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Genetic Differentiation by Nucleic Acid Homology

II. Genotypic Variations Within Two *Mycoplasma* Species

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ABSTRACT

SOMERSON, NORMAN L. (National Institutes of Health, Bethesda, Md.), PAUL R. REICH, BARBARA E. WALLS, ROBERT M. CHANOCK, AND SHERMAN M. WEISSMAN. Genetic differentiation by nucleic acid homology. II. Genotypic variations within two *Mycoplasma* species. J. Bacteriol. **92**:311-317. 1966.—A deoxyribonucleic-ribonucleic acid (DNA-RNA) homology technique was used to determine genetic relatedness among the nucleic acids of eight mycoplasmas which were serologically classified as *Mycoplasma hominis* type 1. The DNA preparations from these organisms were each found to be distinct. No subgrouping of the *M. hominis* type 1 strains could be demonstrated. In contrast, when the nucleic acids from six serologically related mycoplasmas which were isolated from tissue cultures were studied, the DNA from these species could not be distinguished. The DNA buoyant densities of the tissue culture isolates were similar. These isolates were closely related genetically to a porcine mycoplasma, *M. hyorhinis*.

The genetic heterogeneity of four mycoplasmas, classified serologically as *Mycoplasma Hominis* type 1 (17), has been demonstrated by use of the nucleic acid homology technique (15). In the present report, nucleic acids derived from additional isolates serologically classified as *M. hominis* type 1 are shown to be different.

In our earlier publication (15), we also reported that four serologically related mycoplasmas isolated from cell cultures inoculated with human tissue (1) were genetically closely related to one another but distinct from the other human *Mycoplasma* species. These genetically similar mycoplasmas were recently shown to be serologically related to new isolates obtained from "uninoculated" tissue cultures (5). Two of the new mycoplasmas isolated from tissue cultures were included in a more complete analysis in which complementary ribonucleic acid (RNA) synthesized in vitro with template deoxyribonucleic acid (DNA) from each isolate was tested for DNA-RNA hybrid formation with DNA from each strain. Unlike our results with the *M. hominis* type 1 species, we could not distinguish nucleic acids derived from the two new isolates or from the tissue culture isolates we had previously tested.

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MATERIALS AND METHODS

Organisms. Five *M. hominis* type 1 strains were isolated from the oropharynx of patients at D.C. General Hospital in Washington, D.C.; one of these isolates, strain DC63, was recovered from a man with pneumonia (9). A sixth *Mycoplasma*, strain V2785, was isolated from the oropharynx of a volunteer subject. Strains LBD-4 and LBD-5 were cultured from human blood, the former from that of a patient with septicemia (19). Growth inhibition tests (4) were performed on all eight strains, and the results confirmed their close antigenic relationship to *M. hominis* type 1.

The sources of the *Mycoplasma* tissue culture isolates are listed in Table 1. The close serological relationships among these isolates have been reported elsewhere (1, 5).

Two porcine mycoplasmas, *M. hyorhinis* (strain 7) and *M. granularum* (strain 39), were obtained by R. H. Purcell from Dr. Switzer.

Cultivation techniques and DNA preparation. Details concerning the preparation of medium, methods of cultivation, and procedures for DNA extraction have been presented elsewhere (15, 17).

Buoyant density determination. Buoyant densities were determined as outlined in the preceding paper (14).

TABLE 1. *Source of unclassified tissue culture isolates*

Strain	Other designation	Recovered from	Material added to tissue culture	Obtained from (reference)
GDL	Acid-inducing agent	HEp-2	None	Leach (2)
Wistar 3	83810, FS-3	WI-26	None	Hayflick (5)
F-7	T-7, Cincy tumor 7	HEp-2	Thymic granuloma-tumor extract	Somerson and Lewis (*unpublished data*)[a]
F-11[b]	—	WI-38	Hemangioma-cell culture	Hayflick (1)
F-12	—	WI-38	Pharyngeal fibroma-cell culture	Hayflick (1)
F-13	—	HEp-2	Bone marrow	Somerson and Smith (*unpublished data*)[c]

[a] Isolated from tissue culture fluids supplied by Dr. Sabin.

[b] The acid-inducing F-11 strain was used in this study. A strain which did not induce acid formation and which is antigenically related to *M. hominis* type 1 has also been isolated from F-11 cultures (1).

[c] Strain F-13 was isolated from a tissue culture which had received bone marrow. Control HEp-2 cultures were contaminated with mycoplasmas which were serologically indistinguishable from the F-13 isolates. These mycoplasmas are all presumed to have been present in the tissue cultures and not the result of the bone marrow inoculum.

DNA-RNA homology technique. Methods for the synthesis of complementary RNA, for the formation and the detection of DNA-RNA hybrids, and for the application of analysis of variance have been detailed in an earlier publication (15, 16) and briefly reviewed in the preceding report (14).

RESULTS

Genetic heterogeneity among strains of M. hominis type 1 species. Complementary RNA was synthesized with template DNA from each of eight *M. hominis* type 1 isolates and was tested with DNA from each strain for its ability to form DNA-RNA hybrids. The results are listed in Table 2 as observed geometric mean counts. Also shown are the geometric mean counts that would be expected if all the DNA preparations and all the RNA preparations were identical. The interaction values, the ratios of the observed to expected means, ranged from 1.20 to 1.97 for all the homologous DNA-RNA reactions, all approximately equal to or greater than the upper 95% confidence limit (1.22) for indistinguishable nucleic acids. In contrast, the majority of the interaction values for heterologous DNA-RNA reactions were 1.00 or less. With one possible exception (LBD-4 DNA with DC63 RNA), this pattern of interaction values indicated that nucleic acids derived from all of these mycoplasmas could be distinguished from one another.

The relatedness values, measurements of the degree of genetic similarity between two DNA preparations, for the *M. hominis* type 1 strains (Table 3), ranged from 0.17 to 0.90. Except in one case (LBD-4 DNA and DC63 RNA), all of the values fell below the 95% confidence limit for indistinguishable nucleic acid preparations.Thus, at least seven of the eight strains were genetically distinct.

The high relatedness value (0.90) between strains LBD-4 and DC63 resulted mainly from the high yield of the reaction between LBD-4 DNA and DC63 RNA. The interaction value (1.27) for this combination was actually larger than the value for reactions between homologous LBD-4 and DC63 nucleic acids. However, the interaction value for the reciprocal test, DC63 DNA with LBD-4 RNA, was 1.07. These findings suggested that the high relatedness value resulted from a technical error. Therefore, another assay of the degree of relatedness between LBD-4 and DC63 was performed.

RNA was synthesized with template DNA from DC63, LBD-4, and LBD-5; the latter was included as a control nucleic acid which was different from the other two strains. Each RNA was tested with each DNA for its ability to form hybrids, and the results were subjected to statistical analysis (Table 4). The pattern of high interaction values for homologous reactions (1.31 to 1.51), and low values for heterologous reactions (0.79 to 0.94), indicated that nucleic acids derived from strains DC63, LBD-4, and LBD-5 were different. The relatedness values were: DC63 and LBD-4, 0.48; LBD-4 and LBD-5, 0.32; and DC63 and LBD-5, 0.35. All values were below the lower 95% confidence limit (0.77) for indistinguishable nucleic acid preparations. In an additional experiment, each of two preparations of strain DC63 were again shown to be distinguishable from strain LBD-4 (relatedness values, 0.39 and 0.50). The latter results strongly suggest that the original high interaction value found for the DC63 RNA–LBD-4 DNA combination probably resulted from a technical error. Thus, the DC63

TABLE 2. *Mean counts of radioactivity on filters after reaction between DNA from each of eight strains of Mycoplasma hominis type 1 and radioactive RNA synthesized with each of these DNA preparations as primer*

Source of template for RNA[a]	Value	Source of *M. hominis* type 1 DNA tested								Observed row geometric mean
		V2785	DC35	DC63	DC100	DC242	DC926	LBD-4	LDB-5	
V2785	Observed mean count[b]	5,729	2,906	3,087	3,686	3,679	3,343	2,914	3,201	3,484
	Expected mean count[c]	3,703	3,270	3,442	4,105	3,647	3,348	3,027	3,439	
	Interaction value[d]	*1.55*	0.89	0.90	0.90	1.01	1.00	0.96	0.93	
DC35	Observed mean count	247	455	193	242	226	243	262	224	253
	Expected mean count	269	238	250	298	265	243	220	250	
	Interaction value	0.92	*1.91*	0.77	0.81	0.85	1.00	1.19	0.90	
DC63	Observed mean count	2,689	2,246	3,234	2,592	2,696	2,491	2,877	2,236	2,615
	Expected mean count	2,778	2,455	2,583	2,081	2,737	2,512	2,272	2,580	
	Interaction value	0.97	0.92	*1.25*	0.84	0.99	0.99	1.27	0.87	
DC100	Observed mean count	1,534	1,128	1,391	3,154	1,065	1,397	1,027	1,013	1,360
	Expected mean count	1,445	1,277	1,344	1,603	1,424	1,307	1,182	1,342	
	Interaction value	1.06	0.88	1.03	*1.97*	0.75	1.07	0.87	0.75	
DC242	Observed mean count	256	185	320	454	539	229	262	377	310
	Expected mean count	329	291	306	365	324	297	269	306	
	Interaction value	0.78	0.64	1.05	1.24	*1.67*	0.77	0.98	1.23	
DC926	Observed mean count	4,562	3,859	4,363	4,862	4,480	6,199	3,836	4,578	4,546
	Expected mean count	4,829	4,267	4,491	5,355	4,757	4,367	3,949	4,485	
	Interaction value	0.95	0.90	0.97	0.91	0.94	*1.42*	0.97	1.02	
LBD-4	Observed mean count	4,546	4,948	5,104	4,589	5,104	4,910	5,055	4,503	4,839
	Expected mean count	5,142	4,542	4,780	5,702	5,064	4,649	4,204	4,775	
	Interaction value	0.88	1.09	1.07	0.81	1.01	1.06	*1.20*	0.94	
LBD-5	Observed mean count	3,268	3,181	2,963	3,142	3,046	2,296	1,763	4,461	2,923
	Expected mean count	3,105	2,743	2,887	3,443	3,058	2,808	2,539	2,884	
	Interaction value	1.05	1.16	1.03	0.91	1.00	0.82	0.69	*1.55*	
	Observed column geometric mean	1,781	1,574	1,656	1,975	1,755	1,611	1,457	1,655	1,677[e]

[a] DNA (4 μg) was incubated for 16 hr (triplicate assays) with 60,000 to 190,000 counts/min of radioactive complementary RNA.
[b] Geometric mean of counts per minute retained per 4 μg of DNA tested for each set of triplicate experiments.
[c] Geometric mean expected, calculated as the ratio of the product of the row and column means to the grand geometric mean.
[d] The ratio of observed to expected mean. If a given DNA has the same proportional effect on both RNA preparations, and vice versa, the 95% confidence interval for the ratios is 0.82 to 1.22, and the 99% confidence interval is 0.77 to 1.30.
[e] Grand geometric mean.

TABLE 3. *Relatedness values for Mycoplasma hominis type 1 isolates*

Mycoplasma strain	*M. hominis* type 1 relatedness values[a]						
	Mycoplasma strain						
	V2785	DC35	DC63	DC100	DC242	DC926	LBD-4
DC35	.28						
DC63	.45	.29					
DC100	.31	.19	.35				
DC242	.31	.17	.50	.28			
DC926	.43	.33	.54	.35	.31		
LBD-4	.46	.56	.90	.30	.49	.60	
LBD-5	.41	.35	.46	.23	.48	.38	.35

[a] Relatedness value, calculated as the ratio of the product of the observed mean counts in heterologous reactions to the product of the observed mean counts in homologous reactions. The 95% confidence interval calculated for indistinguishable DNA preparations is 0.63 to 1.58; the 99% confidence interval is 0.55 to 1.82.

and LBD-4 strains of *M. hominis* type 1 species could be distinguished from one another, and all eight strains exhibited genetic heterogeneity.

Genetic homogeneity of several unclassified Mycoplasma tissue culture isolates. In an earlier publication, four unclassified mycoplasmas isolated from tissue cultures were shown by DNA-RNA homology to be genetically related (15). We have now included two additional strains in a detailed study of the degree of relatedness among mycoplasmas isolated from uninoculated tissue cultures and from cultures which had received tumor material (5). Complementary RNA was prepared with template DNA from each of six mycoplasmas. In addition, RNA was synthesized with template DNA prepared on two different occasions from the same *Mycoplasma* (GDL). Each RNA preparation was incubated separately with each DNA under conditions necessary for formation and detection of DNA-RNA duplexes. The interaction values (Table 5) calculated for both heterologous and homologous reactions were not significantly different from 1.00. In contrast to the heterogeneity shown with *M. hominis* type 1 strains, all nucleic acids from these tissue culture isolates were indistinguishable.

Relationship of the porcine mycoplasma M. hyorhinis to the unclassified tissue culture isolates. Recent serological evidence has suggested that these tissue culture isolates are related to a porcine species, *M. hyorhinis* (13, 18). Therefore, we incubated nucleic acids derived from two tissue culture isolates, Wistar 3 and F-7, with nucleic acids from two porcine species, *M. hyorhinis* strain 7 and *M. granularum* strain 39. Nucleic acids from *M. granularum* did not react with any of the DNA or RNA preparations

TABLE 4. *Mean counts of radioactivity on filters after reaction between DNA from each of three strains of Mycoplasma hominis type 1 and radioactive RNA synthesized with each of these DNA preparations as primer*

Source of primer for radioactive RNA[a]	Value	*Mycoplasma* DNA						Row geometric mean
		LBD-4		LBD-5		DC63		
		Mean count	Interaction value	Mean count	Interaction value	Mean count	Interaction value	
LBD-4	Observed	8,061		4,730		5,010		5,759
	Expected	5,972		6,027		5,309		
			1.35		0.79		0.94	
LBD-5	Observed	3,838		7,119		3,380		4,520
	Expected	4,686		4,729		4,166		
			0.82		*1.51*		0.81	
DC63	Observed	2,445		2,308		3,137		2,606
	Expected	2,702		2,727		2,402		
			0.91		0.85		*1.31*	
	Observed column geometric mean	4,229		4,268		3,759		4,078[b]

[a] DNA (4 μg) was incubated for 16 hr (triplicate assays) with 120,000 to 190,000 counts/min of radioactive complementary RNA. The design and presentation of this experiment are analogous to those of Table 2. The 95% confidence interval for indistinguishable nucleic acids is 0.94 to 1.06 and the 99% confidence interval is 0.92 to 1.09.

[b] Grand geometric mean.

TABLE 5. *Mean counts of radioactivity on filters after reaction between DNA from each of seven tissue culture isolates and radioactive RNA synthesized with each of these DNA preparations as primer*

Source of template for radioactive RNA[a]	Value	Source of DNA tested							Observed row geometric mean
		Wistar 3	F-7	F-11	F-12	F-13	GDL-1	GDL-2	
Wistar 3	Observed mean count	8,417	14,614	13,070	17,646	11,088	14,821	7,732	12,011
	Expected mean count	8,092	14,412	12,736	17,691	11,529	14,521	8,195	
	Interaction value	*1.04*	1.01	1.03	1.00	0.96	1.02	0.94	
F-7	Observed mean count	7,460	12,600	10,655	15,168	10,155	12,116	6,298	10,237
	Expected mean count	6,897	12,283	10,855	15,088	9,826	12,377	6,985	
	Interaction value	1.08	*1.03*	0.98	1.01	1.03	0.98	0.90	
F-11	Observed mean count	8,358	15,203	13,698	18,220	12,294	15,759	7,696	12,485
	Expected mean count	8,412	14,982	13,239	18,390	11,985	15,095	8,519	
	Interaction value	0.99	1.02	*1.04*	0.99	1.03	1.04	0.90	
F-12	Observed mean count	7,912	13,742	14,280	17,138	11,416	15,200	8,211	12,097
	Expected mean count	8,150	14,516	12,827	17,828	11,612	14,626	8,254	
	Interaction value	0.97	0.95	1.11	*0.96*	0.98	1.04	1.00	
F-13	Observed mean count	8,403	16,831	12,693	18,585	12,139	13,948	2,652	12,544
	Expected mean count	8,452	15,052	13,302	18,477	12,041	15,167	8,555	
	Interaction value	0.99	1.12	0.95	1.01	*1.01*	0.92	1.01	
GDL-1	Observed mean count	6,705	9,984	8,494	14,814	9,182	13,615	7,110	9,595
	Expected mean count	6,465	11,514	10,174	14,133	9,210	11,601	6,547	
	Interaction value	1.04	0.87	0.84	1.05	1.00	*1.17*	1.09	
GDL-2	Observed mean count	6,706	13,792	12,774	16,265	10,601	11,506	9,053	11,135
	Expected mean count	7,502	13,362	11,807	16,401	10,688	13,462	7,598	
	Interaction value	0.89	1.03	1.08	0.99	0.99	0.86	*1.19*	
	Observed column geometric mean	7,675	13,669	12,076	16,779	10,934	13,772	7,773	11,391[b]

[a] DNA (4 μg) was incubated for 8 hr (duplicate assays) with 35,000 to 55,000 counts/min of radioactive complementary RNA. The design and presentation of this experiment are analogous to those in Table 2.

[b] Grand geometric mean.

TABLE 6. *Mean counts of radioactivity on filters after reaction between DNA from strain Wistar-3, F-7, Mycoplasma hyorhinis and radioactive RNA synthesized with each of these DNA preparations as primer*

Source of primer for radioactive RNA[a]	Value	Source of DNA tested						Row geometric mean
		Wistar-3		F-7		*M. hyorhinis*		
		Mean count	Interaction value	Mean count	Interaction value	Mean count	Interaction value	
Wistar 3	Observed	4,657		6,411		1,655		3,669
	Expected	4,713		5,725		1,831		
			0.99		1.12		0.90	
F-7	Observed	5,754		6,968		2,303		4,520
	Expected	5,805		7,052		2,255		
			0.99		0.99		1.02	
M. hyorhinis	Observed	3,054		3,286		1,259		2,329
	Expected	2,992		3,634		1,162		
			1.02		0.90		1.08	
	Observed column geometric mean	4,342		5,275		1,687		3,381[b]

[a] DNA (4 μg) was incubated for 16 hr (triplicate assays) with 90,000 to 120,000 counts/min of radioactive complementary RNA. The 95% confidence interval for indistinguishable nucleic acids is 0.93 to 1.07 and the 99% confidence interval is 0.91 to 1.10.

[b] Grand geometric mean.

TABLE 7. *Buoyant densities of deoxyribonucleic acid obtained from tissue culture isolates*

Source of *Mycoplasma* DNA	Buoyant density (g/cc)
F-7	1.6846
F-11	1.6847, 1.6846
F-12	1.6852, 1.6854
F-13	1.6857
GDL	1.6857

derived from the other three mycoplasmas. The mean counts and interaction values for DNA-RNA reactions among Wistar 3, F-7, and *M. hyorhinis* nucleic acid preparations are shown in Table 6. Three interaction values were outside the 95% confidence interval for indistinguishable nucleic acid preparations. However, the relatedness values for these strains were: Wistar 3 and F-7, 1.14; *M. hyorhinis* and Wistar 3, 0.86; and *M. hyorhinis* and F-7, 0.86; all values were within the 95% confidence interval for indistinguishable nucleic acid preparations. Although we are uncertain as to the reason for the significant deviation of three of the interactions, we can conclude that the three strains are closely related, if not identical.

Buoyant density determination on DNA from tissue culture Mycoplasma isolates. The densities of all *Mycoplasma* tissue culture isolates were very close to each other, ranging from 1.6846 to 1.6857 g/cc (Table 7).

DISCUSSION

The genetic heterogeneity described earlier with four *M. hominis* type 1 strains (15) has now been shown to include eight isolates. We could distinguish each of the eight strains included in the present study from the others, and could not divide these strains into subgroups. A number of differences have been reported among *Mycoplasma* strains serologically classified as *M. hominis* type 1. Kraemer (7) noted that one of four strains of this species produced a lytic reaction in murine lymphoma cell lines. Herderschee, Ruys, and van Rhijn (6) described morphological differences between tissue culture and genital strains of *M. hominis* type 1. Antigenic strain variation was reported by Nicol and Edward (10) and more recently has been demonstrated by the metabolic inhibition technique (12). Possibly, the diversity in the genetic material of *M. hominis* type 1 isolates may be phenotypically expressed as strain variations in pathogenicity (9, 19), or in the ability of these organisms to survive in both the oral and genital tracts of man, as well as in different tissues grown in culture (3, 6, 11).

In contrast to the genetic heterogeneity shown with *M. hominis* type 1 strains, several mycoplasmas isolated from HEp-2 and human diploid tissue cultures appeared to be indistinguishable by the homology technique. However,

they were closely related to a porcine mycoplasma. These organisms were found in uninoculated control tissue cultures and in tissues which had received human tumor material. They were isolated in several laboratories from several different tissues. Antigenic studies of strains Wistar 3, GDL and F-7, F-11 (acid-inducing strain), and F-12 revealed significant cross-reactions among these organisms (5). The inability to distinguish among these isolates was paralleled by a close similarity in DNA buoyant densities.

The origin of these tissue culture isolates is unknown. The isolation of these organisms may result from (i) a latent *Mycoplasma* tissue culture contaminant which, in some cases, becomes unmasked, as Girardi et al. (5) have suggested; (ii) infection of man by a mycoplasma which is closely related to mycoplasmas isolated from lower animal species; or (iii) a simple laboratory contaminant of tissue cultures. The close relationship between other tissue culture isolates, Negroni agent, and *Mycoplasma* strain 880, and the rat species, *M. pulmonis* (8) might be similarly explained.

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