Guanine-Plus-Cytosine Compositions of and Deoxyribonucleic Acid Hybridization Comparisons between *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*

GERALD W. STEMKE,^{1*} DEBRA J. MCINTYRE,¹ KENNETH L. ROY,¹ MARGARET E. STEMLER,² AND JANET A. ROBERTSON²

Department of Microbiology¹ and Department of Medical Microbiology,² University of Alberta, Edmonton, Alberta, Canada T6G 2E9

Among the mycoplasmal species commonly isolated from porcine lungs are *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*. The former organism is a proven agent of enzootic porcine pneumonia, whereas the latter organism is of little or no pathogenicity. These mycoplasmas show considerable antigenic cross-reactivity. The deoxyribonucleic acids of *M. hyopneumoniae* strain 11 and *M. flocculare* strain Ms42^T (T = type strain) each had a guanine-plus-cytosine content of 33 mol%. We also determined the extent of deoxyribonucleic acid sequence homology as reflected in deoxyribonucleic acid-deoxyribonucleic acid hybridization values. Approximately 10% heterologous hybridization was observed. Thus, our data support the previous classification of these organisms as separate species within the genus *Mycoplasma*.

Among the several mycoplasmas commonly isolated from porcine pneumonic lungs are *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare*. Studies have implicated *M. hyopneumoniae* as an etiological agent of porcine mycoplasmal pneumonia (enzootic porcine pneumonia) (16). No convincing evidence implicating *M. flocculare* in porcine mycoplasmal pneumonia has been presented. The taxonomic relationship between *M. hyopneumoniae* (*Mycoplasma suis*) and *M. flocculare* has been described previously (15); there are few biological or cultural differences between these two mycoplasmas, although distinction can be made on the basis of serological properties, growth inhibition, or plate fluorescent antibody tests (15). However, *M. flocculare* cross-reacts serologically in other tests with *M. hyopneumoniae*; multiple peptides of *M. flocculare* react with antiserum to *M. hyopneumoniae* in two-dimensional immunoelectrophoresis (13), whereas whole organism-based enzyme-linked immunosorbent assays show great cross-reactivity (3). Since antibody to *M. flocculare*, a common swine isolate, is also detected with *M. hyopneumoniae* as antigen in a variety of serological tests, antigenic cross-reactivity has proven to be a major hindrance to the serodiagnosis of porcine mycoplasmal pneumonia. In an attempt to learn more of the genetic relationship between these mycoplasmas we characterized their deoxyribonucleic acids (DNAs).

M. hyopneumoniae strain 11 (= ATCC 25095) and *M. flocculare* Ms42^T (= ATCC 27716^T) (T = type strain) were cultured in Friis medium (5) and harvested in the log phase of growth, as determined by the adenosine triphosphate level, using a luciferin-luciferase luminometry method (J. A. Robertson, M. E. Stemler, and G. W. Stemke, Abstr. 5th Int. Cong. Int. Org. Mycoplasmaology, p. 137, 1984) to monitor growth.

M. hyopneumoniae strain 11 was originally designated the type strain (8). Because the current strain 11 in the American Type Culture Collection may not be identical to that original strain, strain J, which is serologically and biochemically indistinguishable from strain 11 (13, 15), was designated the type strain of *M. hyopneumoniae* (15). *Mycoplasma hominis*

ATCC 14027 and *Mycoplasma pneumoniae* ATCC 15531 were grown in bromthymol blue broth (14) and SP4 broth (19), respectively. The cells were collected by centrifugation and then lysed by adding 2% (wt/vol) Sarkosyl in 10 mM ethylenediaminetetraacetate–10 mM tris(hydroxymethyl)aminomethane (pH 8)–10 mM sodium chloride; this was followed by incubation at 50°C for 60 min. Protein was digested with proteinase K (0.2 mg/ml) for 16 h at 37°C. DNA was purified by isodensity ultracentrifugation in cesium chloride-ethidium bromide. The red band was removed and extracted with butanol to remove the dye. The DNA was then precipitated in the cold with 70% ethanol and dissolved in a minimal volume of tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetate. The DNA samples purified in this way had proper ratios of absorbance at 260 nm to absorbance at 280 nm (approximately 2) and were readily digested by appropriate restriction nucleases; these are indications of relative purity.

Guanine-plus-cytosine (G+C) contents were determined by melting point and density methods. The density method of DeLey (1) as modified by Szybalski and Szybalski (18) was used with *Pseudomonas aeruginosa* DNA as the standard. The method of Owen et al. (11) was followed for melting point determinations by using a Beckman model DU-8 spectrophotometer equipped with programmable heating and absorbance recording equipment; calf thymus DNA was used as the standard. The values of the melting point determinations were corrected for the concentration of Na⁺ in the buffer. The results of this experiment are shown in Table 1. Both porcine mycoplasma DNAs had a melting point in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) of 78°C or, within experimental precision, a G+C content of 33 mol%. Similar or identical G+C contents occur in a number of *Mycoplasma* species and are, therefore, not an indication of a close genetic relationship. In fact, most *Mycoplasma* species have G+C contents that range between 24 and 40 mol% (4). The consistent values (42 to 43 mol%) obtained for *M. pneumoniae* by the two methods used in this study are higher than those reported elsewhere (10); in fact, we used the same strain for our determinations that was used in the previous study. If our values are

* Corresponding author.

TABLE 1. G+C contents of mycoplasmal DNAs

DNA source	G+C content (mol%) as determined by the:	
	Melting point method	Specific density method
<i>M. hyopneumoniae</i>	33	32-33
<i>M. flocculare</i>	33	33
<i>M. pneumoniae</i>	42-43	43
Calf thymus	42.4	ND ^a
<i>P. aeruginosa</i>	ND	67

^a ND, Not determined.

correct, they represent the highest G+C contents yet reported for any member of the *Mycoplasmatales*.

To determine the extent of sequence homology between the organisms, DNA-DNA hybridization values were obtained by using a variation of the methods of Denhart (2) and Gillespie and Spiegelman (6) and cellulose nitrate filters. Radioactive probes were prepared by nick translation (12); approximately 0.5 µg of cold DNA was denatured by exposure to NaOH, neutralized, and applied as a dot to filters (type HAWP; Millipore Corp.). The filters were baked at 80°C for 2 h and were presaturated with denatured salmon sperm DNA, which resulted in very low, nonspecific binding of radioactivity. Prokaryotic DNA does not hybridize with eucaryotic DNA so the salmon DNA did not compete specifically. Hybridization was carried out by using an approximately 10-fold excess of unlabeled DNA in 5.6× SSPE (1× SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA [adjusted to pH 7.4]) supplemented with denatured salmon sperm DNA (200 µg/ml) and Denhart solution (2) at 65°C for 24 h. Membranes were washed in 2× SSPE containing 0.1% sodium dodecyl sulfate at 45°C before the radioactivity on the filters was counted. These conditions approximated those recommended by Meinkoth and Wahl (9). The results of this experiment are shown in Table 2. Little binding (1 to 3%, or slightly above the background level) occurred with either *M. hyopneumoniae* or *M. flocculare* to *M. hominis* or to *M. pneumoniae*. *M. hyopneumoniae* and *M. flocculare* showed about 10 to 11% heterologous binding, compared with homologous levels. Although there are several other newer methods for determining hybridization values, for special situations, such as the low levels of hybridization which we found, the filter membrane method remains a satisfactory and well-documented technique.

Our results support the current classification of the four mycoplasmas that we examined as different *Mycoplasma* species; intraspecies hybridization values are normally more than 60% (17). Our values do suggest that the two porcine-derived strains are more closely related to each other than to either *M. hominis* or *M. pneumoniae*, which were chosen as unrelated species. The hybridization values with these latter

TABLE 2. DNA-DNA hybridization values

Radioactive probe DNA	% Hybridization with the following immobilized DNA sources:			
	<i>M. hyopneumoniae</i>	<i>M. flocculare</i>	<i>M. hominis</i>	<i>M. pneumoniae</i>
<i>M. hyopneumoniae</i>	(100) ^a	11.5	2.8	2.6
<i>M. flocculare</i>	9.7	(100)	1.5	1.3

^a Homologous binding values were taken as 100%.

two species were the values expected for mycoplasmas belonging to different or unrelated species (7). Genes for transfer ribonucleic acid and ribosomal ribonucleic acid are closely conserved among mycoplasmas and, indeed, among all procaryotes (one of us [K.L.R., unpublished data] showed that an *Escherichia coli* 16S ribosomal ribonucleic acid probe bound to an *EcoRI* fragment of *M. flocculare* DNA, again emphasizing the conservation of these genes). Thus, a portion of all of the heterologous hybridization values are probably accounted for by complexes with these genetic sequences.

Although we studied only one strain of each of the two porcine mycoplasmas, these strains are representative of their species; we used *M. hyopneumoniae* strain 11, which shows close antigenic cross-reactivity with at least four of seven other strains of *M. hyopneumoniae*, including type strain J (13, 15). *M. flocculare* strain Ms42 is the type strain and the most studied strain of this species. Both organisms grew well in the medium which we used. Additional strains will be examined to determine whether the patterns described above are maintained. These studies should help to establish the relationship between the two porcine mycoplasmas and provide some explanation for the antigenic cross-reactivity between them. Because of the low percentage of heterologous binding, it was not feasible to determine the melting point for hybrid binding and, therefore, not possible to estimate the extent of sequence homologies. However, heterologous binding requires a high degree of sequence homology for at least those pieces which do bind. This, in turn, could translate to proteins and peptides with adequate amino acid sequence homologies to yield significant antigenic cross-reactivity (9).

(A portion of this work was presented at the Fifth International Congress of the International Organization for Mycoplasmaology, Jerusalem, 1984.)

We gratefully acknowledge financial support by the Agricultural Research Council of Alberta (Farming for the Future grants 82-0128 and 83-0013) and by operating grant 7759 from the Medical Research Council, Ottawa, Canada.

LITERATURE CITED

1. DeLey, J. 1970. Re-examination of the association between melting point, buoyant density, and chemical composition of deoxyribonucleic acid. *J. Bacteriol.* **101**:734-738.
2. Denhart, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* **23**:641-643.
3. Freeman, M. J., C. H. Armstrong, L. L. Sands-Freeman, and M. Lopez-Osuna. 1984. Serological cross-reactivity of porcine reference antisera to *Mycoplasma hyopneumoniae*, *M. flocculare*, *M. hyorhinis* and *M. hyosynoviae* indicated by the enzyme-linked immunosorbent assay, complement fixation and indirect hemagglutination tests. *Can. J. Comp. Med.* **48**:202-207.
4. Freundt, E. A., and D. G. ff Edward. 1979. Classification and taxonomy, p. 1-41. In M. F. Barile, and S. Razin (ed.), *The mycoplasmas*, vol. 1. Academic Press, Inc., New York.
5. Friis, N. F. 1975. Some recommendations concerning primary isolation of *Mycoplasma suis pneumoniae* and *Mycoplasma flocculare*. *Nord. Veterinaermed.* **27**:337-339.
6. Gillespie, D., and S. Spiegelman. 1966. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. *J. Mol. Biol.* **12**:829-842.
7. Maniloff, J. 1983. Evolution of wall-less prokaryotes. *Annu. Rev. Microbiol.* **37**:477-499.
8. Mare, C. J., and W. P. Switzer. 1965. *Mycoplasma hyopneumoniae*, a causative agent of virus pig pneumonia. *Vet. Med.* **60**:841-846.
9. Meinkoth, J., and G. Wahl. 1984. Hybridization of nucleic acids

- immobilized on solid supports. *Anal. Biochem.* **138**:267-284.
10. **Neimark, H. C., and J. J. Pere.** 1964. Characterization of pleuropneumonia-like organisms by deoxyribonucleic acid composition. *Proc. Soc. Exp. Biol. Med.* **118**:517-519.
 11. **Owen, R. J., L. R. Hill, and S. P. Lapage.** 1969. Determination of DNA base compositions from melting point profiles in dilute buffers. *Biopolymers* **7**:503-516.
 12. **Rigby, P. W. J., M. Duchemann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**:237-251.
 13. **Ro, L. H., and R. F. Ross.** 1983. Comparison of *Mycoplasma hyopneumoniae* strains by serological methods. *Am. J. Vet. Res.* **44**:2087-2094.
 14. **Robertson, J. A.** 1978. Bromthymol blue broth: improved medium for detection of *Ureaplasma urealyticum* (T-strain mycoplasma). *J. Clin. Microbiol.* **7**:127-132.
 15. **Rose, D. L., J. G. Tully, and R. G. Wittler.** 1979. Taxonomy of some swine mycoplasmas: *Mycoplasma suis* pneumoniae Goodwin et al. 1965, a later, objective synonym of *Mycoplasma hyopneumoniae* Mare and Switzer 1965; and the status of *Mycoplasma flocculare* Meyling and Friis 1972. *Int. J. Syst. Bacteriol.* **29**:83-91.
 16. **Ross, R. F.** 1982. Mycoplasmal diseases, p. 535-549. In A. D. Leman, R. D. Glock, W. L. Mengeling, R. H. C. Penny, E. Scholl, and B. Straw (ed.), *Diseases of swine*, 5th ed. Iowa State Press, Ames.
 17. **Schleifer, K. H., and E. Stackebrandt.** 1983. Molecular systematics of prokaryotes. *Annu. Rev. Microbiol.* **37**:143-187.
 18. **Szybalski, W., and E. H. Szybalski.** 1971. Equilibrium density gradient centrifugation, p. 311-354. In G. L. Contoni and D. R. Davies (ed.), *Procedures in nucleic acid research*, vol. 2. Harper & Row, Publishers, New York.
 19. **Tully, J. G., R. F. Whitcomb, H. F. Clark, and D. L. Williamson.** 1977. Pathogenic mycoplasma: cultivation and vertebrate pathogenicity of a new spiroplasma. *Science* **195**:892-894.