare-cutting restriction endonucleases. The size of the *M. gallisepticum* S6 genome was calculated to be approximately 1,054 kb. The loci of several genes have been assigned to the map by Southern hybridization utilizing specific gene probes.

Phylogenetic studies indicate that mycoplasmas have undergone degenerative evolution arising from a clostral ancestry (38). The reduced genome of mycoplasmas results in a loss of cellular structures, the most notable of which being the absence of a cell wall. Although their apparent simplicity makes the mycoplasmas attractive subjects for genetic analysis, classical approaches have proven relatively difficult to apply. Their complex nutritional requirements and microscopic growth on solid medium have hindered isolation of auxotrophic mutants useful in establishing metabolic pathways. Their use of the universal termination codon UGA as a tryptophan codon (15, 29) makes them attractive subjects for genetic analysis, classical approaches to the examination of the mycoplasma genome is appropriate.

Pulsed-field electrophoretic techniques have proven useful in constructing physical maps of several mycoplasmas (2, 4, 6, 16, 18, 25–27, 35). In an organism in which the isolation and characterization of induced mutants is difficult, physical mapping establishes points of reference to analyze sites of insertional mutagenesis (8, 9, 24, 35, 37) and enables the identification of genes involved in pathogenesis.

Several studies comparing DNA cleavage patterns and random amplified polymorphic DNA and fingerprint analysis have demonstrated that a genotypic heterogeneity exists among strains of *Mycoplasma gallisepticum* (13, 28, 30). We have chosen *M. gallisepticum* S6 for our studies on the basis of the premise that it is widely studied throughout the world. Here we describe the construction of a physical genomic map of *M. gallisepticum* S6 which provides a foundation for continued genetic analysis of this organism.

* M. gallisepticum S6 was cultured at 37°C in Frey’s medium (12). The cells (2 × 10⁹/ml) were pelleted by centrifugation at 12,000 × g for 5 min at 4°C and washed twice with an equal volume of phosphate-buffered saline (130 mM NaCl–10 mM Na₂HPO₄ and 130 mM NaCl–10 mM NaH₂PO₄). DNA was prepared in agarose plugs according to a modification of the procedure described by Krause and Mawn (16). Three milliliters of pelleted mycoplasma culture (6 × 10⁹ cells) was suspended in 125 µl of TBE buffer (10 mM Tris base, 1 mM EDTA, pH 8.0). Then 125 µl of degrading solution (1 mg of protease K per ml in 100 mM EDTA, pH 8.0) and 250 µl of 1.5% low-melting-point agarose (Gibco BRL, Gaithersburg, Md.), melted and equilibrated at 42°C, were added. This mixture was transferred to a plastic mold to form 60-µl agarose plugs. The plugs were transferred to 3.0 ml of degrading solution containing sodium dodecyl sulfate (SDS) at a final concentration of 2.5% and incubated at 42°C for 18 h with rocking. The plugs were then rinsed twice in wash solution (50 mM Tris HCl, 50 mM EDTA) for 15 min at room temperature with rocking. The protease K was inactivated with phenylmethylsulfonyl fluoride, at a final concentration of 10 mM, in wash solution at 37°C for 1 h with rocking. After two additional washes in wash solution, the plugs were stored at 4°C in storage solution (5 mM Tris HCl, 5 mM EDTA).

Restriction endonuclease digestions were performed on agarose-embedded DNA in microcentrifuge tubes. The restriction enzymes used were *Eag* I and *I-Ceu* I (New England BioLabs, Beverly, Mass.) and *Sma* I (Gibco BRL). Each plug was sliced in half and soaked for 2 h at 4°C in reaction buffer, according to the manufacturer’s recommendations, at a final volume of 150 µl. The buffer was replaced with complete reaction mixture containing a restriction endonuclease, and the mixture was incubated at 4°C for an additional 2 h. *Eag* I and *Sma* I digestions were performed with 30 U of enzyme. *I-Ceu* I digestions were performed with 3 U of enzyme and were supplemented with 100 µg of bovine serum albumin per ml. The reactions were incubated at the appropriate temperature for 16 to 20 h. The plugs were soaked twice in 0.5 ml of storage solution for 30 min at 4°C. Double digestions were performed by repeating the procedure described above with the second enzyme.

DNA fragments were separated by field-inversion gel electrophoresis (FIGE) into 1% low electroendosmosis agarose (Gibco BRL) in 0.5× Tris buffer (TBE) (89 mM Tris-borate–89 mM boric acid–2 mM EDTA). Electrophoresis was performed at 4°C in horizontal gels at 6.9 V/cm in 0.5× TBE with field-inversion parameters programmed into the Switchback Pulse Controller (Hoefer Scientific Instruments, San Francisco, Calif.). Multiple-run parameters were programmed with a 3:1 forward-reverse pulse ratio in the reverse mode, an initial run-in time of 5 min, and one of the following pulse time settings: a 1- to 20-s pulse time for 8 h followed by a 1- to 6-s pulse time for 16 h or a 1- to 30-s pulse time for 10 h followed by a 1- to 8-s pulse time for 12 h, depending on the size range of DNA fragments to be separated. Mid Range FFG Marker I and Lambda Ladder PFGE Marker (New England BioLabs) were used as size markers. The average of at least three separate analyses was used to determine fragment sizes (Table 1). On the basis of *Sma* I and *Eag* I single and double digests, we estimate the *M. gallisepticum* S6 genome to be 1,054 kb. DNA fragments resolved by FIGE were transferred to BA-
and a 0.3-kb fragment of the given with standard deviations from the mean. The total size of the M. gallisepticum estimated to be 1,052, 1,057, and 1,052 kb, respectively.

9 of the gyrase A gene in a 2.7-kb SalI gene, encoding elongation factor Tu (40); plasmid pMpP1 (14), containing a 16S rRNA genes and the complete 23S and 5S rRNA genes of Mycoplasma capricolum; plasmid pMP1 (14), containing a 5.0-kb fragment of the P1 cytadhesin gene of M. capricolum; plasmid pMC5 (1), containing a portion of the 16S rRNA genes and the complete 23S and 5S rRNA genes of Mycoplasma capricolum; plasmid pMP1 (14), containing a 5.0-kb fragment of the P1 cytadhesin gene of M. capricolum; and a 0.3-kb fragment of the M. gallisepticum S6 recA gene amplified by PCR using degenerate primers (a gift from K. Dybvig, University of Alabama, Birmingham) as described by Dybvig et al. (10). Plasmids were maintained within transformed E. coli strains and extracted by the alkaline lysis method (17).

PCR was performed with AmpliTaq DNA polymerase (Applied Biosystem/Perkin Elmer, Norwalk, Conn.) in the Perkin Elmer Cetus DNA Thermal Cycler 480 (Applied Biosystem/Perkin Elmer). Amplification reactions for both the M. gallisepticum S6 and Acholeplasma laidlawii (as a positive control) recA fragments were performed in 100–μl final volumes containing 2.5 U of AmpliTaq DNA polymerase, each nucleotide at 250 μM (dATP, dCTP, dGTP, and dTTP), 1.5 mM MgCl2, 400 ng of each degenerate primer, and either 25 ng of S6 genomic DNA or 200 ng of 0.8-μb A. laidlawii recA fragment (11) (gift of K. Dybvig). Reaction mixtures were subjected to three cycles of the following: 1 min at 94°C, 1 min at 37°C, and 1 min at 72°C, with a ramp time of 2 min for adjustment of the temperature from 37°C to 72°C. After these initial cycles, the reaction mixtures were subjected to 30 cycles of the following: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final extension for 10 min at 72°C. M. gallisepticum S6 recA fragment identity was confirmed by sequencing (GenBank accession number, L36389) and hybridization (data not shown) using the positive control A. laidlawii recA fragment as a probe under stringent conditions.

Primers flanking the P1 gene of M. pneumoniae were synthesized from a published nucleotide sequence (34) to amplify a 5.0-μb fragment of the P1 gene. The oligonucleotide primer sequences are 5′-CAA AAA AAC TGC CCT GTC CA-3′ (forward) and 5′-GGG CGT GTA GGG GGT GAG GG-3′ (reverse). Amplification reactions for the M. pneumoniae P1 gene were performed in a 100-μl final volume containing 2.5 U of AmpliTag DNA polymerase, each nucleotide at 250 μM (dATP, dCTP, dGTP, and dTTP), 2.5 mM MgCl2, 350 ng of each primer, and 50 ng of M. pneumoniae genomic DNA. Reaction mixtures were subjected to 20 cycles of the following: 1 min at 94°C, 30 s at 60°C, and 2 min at 72°C, with a final extension for 10 min at 72°C. The amplified M. pneumoniae P1 gene was then cloned into the pCRHI TA cloning vector and transformed into E. coli INVaF according to the manufacturer’s instructions (Invitrogen Corporation, San Diego, Calif.). The identity of the P1 gene was confirmed by nucleotide se-

TABLE 1. M. gallisepticum S6 DNA fragments generated by single and double digestion of agarose-embedded DNA with Smal and Eagl

<table>
<thead>
<tr>
<th>Smal</th>
<th>Eagl</th>
<th>Smal-Eagl size (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment(s)</td>
<td>Size* (kb)</td>
<td>Fragment(s)</td>
</tr>
<tr>
<td>Sm1</td>
<td>356 ± 14</td>
<td>Ea1, Ea2</td>
</tr>
<tr>
<td>Sm2</td>
<td>298 ± 8</td>
<td>Ea3</td>
</tr>
<tr>
<td>Sm3</td>
<td>130 ± 1</td>
<td>Ea4</td>
</tr>
<tr>
<td>Sm4</td>
<td>125 ± 4</td>
<td>Ea5</td>
</tr>
<tr>
<td>Sm5, Sm6</td>
<td>50 ± 1</td>
<td>Ea6</td>
</tr>
<tr>
<td>Sm7</td>
<td>33 ± 1</td>
<td>Ea7</td>
</tr>
<tr>
<td>Sm8</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

* Sizes are averages of at least three determinations for each fragment and are given with standard deviations from the mean. The total size of the M. gallisepticum S6 genome, on the basis of Smal, Eagl, and Smal-Eagl digests, was estimated to be 1,052, 1,057, and 1,052 kb, respectively.
** Band contains two comigrating fragments.
*** Band contains three comigrating fragments.

S85 supported nitrocellulose (Schleicher and Schuell, Inc., Keene, N.H.) by a modified method of Southern (32). To enhance the transfer of large DNA fragments, eletrophoresed DNA was partially depurinated by treatment with 0.25 M HCl for 10 min prior to alkaline denaturation, according to the manufacturer’s recommendations. Southern transfer was carried out for 24 to 24 h, and the blots were baked at 80°C for 1.5 h.

Blots were prehybridized for 2.5 h at 42°C in prehybridization fluid containing 6× SSC (900 mM NaCl, 90 mM sodium citrate, 5× Denhardt’s solution (7), 10 mM EDTA, 0.5% SDS, and 100 μg of denatured salmon sperm DNA per ml, followed by hybridization overnight under stringent conditions (prehybridization fluid with 45% formamide at 42°C), moderately stringent conditions (prehybridization fluid with 45% formamide at 32°C), or conditions of only low stringency (prehybridization fluid with 22.5% formamide at 32°C). Posthybridization washes were performed twice with 2× SSC–0.1% SDS at room temperature for 3 min each time and twice with 0.2× SSC–0.1% SDS at room temperature for 3 min each time. Nitrocellulose filters were dried, overlaid with Fuji RX film (Fisher Scientific, Pittsburgh, Pa.), and exposed for 12 to 16 h at 70°C.

Gene loci were determined by Southern blot hybridization analysis. The following plasmids and PCR-amplified product were used as hybridization probes: the cloned Mycoplasma pneumoniae tuf gene, encoding elongation factor Tu (40); plasmid pSC833 (5), containing the gyrase B gene and the 5′ end of the gyrase A gene in a 2.7-kb SalI fragment from M. pneumoniae (a gift from K. F. Bott, University of North Carolina, Chapel Hill); plasmid pMC5 (1), containing a portion of the 16S rRNA genes and the complete 23S and 5S rRNA genes of Mycoplasma capricolum; plasmid pMP1 (14), containing a 5.0-kb fragment of the P1 cytadhesin gene of M. pneumoniae; and a 0.3-kb fragment of the M. gallisepticum S6 recA gene amplified by PCR using degenerative primers (a gift from K. Dybvig, University of Alabama, Birmingham) as described by Dybvig et al. (10). Plasmids were maintained within transformed E. coli strains and extracted by the alkaline lysis method (17).

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FIG. 1. M. gallisepticum S6 DNA separated by FIGE. (A) Lane 1, Smal; lane 2, Smal plus Eagl; lane 3, Eagl. (B) Lane 1, Eagl plus I-CeuI; lane 2, Eagl. (C) Lane 1, Smal plus I-CeuI; lane 2, Smal. For visual comparison, the lanes were reordered via computer imaging with Adobe Photoshop.
sequence analysis of 0.3 kb of each end of the cloned PCR product.

All DNA probes were labeled by the incorporation of [32P]dATP (DuPont, NEN Research Products, Boston, Mass.) by standard nick translation procedures (29).

*M. pneumoniae* and *M. gallisepticum* S6 genomic DNAs were digested with *Eco*RI and electrophoresed in a 0.8% agarose gel. Southern blot analysis was performed as described above with the 5.0-kb P1 cytadhesin gene as a probe (14). The hybridization pattern for *M. pneumoniae* (14) was consistent with published data (33).

Goh et al. (14) have shown, by hybridization analysis, that the cloned P1 fragment, pMP1, recognizes a single *Eco*RI fragment of *M. gallisepticum* S6. This *Eco*RI fragment has been cloned, sequenced, and shown to be the *M. gallisepticum* homolog of the *M. pneumoniae* P1 gene. This homolog, termed MGP1, hybridized with the same single *M. gallisepticum* EcoRI fragment, suggesting that the P1 homolog in *M. gallisepticum* S6 exists as a single-copy gene, as it does in *M. pneumoniae* and *Mycoplasma genitalium*. MGP1, however, lacks the repeat characteristics of its *M. pneumoniae* and *M. genitalium* counterparts, P1 and MgPa, respectively. As the role of the repeat sequences in P1 and MgPa is currently undefined, the lack of such repeats in MGP1 is of interest, but its significance has yet to be determined.

Digestion of *M. gallisepticum* S6 DNA in agarose plugs with *Sma*I generated eight fragments (Table 1). Seven bands were resolved by FIGE (Fig. 1A, lane 1). An increased intensity of the 50-kb band (Fig. 1A, lane 1) upon staining with ethidium bromide suggested that it was two bands. It was confirmed that Sma5 and Sma6 comigrated as one band by excising the band and digesting it with *Eag*I (data not shown). Double digestion with *Eag*I and *Sma*I confirmed that *Eag*1 and *Eag*2 were in fact comigrating.

*Eag*I and *Sma*I single and double digests (Fig. 1A, lane 2) were analyzed to determine the relative positions of the respective enzyme sites. Upon double digestion of agarose plugs, only nine bands were resolved by FIGE. With *Sma*I and *Eag*I single digests generating eight and seven bands, respectively, double digests should produce fifteen bands. The third and fourth bands from the top in lane 2 (Fig. 1A) exhibited much...
Liu, S.-L., A. Hessel, and K. E. Sanderson. 1993. Genomic mapping with EagI restriction enzyme digestion data, and a previous report of the tuf gene being located adjacent to the complete rRNA operon in M. gallisepticum S6 (31) suggest that the complete 16S-23S-5S rRNA operon exists within the Sm2 fragment, that the separated 16S and 23S-5S rRNA genes exist within the Sm3 fragment, and that the third 16S rRNA gene lies within the Sm7 fragment.

Little information exists on M. gallisepticum regarding the gene locations, operon organizations, or nucleic acid sequences of genes encoding proteins involved in either metabolism or pathogenicity. Here we report the construction of a physical genomic map of M. gallisepticum S6 identifying Sm1, EagI, and I-CeuI restriction sites and the loci of the genes encoding elongation factor Tu, gyrase B, rRNA, RecA, and MGP1. The total genome size is estimated to be 1.054 kb. While the manuscript was in review, an article describing the physical maps of M. gallisepticum R and ATCC 19610 was published (36). These data show EagI and Sm1 restriction sites, transposon insertion sites, and the locations of the 16S rRNA genes. The results from these two studies support the findings of earlier studies (13, 28, 30) that demonstrated genotypic heterogeneity among M. gallisepticum strains. The physical genomic map of M. gallisepticum S6 reported here provides a useful foundation for future genetic analysis of genes involved in the pathogenicity of this strain. This map will prove useful for the placement of loci of other genes, as well as for future transposon mutagenesis studies of M. gallisepticum S6 to identify genes, such as those involved with adherence.

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REFERENCES


