Deoxyribonucleic Acid Homology and Relative Genome Size in *Mycoplasma*

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The deoxyribonucleic acid homologies of *Mycoplasma laidlawii* type A and type B, *M. pulmonis* (strains 47 and 63), and *M. hominis* were determined by membrane methodology. The homology data revealed a difference in genome size between *M. laidlawii* type A and type B. This difference also held with stringent conditions of annealing (high temperature). Little or negligible homology was shown to exist between the *M. laidlawii* strains type A and type B and *M. pulmonis* strains 47 and 63 and *M. hominis*. *M. hominis* showed less than 10% homology to the *M. pulmonis* and *M. laidlawii* strains. Neither of the *M. laidlawii* strains showed more than 2% annealing to the *M. pulmonis* strains. Reaction rate studies are suggested as a means of demonstrating the phylogenetic relationship between the *Mycoplasma* and other microorganisms.

We employed the deoxyribonucleic acid (DNA) membrane method designed by Denhardt (4) to demonstrate DNA-DNA homology among some members of the order Mycoplasmatales. Both binding and competition experiments were employed for the studies reported in this paper and were found to give comparable results. McCarthy and Bolton (7) and Hoyer et al. (5) have shown that competition reactions in addition to the binding reaction are useful in determining the degree of homology that exists between DNA from various sources.

The data obtained from our experiments not only demonstrate levels of DNA homology among some *Mycoplasma* species but, in addition, indicate a difference in genome size between two of the strains investigated.

The possibility of investigating the phylogenetic relationship between the *Mycoplasma* and other microorganisms by reaction rate studies (6) is discussed.

**MATERIALS AND METHODS**

**Microorganisms.** *Mycoplasma laidlawii* types A and B (ATCC 14089 and 14192, respectively), *M. pulmonis* 47 and 63 (obtained from George Kenny, University of Washington), and *M. hominis* were studied. The strain of *M. hominis* has been referred to previously as PPLO-EE (12) and was identified by Wallace Clyde of the University of North Carolina. Radiolabeled and unlabeled DNA were isolated from the *M. laidlawii* species and *M. hominis*; only unlabeled DNA was obtained from the *M. pulmonis* strains because of the difficulty encountered in growing them.

**Medium and cultivation of organisms.** All of the organisms were grown in a dialysate of soy peptone, yeast extract, and NaCl. This broth was supplemented with PPLO Serum Fraction (Difco) for the *M. laidlawii* species and with horse serum for the *M. pulmonis* species. For the cultivation of each organism, the medium was inoculated from a fresh broth culture (4 to 6 hr), and the inoculum was approximately 105 cells per ml. The cultures were grown to late exponential phase (approximately 4 × 108 cells per ml) at 37 C and harvested in the Sorvall refrigerated centrifuge (13).

To the cultures from which labeled DNA was isolated, 3H-thymidine was added during exponential phase, a few generations before harvesting. Approximately 1 mc of 3H-thymidine radioactivity was added per liter of culture. The radiolabeled thymidine was obtained from New England Nuclear Corp., Boston, Mass.; its specific activity was 16.6 c per millimole.

**DNA isolation.** The cells were collected by centrifugation, washed, and resuspended in buffer containing 0.2 M tris(hydroxymethyl)aminomethane, 0.05 M ethylenediaminetetraacetic acid (EDTA), and 1.0 M NaCl. The isolation procedure used was a modification of the method of Saito and Miura (14).

**Melting point determination.** Optical density (OD) measurements were made on gradually heated DNA by use of a recording Gilford spectrophotometer equipped with a heated sample chamber. DNA samples were equilibrated by dialysis with 0.1 × SSC (SSC: 0.15 M NaCl plus 0.015 M sodium citrate). The melting points determined for *M. laidlawii* A, B, *M. pulmonis* 47, 63, and *M. hominis* were 82, 82, 81, 81, and 81 C, respectively.

**Buoyant density.** The buoyant densities of *M. laidlawii* A and B were determined in the Spinco Model E analytical centrifuge with *Escherichia coli* DNA as a reference. The densities measured for A...
and B were 1.694 and 1.693 and corresponded to a guanine plus cytosine (GC) content of 35 and 34%, respectively. These results are in reasonable agreement with chromatographic data obtained in this laboratory.

Preparation of DNA-embedded nitrocellulose membranes. Schleicher & Schuell, (Keene, N. H.) 25-mm diameter membranes were used. Each DNA sample was adjusted to a concentration of 30 μg/ml in 0.1 SSC, heated 10 min at 100 C, quick cooled, and diluted to 3 μg/ml with 6 × SSC. A 5-ml amount of this solution was filtered onto each membrane. The membranes were dried overnight at 60 C (15). From 95 to 100% retention of DNA in the membranes was found when labeled DNA was embedded.

Annealing DNA. Labeled DNA used for the annealing reaction was denatured in the same manner as described in the previous paragraph, except that it was diluted to 15 μg/ml. Volumes of 0.1 ml were used in the reaction mixture. The ratio of membrane-bound DNA to annealing DNA employed in all of these studies was 10:1.

Competition reaction. Membranes embedded with DNA were placed in screw-cap vials containing 1 ml of preincubation mixture (PM) according to the method of Denhardt (4). Incubation at the temperature designated for the experiment was allowed to proceed for 6 hr. The membranes were cooled; a uniform volume (0.4 ml) of the desired concentration of unlabeled, denatured homologous or heterologous DNA was added to each vial along with 0.1 ml of denatured labeled homologous DNA. The contents of the vials were mixed and placed in an oven for 12 hr at a number of temperatures. At the termination of the incubation period, the vials were cooled and the non-annealing DNA was washed from the membranes with three filtrations per side with SSC. The membranes were then dried overnight in a 60-C oven. Scintillation fluid was added, and the samples were counted in a Packard liquid scintillation spectrophotometer series 3000. The proportion of bound radioactive DNA was determined by dividing the counts present in the vial by the total radioactivity administered (counts/min/μg of DNA). The controls, membranes without embedded DNA, contained less than 0.3% of the radioactivity that was annealed to the DNA membranes.

RESULTS AND DISCUSSION

Radiolabeled denatured DNA from M. laidlawii type A and type B (DNA A and DNA B) were annealed to their homologous DNA and with each other at 40, 65, and 75 C. DNA from M. laidlawii types A and B will be referred to in the text as DNA A and DNA B, respectively. The results obtained from these binding experiments are shown in Table 1, and it is observed that the amount of radiolabeled DNA annealing to membrane-bound DNA at 40 and 75 C is less than that found at 65 C. These results are consistent with the facts that are known regarding the annealing reaction of DNA, i.e., that the optimal temperature for the annealing reaction in free solution is about 25 below the Tm of the DNA employed (10), below the optimal temperature, the formation of short stretches of base pairs with accidental sequence complementarity takes place (6), and, at higher temperatures of incubation, the more stringent conditions for base pairing exist (1).

At 65 C, the optimal annealing temperature for comparing homology between DNA A and DNA B when the membrane method is used, it was observed that a difference in the degree of homology occurs when DNA A is annealed to DNA B and vice versa. When labeled DNA A is annealed to DNA B membranes, approximately 71% homology is observed, and, in the reciprocal reaction, when labeled DNA B is annealed to DNA A membranes, about 86% homology is found. Our experiments were repeated several times to verify these observations.

Although the level of binding is lower at 75 C (stringent condition), the 15% difference observed between the two reactions at 65 C is maintained at the higher temperature. The A to B reaction shows 63% homology, whereas the B to A reaction shows 78% homology.

The data from the competition experiments support the findings from the binding experiments. Competition curves are presented in Fig. 1. The competition studies were performed at the three temperatures used to demonstrate the binding reaction. DNA from both strains of M. laidlawii compete with each other at all temperatures studied. Using the formula employed by Brenner et al. (1) to determine relatedness in competition experiments [relatedness in competition experiments = (per cent binding per cent binding in presence of heterologous competitor)/(per cent binding – per cent binding in presence of homologous competitor) × 100], we found the per cent homology at 65 C to be about the same as that determined by the binding experiments. In the competition experiments, when DNA B is the competitor it competes for 88% of the DNA A sites, and, when DNA A is the competitor, it competes for 76% of the DNA B sites.

The interpretation of the results from the binding and competition experiments is that the genome of M. laidlawii A is larger than the genome of M. laidlawii B. From the binding experiments (Table 1), the results showing an 86% reaction when DNA B is annealed to DNA A indicate that 14% of the DNA B base sequences are different from those of DNA A. In the reaction in which DNA A is annealed to DNA A and DNA B membranes, 71% annealing to DNA B occurred, indicating that although DNA A con-
contains 86% of the base sequences of DNA B, 29% of the DNA A base sequences are different from those of DNA B; thus, DNA A would have to be larger than DNA B. This relationship can be expressed as $0.86 M_{(DNA\ B)} = 0.71 M_{(DNA\ A)}$ ($M$ refers to genome size). For the competition data, the values would be 0.88 and 0.76, respectively. The homology method is not sensitive enough to give an absolute value for the size difference, but an estimate can be made. Since the accuracy of the homology determinations is about ±5% for these experiments, the genome of *M. laidlawii* A can be estimated to be between 10 and 20% larger than the genome of *M. laidlawii* B.

The possibility that the difference we observe might be due to change or loss of DNA B during isolation and not to genome size must be considered. The fact that identical procedures of isolation were employed to obtain both types of DNA should, however, rule out this interpretation. In addition, evidence from another laboratory supports our conclusion that the difference found is real and attributable to genome size. Morowitz et al. (11) measured the genomes of these two organisms by autoradiography and showed that the genome of A was 10% larger than the genome of B. It should be noted that McGee et al. (8), using the DNA-agar method to demonstrate homology between the DNA from these two *M. laidlawii* strains, found the converse; they observed that the B to A and A to B reactions showed 67 and 71.5% homology, respectively. However, a critical examination of their data for the A to B reaction showed that their duplicate determinations vary from 60 to 83% homology from which they derive a mean value of 71.5%. If the lower figure were the actual value, then their reactions would be similar to ours except that the percentage values would be lower.

Data from the competition experiments at 75°C demonstrating competition by calf thymus DNA are somewhat puzzling. The calf thymus preparation is from the same stock used for the reactions at 40 and 65°C. As can be seen from Fig. 1, there was no competition at 65°C but there was at 40 and 75°C. The results obtained at the reaction at 40°C would be expected, since various degrees of base pair mismatching could occur at this temperature. At the 75°C incubation, the calf thymus DNA is about 25°C below its melting point in high ionic strength solution, and, in this temperature range, mammalian DNA (single-stranded) tends to form large aggregates or networks as shown by the work of McCarthy and others. The formation of such networks could easily trap other DNA in the solution, promote annealing in solution, and prevent this DNA from annealing to the proper DNA on the filter. Experiments are in progress to study competition at 75°C by DNA from other sources with differing GC contents and molecular weights.

Table 2 shows the homology relationship for the *Mycoplasma laidlawii* strains type A and type B, *M. pulmonis* strains 47 and 63, and *M. hominis*. Since we have not obtained labeled DNA from the pulmonis strains, we used their DNA as the immobilized phase and reacted them with labeled DNA from the other species. The only significant homology exhibited is that between the *M. laidlawii* strains. *M. hominis* showed little homology, less than 10%, to the *M. pulmonis* and *M. pulmonis*
laidlawii strains. Neither M. laidlawii strains showed significant annealing to M. pulmonis strains.

It is apparent from homology studies that, within the species of Mycoplasma, there is much less genetic homology than exists among various genera of bacteria (3, 7). The Mycoplasma have been grouped together primarily by one characteristic they share, i.e., the lack of some cell wall constituents. Because of this property, it was thought that they might be naturally occurring L-forms of bacteria.

McGee et al. (8) reasoned that L-forms and their bacterial parents should exhibit some degree of homology if they were related, since their genomes should contain segments of base sequences that are similar. First they studied a model system of Proteus mirabilis 9 and its stable L-form, and they found that these organisms were indistinguishable with respect to both DNA composition and homology. Then they performed homology tests on the DNA from Mycoplasma pneumoniae and Streptococcus MG, since serological data indicated that these two organisms might be related as L-form to parent. Although the results were negative, they noted that this did not rule out the possibility that the parent might be some other bacterial organism. However, further studies by this same group (9) on several species of Mycoplasma and the presumed bacterial parents showed no genetic relatedness by homology tests. They concluded that it is unlikely that Mycoplasma are the L-forms of some bacteria.

Information could be obtained regarding the genetic character of the Mycoplasma by employing reaction rate studies. Many investigators (2, 10) including McCarthy (6) have noted that the rate of DNA annealing is dependent upon the number of different base sequences in the DNA and that the DNA from a simple genome, such as that of a virus, anneals more rapidly than that of a more complex bacterial or mammalian genome when incubated at the same concentration. McCarthy demonstrated that viral, bacterial, and mammalian DNA each exhibit different reaction rate profiles (DNA annealing per hour) when these rates are studied as a function of temperature.

The determination of the reaction rate profile for the Mycoplasma could be used to demonstrate the phylogenetic relationship of these organisms. If they are a distinct group of organisms, they may exhibit a unique reaction rate profile; if they bridge the evolutionary distance between viruses and bacteria, they may exhibit profiles that range in similarity from the one for viruses to the one for bacteria; and, if they are related to bacteria, then their reaction rate should be similar to the kind of profile exhibited by bacteria.

**ACKNOWLEDGMENTS**

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We wish to thank Gary Hathaway for determining the buoyant densities of M. laidlawii types A and B.

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**LITERATURE CITED**


**TABLE 2. Annealing reaction of radiolabeled DNA to membrane-bound DNA for some Mycoplasma**

<table>
<thead>
<tr>
<th>Source of membrane-bound DNA</th>
<th>Source of radiolabeled DNA</th>
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<tbody>
<tr>
<td></td>
<td>M. laidlawii A</td>
</tr>
<tr>
<td>M. laidlawii A</td>
<td>100</td>
</tr>
<tr>
<td>M. laidlawii B</td>
<td>71</td>
</tr>
<tr>
<td>M. pulmonis 47</td>
<td>1</td>
</tr>
<tr>
<td>M. pulmonis 63</td>
<td>1</td>
</tr>
<tr>
<td>M. hominis</td>
<td></td>
</tr>
</tbody>
</table>

*Expressed as per cent of homology. A 1.5 μg portion of 3H DNA was incubated with 15 μg of membrane-bound DNA at 65 C.*

**FIG. 1.** Competition curves for M. laidlawii types A and B DNA. (a, b, c) Competition curves for M. laidlawii A DNA; (d, e, f) Competition curves for M. laidlawii B DNA. Competitor: M. laidlawii A DNA (●), M. laidlawii B DNA (▲), and calf thymus DNA (■). DNA from each species of Mycoplasma was embedded on nitrocellulose membranes at a concentration of 15 μg per membrane; 1.5 μg of 3H-labeled, heat-denatured DNA was incubated with its homologous membrane-fixed DNA in the presence of unlabeled competitor DNA. Mixtures were incubated according to the method of Denhardt (4) at 40, 65, and 75 C.


