Partial Nucleotide Sequence Similarity within Species of *Mycoplasma* and *Acholeplasma*

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The nucleotide sequence relationships between 18 species of *Mycoplasma* and 3 species of *Acholeplasma* were examined by solution DNA hybridization. Radiolabelled DNAs from strains representing 13 *Mycoplasma* and 2 *Acholeplasma* species were used as hybridization probes. The mycoplasmas were heterogeneous but displayed a low degree of conserved information of the order of 3 to 5 % of the genome. However, several species within each genus showed 13 to 15 % homology. There was no detectable homology between species from the two genera, and *M. pneumoniae* and *M. neurolyticum* appeared to be unrelated to any of the other *Mycoplasma* species or to each other. These results suggest that it may be possible to isolate genes common to most, if not all, *Mycoplasma* and *Acholeplasma* species.

INTRODUCTION

The use of nucleotide sequence homology as a taxonomic tool for bacteria and viruses is accepted practice. Likewise, measurement of nucleic acid hybridization kinetics has become a widely employed technique for detecting viruses in infected and transformed cells (Sharp *et al.*, 1974). Before this technique can be extended to the detection of fastidious micro-organisms in tissue a good understanding of the nucleotide sequence relationships within the microbial group is necessary. Suitable micro-organisms for this type of study are the mycoplasmas, especially those closely cell-associated. Although the relationships between various mycoplasmas have been reported (Reich *et al.*, 1966*a, b*; Somerson *et al.*, 1967; McGee *et al.*, 1967), the work was not exhaustive and utilized either RNA–DNA hybridization or DNA–DNA hybridization in agar columns, which have limits of accuracy that impede the interpretation of low levels of hybridization.

The present study utilized the more quantitative solution hybridization procedure followed by analysis of hybrid formation on hydroxyapatite columns (Britten & Kohne, 1966) in an attempt to provide a base of relationship data upon which to build a molecular detection system. In addition, this study utilized a large number of reciprocal hybridizations with ¹²⁵I-labelled DNA allowing grouping of the micro-organisms into classes based on low degrees of homology.

This study demonstrates that there is little homology among different *Mycoplasma* and *Acholeplasma* species. However, there is a partial conservation of nucleotide sequences between several different species that are found in the same or similar hosts.

METHODS

Growth of mycoplasmas and purification of DNA. Table 1 lists the *Mycoplasma* and *Acholeplasma* strains used in this study, the mol % G+C contents of the various species and their normal hosts. For DNA extraction the organisms were grown in 1·6 l of medium consisting of PPLO broth (Difco) supplemented

Table 1. *Mycoplasma* and *Acholeplasma* species and their sources

Species and type	Strain*	Normal host§	DNA G+C content§ (mol %)
<i>M. anatis</i>	1340	Duck	—
<i>M. arginini</i>	Tissue culture isolate		29
<i>M. arthritidis</i>	PG 6	Rat	30-34
<i>M. bovirhinis</i>	PG 43	Cattle	24-26
<i>M. canis</i>	PG 14	Dog	33
<i>M. fermentans</i>	PG 18	Man	27-29
<i>M. gallinarum</i>	PG 16	Chicken	27-28
<i>M. hominis</i>	PG 21	Man, monkey	27-29
<i>M. hyorhinis</i>	ESCA-1	Swine	27-28
<i>M. iners</i>	PG 30	Chicken, turkey	29
<i>M. maculosum</i>	PG 15	Dog	26-29
<i>M. meleagridis</i>	17529	Turkey	28
<i>M. neurolyticum</i>	Type A	Mouse	22-26
<i>M. orale</i> type 1	ESE-1	Man	24-28
<i>M. pneumoniae</i>	FH-Liu	Man	38-41
<i>M. pulmonis</i>	JB†	Mouse	27-28
<i>M. salivarium</i>	PG 20	Man, monkey	27-32
<i>M. spumans</i>	PG 13	Dog	28-29
<i>A. granularum</i>	No designation‡	Swine	30-32
<i>A. laidlawii</i>	PG 8	Cow, swine, man	31-36
<i>A. oculusi</i>	19-L‡	Goats	—

* Obtained from Dr E. Stanbridge unless otherwise noted.

† From Dr J. Galpin.

‡ From Dr G. Kenny via E. Stanbridge.

§ Information from Normore (1973).

with 10 % (v/v) whole horse serum (Irvine Scientific, Santa Ana, Calif., U.S.A.), 0.5 % (w/v) yeast extract and 100 units penicillin ml⁻¹. Confirmation of growth and the absence of contamination were checked by plating 0.1 ml of the broth culture on PPLO agar supplemented as above. Before harvesting, cultures were Gram-stained to check for contamination. Uncontaminated cultures were centrifuged (40 min, 33 000 g) and the pellet was resuspended in 0.15 M-NaCl (10 ml) with sodium dodecyl sulphate (SDS, 1 %, w/v, final concn), 1 mM-EDTA and 100 µg Proteinase K ml⁻¹. This solution was incubated for 1 h at 37 °C then extracted once with an equal volume of redistilled phenol saturated with 0.15 M-NaCl. The aqueous phase was adjusted to 8 M-urea, 0.1 % SDS using 10 M-urea in 0.24 M-phosphate buffer (equimolar Na₂HPO₄ and NaH₂PO₄, pH 6.8). The DNA/urea solution was run through a hydroxyapatite column (3 ml bed volume) which was washed with 50 ml 8 M-urea in 0.24 M-phosphate buffer followed by 300 ml 0.12 M-phosphate buffer. The DNA was then eluted with 0.48 M-phosphate buffer and centrifuged to equilibrium twice in a CsCl gradient (Padgett *et al.*, 1978). After the second gradient, fractions containing DNA, as determined by the absorbance at 260 nm, were pooled and dialysed against 2 l 1 mM-EDTA.

Radiolabelling of DNA. DNA was labelled with ¹²⁵I using a modification of the procedure of Commerford (1971). Prior to iodination the DNA was sheared by sonication for 2 min at 0 to 1 °C with the microprobe of a Branson W140 Sonifier (Heat Systems, Plainsview, N.Y., U.S.A.). The resulting fragment size averaged 350 nucleotide pairs as determined by sedimentation in a 5 to 20 % (w/v) alkaline sucrose density gradient centrifuged at 40 000 rev. min⁻¹ in a Beckman SW50.1 rotor for 17 h. The sheared DNA was precipitated in ethanol, resuspended in water at 500 µg ml⁻¹, boiled for 5 min and cooled rapidly.

The iodination reaction mixture consisted of 2.5 µg single-stranded DNA in 16 µl water, 3 µl 1.0 M-sodium acetate (pH 5.0), 10 µl Na¹²⁵I (protein iodination grade; Amersham; 100 mCi ml⁻¹, 3.7 GBq ml⁻¹) and 1 µl 0.2 mM-TiCl₃. This mixture was incubated for 30 min at 60 °C and then the reaction was quenched by adding 200 µl 0.12 M-phosphate buffer. The mixture was chromatographed on an 8 ml Sephadex G-100 column equilibrated with 0.12 M-phosphate buffer. Ten 1 ml fractions were collected, the iodinated DNA eluting in fractions 3 to 4. The eluted labelled DNA in 0.12 M-phosphate buffer was incubated for an additional hour at 60 °C to destroy the heat-labile iodouracil formed during the catalysed reaction, concentrated with 2-butanol and rechromatographed on Sephadex G-100. The eluted DNA was used in the hybridization experiments directly. Specific activities of 1.2 MBq (µg DNA)⁻¹ were obtained by this procedure.

Hybridization procedures. Sheared ¹²⁵I-labelled mycoplasma DNA (approx. 1.5 ng, 117 Bq) was mixed

with sheared unlabelled mycoplasma DNA ($20 \mu\text{g ml}^{-1}$), 0.48 M-phosphate buffer and 1 mM-EDTA (total vol. $150 \mu\text{l}$). The reaction mixture was placed in a 1.5 ml Eppendorf micro-test tube and denatured at 100°C for 5 min. After denaturation a sample was taken immediately (zero time) and the reaction mixture was overlaid with $150 \mu\text{l}$ mineral oil to prevent evaporation. The hybridizations were carried out at 60°C . Under these conditions of temperature and DNA concentration the reaction rate was approximately $1.25 C_0 t \text{ h}^{-1}$. ($C_0 t$ is the product of DNA concentration and time expressed as $\text{mol nucleotide l}^{-1} \text{ s}^{-1}$; Britten & Kohne, 1966.) Two further samples were taken at times when the reaction was expected to be complete ($C_0 t = 25$ and $C_0 t = 50$). Each sample was prepared for column separation by immediate dilution into 2.5 ml 0.14 M-phosphate buffer containing 0.4 % (w/v) SDS, then passed through a hydroxyapatite column equilibrated to 60°C (Britten & Kohne, 1966). The 0.14 M-phosphate buffer eluate containing the single strands was collected and the double-stranded (reassociated) DNA bound to the column was eluted with 0.3 M-phosphate buffer. The column effluents were adjusted to equal volumes (8 ml) and buffer concentrations (0.12 M) by the addition of water, then mixed with 10 ml Aquasol 2 scintillation fluid (New England Nuclear) and counted in a liquid scintillation counter. Percentage reassociation was calculated by subtracting the background counts and dividing the counts in the 0.3 M-phosphate buffer eluate by the total number of counts in the sample. Under these reaction conditions there was less than 2 % ^{125}I -labelled DNA self-reassociation.

Thermal elution of DNA hybrids. The hybridization reactions (reaction vol. $300 \mu\text{l}$) were run to a $C_0 t$ greater than 10 times the $C_0 t_{\frac{1}{2}}$ of the micro-organism in order to ensure that at least 90 % of the unlabelled 'driver' DNA had reannealed. A $120 \mu\text{l}$ portion was removed, diluted into 0.14 M-phosphate buffer and applied to a hydroxyapatite column equilibrated to 60°C . The column temperature was lowered to 26°C and thermal elution was started after equilibrating the column with an elution buffer (4 M- NaClO_4 in 0.14 M-phosphate buffer; Sugino & Kingsbury, 1976) which reduces the T_m of the DNA approximately 13°C to prevent column overheating. The column temperature was raised in 4°C increments. After each temperature equilibration the column was washed with 3.0 ml elution buffer at column temperature. Following thermal elution the column was washed with 0.3 M-phosphate buffer to ensure that all the DNA had been eluted. Samples were counted after adding 5 ml water and 10 ml Aquasol 2. The temperature at which 50 % of the DNA had eluted was considered the elution mid-point, $T_{m(e)}$. This value was corrected by the use of DNA standards (Sugino & Kingsbury, 1976) and used for the estimation of the accuracy of DNA reannealing and the determination of the G + C content of the DNA.

RESULTS

Hybridization of ^{125}I -labelled DNA with homologous unlabelled DNA

Each of 15 ^{125}I -labelled DNA preparations was characterized prior to use by estimating the extent of the homologous reaction (Table 2), the kinetics of the homologous reaction (results not shown), as well as the melting profiles of the homologous hybrids (Table 2). The extent of the reactions varied between 61 % for *M. neurolyticum* and 88 % for *M. canis*. In all but two cases the probes used were capable of greater than 70 % homologous reaction. In all instances the thermal denaturation temperatures were those expected, and the calculated G + C contents of the DNAs are included in Table 2. The lack of full reactivity in two of the probes is probably a result of iodinated DNA fragments not large enough to form stable double-stranded DNA hybrids.

Where the $C_0 t_{\frac{1}{2}}$ was accurately determined, the reaction rate was almost exactly that expected for genomes the size of the mycoplasmas (Kingsbury, 1969). Based on these results the heterologous reactions were carried out under conditions which would have given completed homologous reactions ($10 \times C_0 t_{\frac{1}{2}}$).

Hybridization of ^{125}I -labelled DNA with heterologous DNA

Iodinated DNAs from strains representing 13 *Mycoplasma* species and 2 *Acholeplasma* species (Table 2) were used as hybridization probes and reacted with a 17000-fold excess of unlabelled homologous or heterologous DNA. The reaction conditions selected (60°C , 0.48 M-phosphate buffer) were relaxed enough to allow similar but not identical sequences to hybridize. In all cases the incubation conditions were chosen to drive the homologous hybridization to completion. Table 3 lists the results of 284 hybridization reactions. In these experiments unlabelled DNA from 21 different species was used to drive the 15 different

Table 2. *Extent of hybridization and thermal stability of homologous hybrids of ^{125}I -labelled DNA from Mycoplasma and Acholeplasma species*

Source of labelled DNA	Percentage hybridization*	Hybrid $T_{m(e)}\dagger$ ($^{\circ}\text{C}$)	DNA G+C content $\ddagger$ (mol %)
<i>M. bovirhinis</i>	78	59	27
<i>M. canis</i>	88	63	34
<i>M. fermentans</i>	85	60	29
<i>M. gallinarum</i>	85	59	27
<i>M. hominis</i>	72	59	27
<i>M. hyorhinis</i>	80	59	27
<i>M. iners</i>	85	60	29
<i>M. maculosum</i>	71	59	27
<i>M. meleagridis</i>	83	60	29
<i>M. neurolyticum</i>	61	56	23
<i>M. orale</i>	82	59	27
<i>M. pneumoniae</i>	77	66	40
<i>M. pulmonis</i>	87	59	27
<i>A. laidlawii</i>	87	61	33
<i>A. oculusi</i>	63	59	27

* Expressed as the percentage of the probe in a double-stranded form following reaction with a 17000-fold excess of unlabelled DNA.

$\dagger$ The temperature of elution of 50 % of the double-stranded DNA from a hydroxyapatite column, determined as described in Methods. The elution buffer used in these experiments depressed the T_m of the DNA approximately 13 $^{\circ}\text{C}$ below that observed in standard saline citrate (SSC).

$\ddagger$ Calculated from the experimentally determined $T_{m(e)}$ values by use of the established relationship between base composition and T_m (Normore, 1973).

labelled probes. The results represent normalized relatedness determined by correcting the homologous reaction to 100 % and using the same factor to correct the other reactions with that probe.

The results indicate that there was no significant homology between any of the species examined, a result expected from earlier more limited studies (Reich *et al.*, 1966*a, b*; Somerson *et al.*, 1967; McGee *et al.*, 1967; Neimark, 1970). However, there was 13 % homology between *M. canis* (isolated from a dog) and *M. bovirhinis* (isolated from cattle) while the *M. bovirhinis* probe failed to react significantly with DNA from any other species tested. The probe derived from *M. canis* behaved in an identical manner and the reciprocal reactions between the two were almost identical.

Very little homology was found between the human mycoplasmas *M. fermentans*, *M. hominis*, *M. orale* type 1 and *M. salivarium*. ^{125}I -labelled DNA from *M. gallinarum* showed 15 % homology with DNA from *M. iners*, another bird mycoplasma, while the DNA from the bird mycoplasmas hybridized to DNA from strains of most other species to a lesser extent.

There was no detectable hybridization of ^{125}I -labelled DNA from *M. hyorhinis* (a swine mycoplasma) to DNA from any other *Mycoplasma* species, even those with similar G+C contents. There was also no detectable hybridization of ^{125}I -labelled DNA from *M. neurolyticum* (isolated from mice) or from *M. pneumoniae* (isolated from man) to DNA from any other *Mycoplasma* species; however, this would be expected because of their extreme G+C contents (23 and 40 mol %, respectively).

Both *Acholeplasma* DNA probes showed no detectable hybridization with DNA from any of the *Mycoplasma* species and there was only about 10 % homology between the three species examined.

Table 3. DNA homologies among *Mycoplasma* and *Acholeplasma* species

Normalized percentage homology*															
	Source of labelled DNA														
Source of unlabelled DNA	<i>M. bovirhinis</i>	<i>M. canis</i>	<i>M. fermentans</i>	<i>M. gallinarum</i>	<i>M. hominis</i>	<i>M. hyorhinis</i>	<i>M. iners</i>	<i>M. maculosum</i>	<i>M. meleagridis</i>	<i>M. neurolyticum</i>	<i>M. orale</i>	<i>M. pneumoniae</i>	<i>M. salivarium</i>	<i>A. laidlawii</i>	<i>A. oculusi</i>
<i>M. anatis</i>	—	7	—	—	<2	<2	5	—	—	—	<2	—	<2	<2	<2
<i>M. arginini</i>	5	5	<2	4	10	<2	4	4	5	<2	6	<2	7	<2	<2
<i>M. arthritis</i>	—	3	—	4	5	<2	<2	<2	—	—	5	—	6	<2	<2
<i>M. bovirhinis</i>	100	14	4	7	<2	<2	4	<2	5	<2	<2	<2	<2	<2	<2
<i>M. canis</i>	13	100	4	—	<2	<2	5	4	5	<2	<2	<2	3	<2	<2
<i>M. fermentans</i>	5	6	100	8	4	<2	9	7	8	<2	6	<2	3	<2	<2
<i>M. gallinarum</i>	5	7	8	100	<2	<2	17	7	12	<2	<2	<2	5	<2	<2
<i>M. hominis</i>	4	5	<2	5	100	<2	<2	<2	5	<2	6	<2	8	<2	<2
<i>M. hyorhinis</i>	<2	5	<2	4	<2	100	<2	<2	4	<2	<2	<2	3	<2	<2
<i>M. iners</i>	5	6	7	15	<2	<2	100	7	16	<2	<2	<2	<2	<2	<2
<i>M. maculosum</i>	4	6	5	8	<2	<2	6	100	6	<2	<2	<2	<2	<2	<2
<i>M. meleagridis</i>	5	5	5	8	13	<2	12	6	100	<2	<2	<2	<2	<2	<2
<i>M. neurolyticum</i>	4	4	<2	4	<2	<2	4	<2	4	100	<2	<2	<2	<2	<2
<i>M. orale</i>	4	5	4	4	7	<2	4	<2	5	<2	100	<2	12	<2	<2
<i>M. pneumoniae</i>	<2	<2	<2	—	<2	<2	<2	<2	<2	<2	<2	100	<2	<2	<2
<i>M. pulmonis</i>	—	3	—	4	<2	<2	<2	<2	—	<2	<2	—	<2	<2	<2
<i>M. salivarium</i>	<2	6	<2	5	7	<2	4	<2	5	<2	10	<2	100	<2	<2
<i>M. spumans</i>	—	3	—	7	7	<2	4	7	—	<2	5	—	5	<2	<2
<i>A. granularum</i>	—	<2	—	—	—	—	<2	—	—	—	<2	—	<2	16	10
<i>A. laidlawii</i>	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	100	10
<i>A. oculusi</i>	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	14	100

* Calculated as $100 \times (\text{c.p.m. bound in heterologous reaction}) / (\text{c.p.m. bound in homologous reaction})$.

DISCUSSION

Previously (Reich *et al.*, 1966*a, b*; Somerson *et al.*, 1967; McGee *et al.*, 1967; Neimark, 1970) the heterogeneity and lack of significant homology among mycoplasmas has been clearly demonstrated. However, the techniques used in these earlier studies could have easily failed to detect relationships of the order of 1 to 5 %. The use of reassociation in free solution with subsequent analysis on hydroxyapatite establishes conditions which favour more quantitative hybrid formation. In addition, this procedure does not suffer from the problems of DNA hybrid loss associated with agar columns and nitrocellulose filters. The results reported here show that there is some nucleotide sequence similarity within each of the two genera and probably no relationship between them. The nucleotide sequence similarity is so slight, however, that it does not suggest that the mycoplasmas, grouped together on the basis of their lack of a cell wall, have evolved from a common ancestor. The results presented here suggest that the evolution of each species may have been an independent event and that the only detectable relationships exist in a few combinations found in similar hosts (such as *M. iners* and *M. gallinarum*). The 16S ribosomal RNA 'signatures' of mycoplasmas (Woese *et al.*, 1980) also suggest that they evolved independently from ancestral Gram-positive bacteria. This independent and apparently rapid evolution has presented the taxonomist with numerous problems in the classification of the mycoplasmas.

The findings reported here may serve as a base for the development of a molecular detection system. There appear to be enough common sequences for group-specific, but not species-specific, hybridization probes to be obtained. If the group-specific nucleotide sequences are similar and can be identified and purified, they have the potential of serving

as sensitive *Mycoplasma*- or *Acholeplasma*-specific, yet not species-specific, hybridization probes to detect the presence of these organisms in tissues and tissue cultures. Preliminary experiments done in this laboratory suggest that there is considerable conservation of the 23S ribosomal RNA sequences. If, indeed, the ribosomal RNA sequences are conserved within each genus then a workable detection scheme becomes feasible. In many instances such detections need not be accurate at the species level initially, since knowledge of the very existence of the micro-organisms in the sample may be adequate. The utility of this method of detection and classification of new isolates has yet to be proven; however, the experience gained from dealing with virus-infected cells suggests that it could develop into a very powerful technique for the detection of fastidious organisms.

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REFERENCES

- BRITTEN, R. J. & KOHNE, D. E. (1966). Nucleotide sequence repetition in DNA. *Yearbook of the Carnegie Institution of Washington* **65**, 78–89.
- COMMERFORD, S. L. (1971). Iodination of nucleic acids *in vitro*. *Biochemistry* **10**, 1993–1999.
- KINGSBURY, D. T. (1969). An estimate of the genome size of various microorganisms. *Journal of Bacteriology* **98**, 1400–1401.
- MCGEE, Z. A., ROGUL, M. & WITTLER, R. G. (1967). Molecular genetic relationships among mycoplasma, L-forms and bacteria. *Annals of the New York Academy of Sciences* **143**, 21–30.
- NEIMARK, H. C. (1970). Division of mycoplasmas into subgroups. *Journal of General Microbiology* **63**, 249–263.
- NORMORE, W. M. (1973). Guanine-plus-cytosine (GC) composition of the DNA of bacteria, fungi, algae and protozoa. In *Handbook of Microbiology*, vol. 2, pp. 654–657. Edited by A. I. Laskin & H. A. Lechevalier. Cleveland: CRC Press.
- PADGETT, R. A., MOORE, D. A. & KINGSBURY, D. T. (1978). Herpes simplex virus nucleic acid synthesis following infection of non-permissive XC cells. *Journal of General Virology* **40**, 605–614.
- REICH, P. R., SOMERSON, N. L., ROSE, J. A. & WEISSMAN, S. M. (1966*a*). Genetic relatedness among mycoplasmas as determined by nucleic acid homology. *Journal of Bacteriology* **91**, 153–160.
- REICH, P. R., SOMERSON, N. L., HYBNER, C. J., CHANOCK, R. M. & WEISSMAN, S. M. (1966*b*). Genetic differentiation by nucleic acid homology. I. Relationships among *Mycoplasma* species of man. *Journal of Bacteriology* **92**, 302–310.
- SHARP, P. A., PETTERSSON, U. & SAMBROOK, J. (1974). Viral DNA in transformed cells. I. A study of the sequences of Adenovirus 2 DNA in a line of transformed rat cells using specific fragments of the viral genome. *Journal of Molecular Biology* **86**, 709–726.
- SOMERSON, N. L., REICH, P. R., CHANOCK, R. M. & WEISSMAN, S. M. (1967). Genetic differentiation by nucleic acid homology. III. Relationships among mycoplasma, L-forms and bacteria. *Annals of the New York Academy of Sciences* **143**, 9–20.
- SUGINO, W. M. & KINGSBURY, D. T. (1976). DNA homologies between strains of Herpes simplex virus. *Virology* **71**, 605–608.
- WOESE, C. R., MANIHOFF, J. & ZABLEN, L. B. (1980). Phylogenetic analysis of the mycoplasmas. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 494–498.