Division of Mycoplasmas into Subgroups

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SUMMARY

The deoxyribonucleic acid (DNA) base compositions of 12 recently isolated or new species of mycoplasmas fell within the range known for the *Mycoplasma* group. By DNA-DNA hybridization a new sterol-nonrequiring strain, s-743, was unrelated to *Mycoplasma laidlawii* or *M. granularum*, the latter two strains showing moderate cross-reactivity; swine strain B3 appeared to be related to *M. mycoides* var. *capri*. From these results and information in the literature, the mycoplasmas have been divided into six provisional subgroups on the basis of physiological characters, DNA base composition and nucleic acid hybridization. Some speculations are made on the significance of heterogeneity among mycoplasmas.

INTRODUCTION

Earlier work has emphasized the heterogeneity of mycoplasmas (Neimark & Pickett, 1960). Studies showing that the guanine + cytosine (GC) contents of *Mycoplasma* DNA range widely from approximately 23 to 40 moles % established that this heterogeneity was fundamental (Neimark & Pene, 1965). Studies also revealed that a cluster of mycoplasmas exists with GC contents lower than those of any known aerobic bacteria (Jones & Walker, 1963; Neimark & Pene, 1965). Data now available on the DNA base compositions of most known mycoplasmas (McGee, Rogul & Wittler, 1967; Neimark, 1967; Bak & Black, 1968; Kelton & Mandel, 1969; Williams, Wittler & Burris, 1969) indicate that heterogeneity in base composition extends through both fermentative and nonfermentative strains and divides the mycoplasmas into subgroups that are incompatible in a single genus (Neimark, 1967). Nucleic acid hybridization studies have been carried out between several mycoplasmas (Somerson, Reich, Chanock & Weissman, 1967; McGee *et al.* 1967; Peterson & Pollock, 1969). The present paper reports DNA base compositions of some recently isolated mycoplasmas (some representing new species) and results of DNA-DNA hybridization between selected mycoplasmas. Six subgroups are proposed and speculations made on the implications of divisions within the mycoplasmas. These results were presented in preliminary form (Neimark, 1969).

METHODS

Organisms, media and growth conditions. The organisms used in this study were kindly supplied by several colleagues and, whenever possible, were obtained from the laboratories which originally isolated or identified them. Strains s-410 and s-743 (Tully & Razin, 1969) and B3 (Dinter, Danielsson & Bakos, 1965) were provided by

Dr J. Tully; goat 145 by Dr J. Tully and Dr R. Del Giudice; UM30847 and *Mycoplasma arginini* strain G230 (Barile *et al.* 1968) by Dr M. Barile; strains 747v and s3-7 (Davidson & Thomas, 1968) by Dr M. Davidson; *M. agalactiae* var. *bovis* (Hale, Helmboldt, Plastringe & Stula, 1962) by Dr A. Mosher. The latter strain does not produce acid from glucose in amounts that can be detected by change in pH indicator, even after prolonged serial passage on media containing glucose. *Mycoplasma gallisepticum* strain s6, obtained from Dr H. Adler, and *M. granularum*, from Dr W. Switzer, were used in hybridization studies. The identity of the *M. granularum* strain used in this study was kindly confirmed by Dr J. Tully. Each strain was cloned three times. DNA from Connecticut goat, designated *M. mycoides* var. *capri* (Jonas & Barber, 1969), VOM strain, and Mexican strain was prepared through the courtesy of Dr J. J. Callis and Dr T. Barber, United States Department of Agriculture, Plum Island Animal Disease Laboratory.

Media and growth conditions were essentially as described previously (Neimark & Pickett, 1960; Neimark, 1967). Neither inhibitory agents nor antibiotics were employed.

Labelled DNA was produced by growing mycoplasmas in liquid media containing 0.5 μ Ci/ml. of thymidine-methyl- 3 H.

Enzymes and isotopes. Ribonuclease (Worthington Biochemical Corporation, Freehold, New Jersey) was heated at 85° for 10 min. to inactivate any contaminant deoxyribonuclease. Pronase (Calbiochem, Los Angeles, California) was self-digested for 2 h. at 37° (Saito & Miura, 1963; Gillespie & Spiegelman, 1965). Thymidine-methyl- 3 H (specific activity approximately 15 Ci/mmol) was purchased from Schwartz BioResearch, Orangeburg, New York.

Isolation of DNA. Cells were collected from broth by centrifugation at approximately 20,000 g, washed once with cold saline-EDTA (0.15 M-NaCl+0.1 M- Na_2 ethylenediamine tetra-acetate, pH 8.0), and their DNA isolated by the method of Marmur (1961).

Cells from strains 747v and s3-7 could be obtained only in small amounts, and their DNA was partially purified. After suspensions had been lysed with 1 % sodium dodecyl sulphate, they were diluted with an equal volume of saline-EDTA and digested with Pronase (50 μ g./ml.) for 12 to 16 h. at 37°. The lysate was then deproteinized by shaking with chloroform-isoamyl alcohol, the aqueous phase precipitated with 2 vol. ethanol, and the DNA dissolved in a small volume of SSC (0.15 M-NaCl+0.015 M-sodium citrate).

Determination of DNA base composition. The average GC content of DNA was estimated from buoyant density in a CsCl density gradient as described by Schildkraut, Marmur & Doty (1962) using *Escherichia coli* DNA as reference (taken to have a buoyant density of 1.710 g./cm. 3). All samples were analysed at least twice.

Immobilization of DNA on filters. DNA was bound to membrane filters by the procedures of Gillespie & Spiegelman (1965) and Denhardt (1966), with minor modifications. Native DNA (50 μ g./ml.) was denatured by boiling for 8 min. and then quickly cooled by immersion in an ice bath and dilution with 9 vol. cold sixfold concentrated SSC. Denatured DNA was filtered through 25 mm. membrane filters (HA, 0.45 μ m; Millipore Corporation, Bedford, Massachusetts) prewashed in sixfold concentrated SSC. Filters with retained DNA were washed with sixfold concentrated SSC, dried overnight in a desiccator at 22°, and baked in a vacuum oven for 2 h. at 80°.

Labelled DNA and competitor DNA were sheared by sonic oscillation (Biosonic II, microtip probe; Will Scientific, New York) and then denatured.

DNA-DNA duplex formation. Filters with immobilized DNA were preincubated in the medium of Denhardt (1966) for 2 to 6 h. at the temperature to be used for hybridization, drained and blotted, and then placed in vials which contained 1 µg, homologous or heterologous labelled DNA fragments in twofold concentrated SSC. For competition experiments (Hoyer, McCarthy & Bolton, 1964), a large excess of unlabelled DNA fragments was added to the reaction mixture (final volume of 0.5 ml.) to compete with labelled DNA fragments for binding sites on the immobilized DNA. The ratio of membrane-bound to labelled DNA was 10:1 or, in some cases, 40:1. Hybridization was carried out for 12 to 16 h. at 25° below the optical melting temperature (T_m) of the DNA (Marmur & Doty, 1961, 1962); where DNA samples differed in T_m , the higher temperature, which provided more stringent test conditions, was used. Filters were then washed on each side with twofold concentrated SSC at the temperature of hybridization, dried at 60° and counted in a Picker Liquimat or Beckman liquid scintillation counter.

RESULTS

DNA base compositions. For several recently isolated mycoplasmas the GC contents of carbohydrate-fermenting strains ranged from 26 to 31 moles % and those for nonfermentative strains ranged from 26 % to 32 % (Table 1). In addition, GC contents of 35.2 % and 32.4 % were found for *Mycoplasma gallisepticum* s6 and *M. granularum*. No satellite bands were observed for any of these strains.

Table 1. *DNA base compositions of some recent isolates and new Mycoplasma species*

Species or strain	Origin	Density* (g./cm ³)	GC content (mole %)
A. Fermentive strains			
S-410	Cell culture	1.6907	31.3
S-743	Cell culture	1.6907	31.3
Connecticut goat	Goat	1.6892	29.8
VOM goat	Goat	1.687	27.6
Mexican goat	Goat	1.6868	27.4
UM 30847	Goat	1.686	26.5
B 3	Swine	1.6857	26.3
B. Nonfermentative strains			
<i>M. agalactiae</i> . var <i>bovis</i> †	Cow	1.692	32.7
Goat 145	Goat	1.689	29.6
<i>M. arginini</i> G 230	Several animals	1.688	28.6
747V	Primate	1.687	27.6
S 3-7	Primate	1.686	26.5

* Average of two determinations.

† This strain does not produce acid from glucose in amounts that can be detected by pH change.

DNA-DNA hybridization. The sterol-nonrequiring strains S-410 and S-743 have identical average base compositions of 31.3 moles % GC (Table 1). Strain S-743 was tested against the other known sterol-nonrequiring mycoplasmas, *Mycoplasma laidlawii* and *M. granularum*, to determine its relationship to them. Reciprocal hybridiza-

tion tests between s-743 and *M. granularum* showed only very low levels of DNA binding (Table 2). In competition reactions between s-743 and *M. laidlawii* A (Table 3), the addition of homologous unlabelled DNA fragments reduced binding of labelled DNA by approximately two-thirds in each case, whereas addition of heterologous fragments produced no significant decrease in binding. Thus there is little or no genetic relatedness between s-743 and *M. laidlawii* A or *M. granularum*. One-way crosses between DNAs from s-743, *M. pneumoniae* and *M. gallisepticum* also showed only a very low level of duplex formation (Table 2).

Table 2. *DNA-DNA hybridization tests between Mycoplasma strains*

Relations among strains are expressed as % relative to the homologous reaction which is designated 100%. Averages of duplicate trials are reported.

Source of DNA on filter	Source of ³ H-labelled DNA fragments	DNA bound (%)
s-743	s-743	100
<i>M. granularum</i>	s-743	3
<i>M. gallisepticum</i> s6	s-743	5
<i>M. pneumoniae</i> FH	s-743	3
<i>M. granularum</i>	<i>M. granularum</i>	100
s-743	<i>M. granularum</i>	6
<i>M. laidlawii</i> A	<i>M. granularum</i>	34
<i>M. laidlawii</i> A	<i>M. laidlawii</i> A	100
<i>M. granularum</i>	<i>M. laidlawii</i> A	32

Table 3. *DNA competition reactions between selected Mycoplasma strains*

Relations among strains are expressed as % relative to the homologous reaction which is designated 100%. Averages of duplicate trials are reported.

Source of DNA on filter	Source of ³ H-labelled DNA fragments	Competitor DNA fragments added (50 µg.)	DNA bound (%)
A. s-743 against <i>M. laidlawii</i> A			
s-743	s-743	None	100
s-743	s-743	s-743	33
s-743	s-743	<i>M. laidlawii</i>	98
<i>M. laidlawii</i> A	<i>M. laidlawii</i> A	None	100
<i>M. laidlawii</i> A	<i>M. laidlawii</i> A	<i>M. laidlawii</i> A	30
<i>M. laidlawii</i> A	<i>M. laidlawii</i> A	s-743	105
B. Strain B3 against VOM strain of <i>M. mycoides</i> var. <i>capri</i>			
B3	B3	None	100
B3	B3	B3	34
B3	B3	VOM	33
VOM	B3	None	73
VOM	B3	B3	25
VOM	B3	VOM	22

Duplex formation between *Mycoplasma laidlawii* and *M. granularum* DNA was observed at levels of about one-third that of the homologous reactions (Table 2). For comparisons between strain B3 and the vom strain of *M. mycoides* var. *capri*, the DNA of strain vom was prepared under quarantine at the United States Department of Agriculture Plum Island Animal Disease Laboratory by the author and was sterility

tested by Plum Island Laboratory staff. Under these conditions it was not possible to prepare radio-labelled DNA, and therefore reciprocal hybridization tests could not be done. However, DNA fragments of B3 and VOM were approximately equivalent in their competition with labelled B3 fragments for sites on membrane-bound DNA of B3 and VOM (Table 3). This clearly demonstrates that strain B3 and *M. mycoides* var. *capri* are genetically related.

DISCUSSION

The DNA base compositions reported here fall within the known range of 22.8 to 41.0 moles % for the mycoplasmas. A GC content of 23 % would appear to be near a lower limit, considering that GC contents of some mycoplasmas are amongst the lowest known for prokaryotic or any other organisms (Jones & Walker, 1963; Neimark & Pene, 1965) and because of limits to the degeneracy of the genetic code.

Sterol-nonrequiring mycoplasmas. The sterol-nonrequiring organisms *Mycoplasma laidlawii* and *M. granularum* are physiologically distinct from all other mycoplasmas (Edward, 1967; Tully & Razin, 1968). Gel-electrophoresis patterns revealed some similarity in their proteins, and serological tests showed some sharing of antigens (Tully & Razin, 1968). My finding of a moderate amount of DNA duplex formation between *M. laidlawii* A and *M. granularum* agrees partially with the results of Pollock & Bonner (1969), who reported that DNA from *M. granularum* strain BTS-39 bound DNA from *M. laidlawii* A to somewhat less than one-fourth that of the homologous DNA. *M. laidlawii* and *M. granularum* are thus related.

Tully & Razin (1969) showed that two new sterol-nonrequiring isolates, S-410 and S-743, differed from *Mycoplasma laidlawii* and *M. granularum* by serological tests, their inability to synthesize pigmented carotinoids, and gel-electrophoresis patterns of cellular proteins; they also differed serologically from the known sterol-requiring mycoplasmas. My DNA hybridization experiments showed only very low levels of homology between S-743 and *M. laidlawii* or *M. granularum*. Isolate S-743 is distinct from *M. laidlawii* and *M. granularum* and is more distant from these two organisms than they are from one another. Tully and Razin (personal communication) propose to nominate this organism as a new species. The occurrence of additional sterol-nonrequiring mycoplasmas distinct from *M. laidlawii* gives further support for the establishment of a new genus in the order Mycoplasmatales (Edward *et al.* 1967; Tully & Razin, 1969). Recently, Edward & Freundt (1969) formally proposed a second family and genus within the Mycoplasmatales to recognize the distinction between the sterol-nonrequiring organisms and the rest of the group.

Fermentative mycoplasmas of low GC content. Strain B3 has been distinguished from mycoplasmas isolated from pigs (Dinter *et al.* 1965), and although a number of random samples of porcine and avian sera contained antibody to B3, no serological relationship with any strain was demonstrated (Taylor-Robinson & Dinter, 1968). Strain B3 is closely related to *Mycoplasma mycoides* var. *capri*, as indicated by the proximity of their GC contents and the high level of duplex formation between B3 and VOM DNA; Razin (1968) has also noted the similarity. Strain B3 and the strains of *M. mycoides* var. *capri* examined here are all rapidly growing, fermentative organisms and, with *M. mycoides* var. *mycoides*, form a cluster of organisms with GC contents within a 4 % span. Strain UM30847 is related to B3 and VOM and appears to be a member of this cluster (Neimark, 1969) even though growth inhibition tests by the author of UM30847

and B3 with *M. mycoides* var. *mycoides* and var. *capri* antisera were negative (sera kindly provided by Dr J. J. Callis, Plum Island Animal Disease Laboratory).

Although these organisms form a cluster they are distinguishable by DNA homology and serological procedures (Jonas & Barber, 1969). These differences may be similar to the species heterogeneity found in *Mycoplasma laidlawii* (Peterson & Pollock, 1969), *M. hominis* and *M. salivarium*, but not in *M. pneumoniae* (Reich *et al.* 1966; Somerson *et al.* 1966) or *M. hyorhinae* (Somerson *et al.* 1966).

Mycoplasma agalactiae and *M. agalactiae* var. *bovis*. Although the biological properties of these organisms are not inconsistent (Leach, 1967), no relationship has been demonstrated (Jain, Jasper & Dellinger, 1967); the similarities of gel-electrophoresis patterns (Razin, 1968) and GC content, shown here, are evidence of a relationship. Their classification as nonfermentative also must be re-examined, since *M. agalactiae* consistently produces slight acidity in glucose broth (Cottew *et al.* 1968) and strains of *M. agalactiae* var. *bovis* are known to produce an acid pH change in media containing glucose.

Fig. 1. Distribution of mole % GC contents among carbohydrate fermenting and nonfermenting mycoplasmas. The numbers over the bars refer to the subgroups discussed in the text. Bar No. 1 is *Mycoplasma pneumoniae*. The bracket, 2, indicates the span where sterol-nonrequiring organisms occur. Bars 3, 4, 5 and 6 are the organisms that mark the upper or lower ends of the other provisional subgroups.

Division of the mycoplasmas into subgroups. Values for the DNA base compositions of most of the recognized fermentative and nonfermentative mycoplasmas are in the literature (Table 4). The GC contents of the mycoplasmas form an almost continuous span from 23 to 35 %, followed by a gap with *Mycoplasma pneumoniae* set apart. The range of GC values for the fermentative and nonfermentative strains is shown graphically in Fig. 1. The arrangement of organisms by ability to ferment carbohydrates may reflect an evolutionary separation within the mycoplasmas (Neimark & Pickett, 1960). However, it should be recognized that the division is based only on results of fermentation tests and there may exist mycoplasmas that are phenotypically nonfermentative but genotypically fermentative; a possible example is the sterol-nonrequiring strain which cannot ferment glucose but which was identified as *M. laidlawii* (Leach, 1967; Tully & Razin, 1968), a carbohydrate-fermenting species.

Table 4. DNA base compositions of *Mycoplasma* strains

Species or designation	Strain	GC (mole %) by		Chroma- tography	References
		Density	T _m		
A. Fermentative Mycoplasma					
<i>M. pneumoniae</i> FH		40.8	39.0	—	Neimark & Pene, 1965; Neimark, 1967
	FH: 15531 ^a	—	39.9	—	Williams <i>et al.</i> 1969
	Bru: 15377	—	39.3 38.6	—	McGee <i>et al.</i> 1965; McGee <i>et al.</i> 1967
<i>M. gallisepticum</i>	801, PG 31, 1010	35.7	—	—	Kelton & Mandel, 1969
	A 5969	34.0	35.0	—	Marmur & Doty, 1962; Schildkraut <i>et al.</i> 1962
	S 6	35.2 35.2 34.6 ^b	—	—	Kelton & Mandel, 1969; Neimark, this paper; Reich <i>et al.</i> 1966
	So: 15302	—	32.5, 32.0	—	Rogul <i>et al.</i> 1965; McGee <i>et al.</i> 1967
	I 150	34.7	—	—	Kelton & Mandel, 1969
	A 5969	34.7 ^b 33.7	— 33.4	—	Morowitz <i>et al.</i> 1962; Morowitz <i>et al.</i> 1967
	PG 31: 19610	—	31.8	—	Williams <i>et al.</i> 1969
<i>M. laidlawii</i> A	—	35.7	31.7	—	Neimark, 1967
	—	35.7	30.9	—	Neimark & Pene, 1965
	—	—	34.1	—	Morowitz <i>et al.</i> 1967
	PG 8: 23206	—	33.0	—	Williams <i>et al.</i> 1969
	I 4089	35	32.5	—	Peterson & Pollack, 1969; McGee <i>et al.</i> 1967
	PG 10	34.2	—	—	Kelton & Mandel, 1969
<i>M. laidlawii</i> B	PG 9	33.6 ^b	—	33.3	Reich <i>et al.</i> 1966; Chelton <i>et al.</i> 1968
	PG 9: 23217	—	32.4	—	Williams <i>et al.</i> 1969
	I 4192	34	32.3	—	Peterson & Pollock, 1969; McGee <i>et al.</i> 1967
	—	33.7	33.2	—	Morowitz <i>et al.</i> 1967
<i>M. synoviae</i>	I 853	34.2	—	—	Kelton & Mandel, 1969
<i>M. granularum</i>	—	32.4	—	—	Neimark, this paper
	—	32.1	—	—	Kelton & Mandel, 1969
	BTS-39: 19168	—	30.4	—	McGee <i>et al.</i> 1967
S-410/S-743	—	31.3	—	—	Neimark, table 1 ^c
Avian serotype C	TU	30.5	—	—	Kelton & Mandel, 1969
	C	29.1	—	—	Kelton & Mandel, 1969
<i>M. edwardii</i>	PG 24 (C 21): 23462	—	29.2	—	Williams <i>et al.</i> 1969
Connecticut goat	—	29.8	—	—	Neimark, table 1
<i>Mycoplasma</i> sp. (goat) ^d	C 30 KS-1: 15718	—	28.9	—	Williams <i>et al.</i> 1969
<i>M. canis</i>	PG 14	29.1	28.4	—	Kelton & Mandel, 1969; McGee <i>et al.</i> 1967

Table 4 (*cont.*)

Species or designation	Strain	GC (mole %) by			References
		Density	T_m	Chromatography	
<i>M. pulmonis</i>	Sabin type C:	—	29.2	—	Williams <i>et al.</i> 1969
	23115°	—	—	—	—
	Negroni	—	28.3	—	McGee <i>et al.</i> 1967
	ASH(PG 34):	—	27.9	—	Williams <i>et al.</i> 1969
Avian serotype D	19612	—	—	—	—
	KON:14267	—	27.5	—	McGee <i>et al.</i> 1967
	PSU-4	28.6	—	—	Kelton & Mandel, 1969
	NY	28.1	—	—	Kelton & Mandel, 1969
<i>M. bovirhinis</i>	PG I I	28.1	—	—	Kelton & Mandel, 1969
	PG II:19852	—	30.4	—	Williams <i>et al.</i> 1969
	PG II:14173	—	29.6	—	McGee <i>et al.</i> 1967
<i>M. fermentans</i>	PG 18(G):19989	—	28.7	—	Williams <i>et al.</i> 1969
	G2	27.6	—	—	Neimark, 1967
	G	27.5 ^b	—	—	Reich <i>et al.</i> 1966
	GII:15474	—	27.8	—	McGee <i>et al.</i> 1967
<i>M. hyorhinis</i>	GDL	—	27.8	—	McGee <i>et al.</i> 1967
	BTS-7:17981	—	27.3	—	McGee <i>et al.</i> 1967
<i>Mycoplasma</i> sp. (sheep)	67-166:23243	—	27.2	—	Williams <i>et al.</i> 1969
<i>M. mycoides</i> var. <i>capri</i>	VOM	27.6	—	—	Neimark, table 1
	PG 3	—	—	26-24	Jones & Walker, 1963;
				24.8	Jones <i>et al.</i> 1965;
				25.0	Walker, 1967
Mexican goat	—	27.4	—	—	Neimark, table 1
Goat UM 30847	—	26.5	—	—	Neimark, table 1
<i>M. mycoides</i> var. <i>mycoides</i>	T3	—	—	30.0	Chelton <i>et al.</i> 1968
	GLADYS DALE	26.5	26.8	—	Neimark, 1967
	V5	—	26.1	—	McGee <i>et al.</i> 1967
California calf	—	26.5	23.6	—	Neimark & Pene, 1965;
		26.5	23.5	—	Neimark, 1967
Avian serotype F	SA	26.5	—	—	Kelton & Mandel, 1969
B3 (Dinter)	—	26.3	—	—	Neimark, table 1
<i>M. felis</i>	CO:23391	—	25.2	—	Williams <i>et al.</i> 1969
California goat	Kid	25.5	24.1	—	Neimark & Pene, 1965
Avian serotypes group I, J, K, N, Q, R	(J) 693	25.5	—	—	Kelton & Mandel, 1969
	(I) 695	24.5	—	—	Kelton & Mandel, 1969
	(K) 1805	24.5	—	—	Kelton & Mandel, 1969
		—	—	—	—
<i>M. bovirhinis</i>	PG 43	24.5	—	—	Kelton & Mandel, 1969
	PG 43 (5M331):	—	25.4	—	Williams <i>et al.</i> 1969
	19884	—	—	—	—
<i>M. neurolyticum</i>	Type A:19988	—	26.2	—	Williams <i>et al.</i> 1969
	Type A	22.8	—	—	Neimark, 1967
	KSA:15049	—	23.0	—	McGee <i>et al.</i> 1967

Table 4 (cont.)

Species or designation	Strain	GC (mole %) by		Chromatography	References
		Density	T _m		
B. Nonfermentative strains					
<i>M. agalactiae</i> ^t	—	34·2	33·6	—	Neimark, 1967
<i>M. agalactiae</i> var. <i>bovis</i> ^t	DONETTA	32·7	32·9	—	Neimark, table 1; Morowitz <i>et al.</i> 1967
Avian serotype L	694	32·1	—	—	Kelton & Mandel, 1969
<i>M. arthritis</i>	PG 27 ^g	32·6 ^b	—	—	Reich <i>et al.</i> 1966
	—	32·6	30·0	—	Morowitz <i>et al.</i> 1967
	07	32·1	—	—	Kelton & Mandel, 1969
	39	32·1	—	—	Kelton & Mandel, 1969
	H 606:13988	—	31·9	—	McGee <i>et al.</i> 1967
	CAMPO:14152 ^g	—	31·7	—	McGee <i>et al.</i> 1967
	PG 6:19611	—	31·3	—	Williams <i>et al.</i> 1969
Goat 145	—	29·6	—	—	Neimark, table 1
<i>M. maculosum</i>	PG 15	29·6	—	—	Kelton & Mandel, 1969
	PG 15:19327	—	26·7	—	McGee <i>et al.</i> 1967
<i>M. iners</i>	O	29·6	—	—	Kelton & Mandel, 1969
	PG 30	29·1	—	—	Kelton & Mandel, 1969
	PG 30(M): 19705	—	29·1	—	Williams <i>et al.</i> 1969
<i>M. spumans</i>	PG 13	29·1	—	—	Kelton & Mandel, 1969
	PG 13 (C 48): 19526	—	28·6	—	Williams <i>et al.</i> 1969
	PG 13:15147	—	28·4	—	McGee <i>et al.</i> 1967
<i>M. arginini</i>	G 230	28·6	—	—	Neimark, table 1
<i>M. meleagridis</i>	529	28·6	—	—	Kelton & Mandel, 1969
	N	28·1	—	—	Kelton & Mandel, 1969
<i>M. salivarium</i>	PG 20 (H 110): 23064	—	31·4	—	Williams <i>et al.</i> 1969
	PG 20	28·5 ^b	—	—	Reich <i>et al.</i> 1966
	MANIRE	—	27·3	—	McGee <i>et al.</i> 1967
Primate 747v	—	27·6	—	—	Neimark, table 1
<i>M. hominis</i>	H 39	31·6	33·7	—	Morowitz <i>et al.</i> 1967
<i>M. hominis</i> type 1	PG 21 (H 50): 23114	—	29·2	—	Williams <i>et al.</i> 1969
	H 34:15056	—	29·2	—	McGee <i>et al.</i> 1967
	4387:14027	—	28·7	—	McGee <i>et al.</i> 1967
	4330	27·3	—	—	Kelton & Mandel, 1969
<i>M. gateae</i>	CS:23392	—	28·5	—	Williams <i>et al.</i> 1969
T strains (seven human serotypes)	—	—	28·5–27·7	—	Bak & Black, 1968

Table 4 (cont.)

Species or designation	Strain	GC (mole %) by			References
		Density	T_m	Chromatography	
<i>M. gallinarum</i>	54-537	27.0	—	—	Kelton & Mandel, 1969
	PG 16:15319	—	28.0	—	Rogul <i>et al.</i> 1965;
			27.5		McGee <i>et al.</i> 1967
	PG 16:19708	—	27.0	—	Williams <i>et al.</i> 1969
	PG 16	26.5	—	—	Kelton & Mandel, 1969
<i>M. orale</i> type 1 ^h	CH 19299:	27.5 ^b	27.8	—	Reich <i>et al.</i> 1966;
	23714				Williams <i>et al.</i> 1969
	823 B:15539	—	26.6	—	McGee <i>et al.</i> 1967
<i>M. pharyngis</i> ^h	LGM:19524	—	27.0	—	McGee <i>et al.</i> 1967
	PATT:15544	—	23.9	—	Williams <i>et al.</i> 1969
<i>M. orale</i> type 2	DC 1600	26.5 ^b	26.4	—	Reich <i>et al.</i> 1966;
					Williams <i>et al.</i> 1969
	CH 20247:23636	—	26.1	—	Williams <i>et al.</i> 1969
<i>Mycoplasma</i> sp. (human)	NAVEL:15497	—	26.8	—	Williams <i>et al.</i> 1969
Primate s3-7	—	26.5	—	—	Neimark, table 1
Avian serotype M [†]	R49	25.0	—	—	Kelton & Mandel, 1969
Avian serotype E	HPR-15	24.0	—	—	Kelton & Mandel, 1969

^a Numbers after colons in strain designations are American Type Culture Collection numbers.

^b Calculated from data in reference cited, taking *Escherichia coli* density as 1.710 g./cm³.

^c Table 1 refers to results reported in this paper.

^d This strain was believed to be a subculture of Cordy & Adler's goat pathogen (Williams *et al.* 1969); another subculture appears in the table as California goat, kid strain.

^e Sabin type C, *Mycoplasma histotropicum*, has been shown to be identical with *M. pulmonis* (Lemcke, Forshaw & Fallon, 1969).

^f These strains have been reported to produce slight acidity in glucose broth (see text).

^g Formerly *M. hominis* type 2.

^h The names *M. pharyngis* and *M. orale* type 1 are synonyms; the epithet *orale* is favoured (D. G. ff. Edward & E. A. Freundt, personal communication).

ⁱ Avian strain R 49, from which all serotype M cultures are derived, is a mixed culture that contains *M. granularum* and an unknown fermenter (J. Fabricant, personal communication). However, density gradient centrifugation of DNA from R49 did not reveal significant contamination.

Mycoplasma neurolyticum and *M. pneumoniae*, at the extremes of the span of base compositions of fermentative organisms, are approximately 17 % apart in GC content, and both differ by more than 10 % from several other fermentative mycoplasmas. Since bacteria with GC contents differing by more than 10 % would be expected to have little genetic relatedness (Sueoka, 1961), the fermentative mycoplasmas may contain at least two unrelated subgroups.

Mycoplasma pneumoniae is not related physiologically or by nucleic acid homology to any of the nearby sterol-nonrequiring mycoplasmas (McGee *et al.* 1967; Table 2). Only rather low-level DNA reactions were found by the DNA-agar method between *M. pneumoniae* and *M. gallisepticum* (separated by ca. 5 % in GC content) and

M. fermentans (differing by ca. 12 %) (McGee *et al.* 1967); a DNA-RNA method did not detect any reaction between *M. pneumoniae* and *M. fermentans* (Reich *et al.* 1966). Consequently, *M. pneumoniae* is distinct from the middle and low-range GC mycoplasmas. Even excluding *M. pneumoniae* and the sterol-nonrequiring subgroup, the fermentative mycoplasmas still are heterogeneous with a span of 12 %; probably *M. gallisepticum* (GC ca. 35 %) at the upper extreme is unrelated to *M. neurolyticum* at the lower extreme.

The nonfermentative mycoplasmas span a range of 10 % in GC content, and here, too, the organisms at opposite ends of the span would fall into separate subgroups; the range of GC content will be contracted if *M. agalactiae* (and possibly *M. agalactiae* var. *bovis*) is proved to ferment carbohydrates. This division contains the human T strain mycoplasmas (Shepard, 1967; Taylor-Robinson, Williams & Haig, 1968), of which seven have the same base composition but are serologically distinct (Bak & Black, 1968).

Of those nonfermentative organisms examined for DNA-RNA homology, *Mycoplasma salivarium*, *M. orale* types 1 and 2, *M. hominis* and *M. arthritidis* were readily separated, although low-level cross-reactions were demonstrated, in agreement with serological findings (Reich *et al.* 1966). These organisms fall within a span of 26 to 32 % in GC content and utilize arginine (Barile, Schimke & Riggs, 1966); they also show low-level DNA homology with *M. fermentans*, an arginine-utilizing, carbohydrate-fermenting Mycoplasma (Reich *et al.* 1966). DNA homology studies (McGee *et al.* 1967; Pollock & Bonner, 1969; Peterson & Pollock, 1969) reveal no reaction between fermentative and nonfermentative organisms except for a low-level cross-reaction between *M. fermentans* and *M. gallinarum* (McGee *et al.* 1967).

To provide 'working' units for studying relationships, the mycoplasmas can be divided tentatively into six subgroups:

- (1) *Mycoplasma pneumoniae*. GC content ca. 40 %. Set apart from all other mycoplasmas.
- (2) Sterol-nonrequiring mycoplasmas. Contains *M. laidlawii*, *M. granularum*, and strains s-743/s-410. GC ca. 31 to 35 %.
- (3) High GC fermentative organisms. GC ca. 35 % and less. Contains *M. gallisepticum*.
- (4) Low GC fermentative organisms. GC ca. 23 % and greater. Contains *M. neurolyticum*.
- (5) High GC nonfermentative organisms. GC ca. 34 % and less. Contains *M. agalactiae*.
- (6) Low GC nonfermentative organisms. GC ca. 24 % and greater. Contains strain HPR-15, avian serotype E. If *M. agalactiae* and *M. agalactiae* var. *bovis* prove to be fermentative, the distinction between subgroups 5 and 6 will depend on there being no DNA homology between strain HPR-15 and *M. arthritidis*.

The demonstration of homology between fermentative and nonfermentative mycoplasmas would be less surprising than the finding of a relationship between organisms at extremes of the GC range, for the latter would contradict accepted principles of molecular genetics.

Serology and homology studies generally agree in instances where comparable data are available (Reich *et al.* 1966; Somerson *et al.* 1967). *Mycoplasma gallisepticum* and *M. synoviae* might be compared further, since they are serologically related (Olsen,

Yamamoto & Ortmayer, 1965) and their GC contents are not far apart; *M. hominis* and *M. gateae* also share antigens (Cole, Golightly & Ward, 1967) and differ by about 1% in GC content. Serological cross-reactions are known for mycoplasmas that otherwise appear unrelated: for example, between *M. pneumoniae* and *M. mycoides* (Lemcke, Marmion & Plackett, 1967) and between *M. iners* and avian serotype E (Kelton & Mandel, 1969). Kenny (1969) reported that five fermentative organisms with GC contents of 23 to 28% were serologically related, while *M. pneumoniae* and *M. gallisepticum* were distinct; this supports the proposed subgroup divisions.

Significance of Mycoplasma subgroups. Genetic heterogeneity is a major feature of the mycoplasmas, but its origins are obscure. This diversity may indicate that mycoplasmas are derived from a variety of sources or, alternatively, may merely reflect breadth developed through evolution from a single source. Whether mycoplasmas share ancestors with or are ancestral to bacteria, or whether they developed from bacteria is uncertain; at present there is no evidence that they arose by simple discontinuous events from bacteria. The hypothesis that mycoplasmas are L-forms of bacteria is not supported by homology studies (McGee, Rogul, Falkow & Wittler, 1965; Somerson *et al.* 1967; McGee *et al.* 1967; Neimark, 1967). Also, the mycoplasmas with uniquely low GC contents do not appear to be stable L-forms of known bacteria (Jones & Walker, 1963; Neimark & Pene, 1965).

Theoretically the mycoplasmas could have developed through mutants with alterations in GC content; reports of such mutants in bacteria have not been confirmed (see Neimark, 1967; and Mandel, 1969). One stable L-form of *Streptococcus faecalis* lacks some 4 to 6% of DNA sequences present in the parent (Hoyer & King, 1969); such deletion mutants could conceivably have been factors in the development of mycoplasmas.

On the other hand, if the Mycoplasma are a true biological group, it should be possible to demonstrate some common fundamental properties in addition to the absence of a cell wall. One property that has been examined is genome size. Values for seven strains ranged from 4.4×10^8 to 1.2×10^9 daltons (Bode & Morowitz, 1967; Morowitz, Bode & Kirk, 1967; Ryan & Morowitz, 1969). Bak, Black, Christiansen & Freundt (1969) determined genome sizes in 12 mycoplasmas, and their results were partly in accord with those of Morowitz *et al.* (1967); however, the mycoplasmas fell into only two groups, each composed of strains with identical genome size. One group containing the sterol-nonrequiring organisms had genome sizes of about 1.0×10^9 daltons (cf. *Haemophilus influenzae*, 7.0×10^8 to 1.0×10^9 daltons). All the serum-requiring strains examined were in the second group, with genome sizes of about 4.6×10^8 daltons, a value half that for the sterol-nonrequiring strains and smaller than any known for bacteria. The finding of identical genome size may reflect common phylogenetic origin (Bak *et al.* 1969; Peterson & Pollock, 1969; Mandel, 1969); the discontinuity between the two groups implies that organisms in each may have arisen or developed independently. These findings, if confirmed and shown to be representative of the entire Mycoplasma group, would indicate that the extensive genetic heterogeneity observed in GC content does not extend to genome size and would provide a fundamental trait with which homogeneous divisions among the mycoplasmas could be established. If the mycoplasmas still to be examined should fall into additional groups, then the possibility would have to be considered that the Mycoplasma group is composed of clusters of organisms that arose and evolved independently.

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