Nucleic Acid Homologies of Selected Bacteria, L Forms, and *Mycoplasma* Species¹

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ABSTRACT

ROGUL, M. (Walter Reed Army Institute of Research, Washington, D.C.), Z. A. MCGEE, R. G. WITTLER, AND STANLEY FALKOW. Nucleic acid homologies of selected bacteria, L forms, and *Mycoplasma* species. *J. Bacteriol.* **90**:1200-1204. 1965.—The molar per cent of guanine plus cytosine (G + C) in the deoxyribonucleic acids (DNA) of *Proteus mirabilis*, strain 9, and its stable L form was determined by thermal denaturation and found to be approximately 39.5% G + C. The DNA homologies of this bacterium and its L form were estimated by the agar-column technique and were equivalent in their abilities to anneal and form specific duplexes. The next series of comparisons were performed between two *Mycoplasma* species and their often suggested bacterial parent. The G + C ratios of *M. gallisepticum* (32.7%), *M. gallinarum* (28.1%), and *Haemophilus gallinarum* (41.9%) varied to a high degree. In the homologous system, the denatured DNA of *H. gallinarum* trapped in agar bound approximately 40% of its sheared, denatured, and H³-labeled DNA. In comparison, the nucleic acids of *M. gallinarum* and *M. gallisepticum* were incapable of binding the labeled DNA of *H. gallinarum*. These findings provided evidence that the two strains of *Mycoplasma* were not derived from *H. gallinarum*.

The only universally accepted criterion differentiating stable L forms from species of *Mycoplasma*, commonly termed pleuropneumonia-like organisms (PPLO), is the knowledge that the L form was previously derived from a bacterium, whereas the origin of *Mycoplasma* species is, as yet, unresolved. Otherwise, strains from these two groups are frequently indistinguishable in their morphological and cultural characteristics. Heretofore, attempts to relate species of *Mycoplasma* with bacteria by using conventional taxonomic criteria have been unsuccessful (Freundt, 1958; Smith and Rothblat, 1960; Smith, 1964). Evidence for the relatedness of certain mycoplasmas to bacteria, derived from transformation experiments and observations of reversions from mycoplasmas to bacteria, still remains equivocal (Wittler, Cary, and Lindberg, 1956; Smith, Peoples, and Morton, 1957; Kelton, Gentry, and Ludwig, 1960).

In recent years, techniques and information have become available which have further aided in relating and distinguishing various groups of

organisms. It has been shown that most organisms which are capable of genetic interchange also have similar molar per cent base ratios [expressed as guanine plus cytosine (G + C) ratio] in their deoxyribonucleic acids (DNA) (Catlin and Cunningham, 1961; Marmur, Seaman, and Levine, 1963; Catlin and Cunningham, 1964). The converse of this statement is not necessarily true and only serves as a predictive hypothesis in aiding systematic classification. In addition, the agar-column hybridization technique (McCarthy and Bolton, 1963) has made possible a method for determining the ability of denatured nucleic acid fragments to bind and form specific duplexes with complementary nucleotide sequences of long, single-stranded nucleic acids immobilized in agar. The comparison of binding abilities of DNA fragments to DNA strands from homologous and heterologous organisms has then been interpreted in terms of complementary nucleotide sequence and genetic homology.

It was then assumed that if mycoplasmas are naturally evolved L forms of bacteria their base composition and duplex formation of DNA might have the same or similar relationships as a bacterium and its artificially produced stable L form.

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In the present study, we have used the G + C ratio (as determined by thermal denaturation) and per cent DNA duplex formation to predict and determine the relative homology between a bacterium, *Proteus mirabilis* strain 9, and its stable L form, *P. mirabilis* L-9. The same rationale was used to determine whether there was any genetic homology between *Haemophilus gallinarum* and *M. gallinarum* or *M. gallisepticum* (Edward and Kanarek, 1960). These organisms were chosen for study because they are all inhabitants of chickens, they have been implicated in fowl coryza, they have similar saccharolytic properties, and evidence has been presented concerning the reversion and transformation of avian mycoplasmas to *H. gallinarum* (McKay and Taylor, 1954; McKay and Truscott, 1960).

MATERIALS AND METHODS

Organisms and cultural conditions. The organisms were obtained from the American Type Culture Collection (ATCC). *P. mirabilis* 9 (ATCC 14273) was grown in Heart Infusion (HI) Broth (Difco) containing 0.5% glucose and 0.5% yeast extract (Difco). Radioactive labeling of the bacterial DNA was accomplished by adding 100 μ C of C^{14} -uracil to a minimal broth containing inorganic salts (Johnson, Falkow, and Baron, 1964), 20 μ g/ml of nicotinic acid, 0.05% Casamino Acids, and 0.3% glycerine. Controls for purity of culture were performed by examination of Gram-stained films from harvest broths and subcultures on Phenylethanol Blood Agar (Difco), blood-agar, and MacConkey Agar (Difco).

The stable L form of *P. mirabilis* 9 (ATCC 14166) was cultivated in HI Broth containing 16% human serum and 4% (v/v) of a 10% (w/v) solution of yeast extract (Oxoid). The purity of harvest broths was monitored by examination of Gram-stained films and cultivation on Phenylethanol Blood Agar, blood-agar, MacConkey Agar, and Heart Infusion Agar with the same supplements as the broth.

H. gallinarum (ATCC 14385) was grown in 2.0% Tryptose Broth (Difco) containing 0.8% NaCl, 0.5% dextrose, 0.1% NaHCO_3 , and 5.0 μ g/ml of reduced nicotinamide adenine dinucleotide (NADH_2). A 600-ml amount of this medium was supplemented with 2 ml of H^3 -thymidine for the purpose of labeling the bacterial DNA. Controls consisted of Gram-stain examination and demonstration of satellite phenomenon on blood-agar around a staphylococcus streak.

M. gallinarum PG-16 (ATCC 15319) and *M. gallisepticum* S-6 (ATCC 15302) were cultivated in PPLO Broth (Difco) or HI Broth, each containing 10% horse serum and yeast extract (Oxoid). Purity of cultures was determined by examination of Gram-stained films from final harvest broths and subculture on PPLO Agar and blood-agar.

The identities of *H. gallinarum*, *M. gallinarum*, and *M. gallisepticum* (samples from final harvest)

were kindly confirmed by H. W. Yoder, Jr., of the Veterinary Medical Research Institute, Iowa State University, by using appropriate cultural, chemical, and serological tests.

All cultures were incubated in a rotary shaking incubator at 37 C. Antibiotics or other bacterial inhibitors which might have masked occult contamination were not used in any of the media.

DNA extraction. The DNA extraction method of Marmur (1961) was used throughout this study and was occasionally implemented by 3 mg/ml of Pronase (Calbiochem) in 1 to 2% sodium lauryl sulfate for the purpose of purifying residual nucleoprotein.

Determination of DNA base composition. The guanine plus cytosine content of the DNA preparations was determined by the thermal denaturation method of Marmur and Doty (1962) by use of a Gilford model 2000 spectrophotometer with a Beckman DU monochromator. The temperature probe was standardized by a stem-corrected Bureau of Standards Fisher thermometer. Absorbance was corrected for the thermal expansion of water. The melting temperature (T_m) was chosen as that point midway between the optical density (OD) at 69 C (approximate T_m of DNA containing only adenine plus thymine bases) and the OD at maximal hyperchromicity.

A standard curve was derived by determining the T_m of DNA extracted from *Escherichia coli*, *Bacillus cereus*, *P. mirabilis*, *Pseudomonas fluorescens*, and *Diplococcus pneumoniae*. These strains were chosen because their DNA base compositions have been chemically determined and listed (Marmur and Doty, 1962).

DNA quantification and hybridization methods. The isotope-labeled DNA preparations were sheared in 5- to 10-ml portions in a 25-ml plastic cup by use of a Branson sonifier, model LS-75, for 4 min and 45 sec at a setting of 3. At this setting the sonic oscillations occur at a frequency of 20 kc/sec maintained at a medium output of approximately 13 w. The resulting DNA fragments have a molecular weight of about 300,000 as determined by sedimentation at 37,000 rev/min in the Beckman model E analytical ultracentrifuge. After sonic treatment the fragments were denatured by heating at 100 C for 10 min in a solution of 0.3 M NaCl and 0.03 M sodium citrate. Denatured DNA was trapped in agar and quantitated by the procedures of McCarthy and Bolton (1963, 1964). Agar-column hybridizations were also performed according to their methods. The amounts of free and bound radioactive DNA were determined in a Nuclear-Chicago liquid scintillation counter, 720 series.

RESULTS AND DISCUSSION

The pertinent data of this study are presented in Table 1. The first series of experiments were performed with *P. mirabilis* 9 and its stable L form to determine whether any significant difference occurs in G + C content or duplex

TABLE 1. Guanine plus cytosine per cent composition and duplex formation of DNA

Radioactively labeled DNA	DNA trapped in agar	Per cent of labeled DNA bound*	Per cent of DNA bound relative to bacterial form	Per cent G ⁺ +C of DNA (from T _m)†
DNA-C ¹⁴ from <i>Proteus mirabilis</i> 9, 20 µg	DNA from <i>P. mirabilis</i> 9, 200 µg	77.6	100	39.5 ± 0.2
		78.1	100	
		77.8	100	
	DNA from L form of <i>P. mirabilis</i> 9, 200 µg	69.4	89.2	39.7 ± 0.3
		83.8	107.7	
		83.3	107.0	
		78.8	101.3	
	Agar blank	2.4	(3.1)	
		3.6	(4.7)	
DNA-H ³ from <i>Haemophilus gallinarum</i> , 20 µg	DNA from <i>H. gallinarum</i> , 200 µg	41.0	100	41.9 ± 0.2
		38.8	100	
		39.9	100	
	DNA from <i>Mycoplasma gallisepticum</i> , 200 µg	1.4	3.5	32.7 ± 0.1
		3.6	9.1	
		2.5	6.3	
	DNA from <i>M. gallinarum</i> , 200 µg	2.2	5.6	28.1 ± 0.1
		1.4	3.6	
		1.8	4.6	
	Agar blank	1.5	(3.6)	
		2.7	(6.6)	

* Figures in italics represent the average for each set of determinations.

† Mean derived from at least three determinations.

formation of DNA between a bacterium and its stable morphological variant (L form). It is obvious that these two forms of *P. mirabilis* are indistinguishable by the methods used. The close quantitative base composition of the two morphological forms is in good agreement with the finding that the DNA of *Sphaerophorus necrophorus* and its "large body" form both have G + C contents of 31% (Dowell, Loper, and Hill, 1964). Panos (1965) was also unable to find any alteration in the molar base ratios of DNA from a group A streptococcus or its stable L form. It then follows that if certain mycoplasmas are stable L forms of bacteria, the DNA of both forms should have an overall similar base composition and high degree of nucleic acid homology. The first mycoplasma-bacterial series of experiments were performed with *H. gallinarum*, *M. gallisepticum*, and *M. gallinarum*. The marked difference in DNA G + C content between *H. gallinarum* and the *Mycoplasma* species *M. gallisepticum* and *M. gallinarum* suggests that there is little, if any, genetic homology between these mycoplasmas and the bacterium.

Sueoka (1961) concluded that, since the DNA

molecules of any specific microorganism are unimodal in distribution and have an average G + C content which is narrow in range, a difference of 10% G + C between two bacterial species is predictive of few DNA molecules in common. Although this is essentially true, it has been demonstrated that the per cent base composition can vary somewhat in small areas of the DNA molecules of a bacterial strain (Guild, 1963), and denatured nucleic acid fragments of organisms with slightly different G + C ratios can anneal with portions of nucleic acids immobilized in agar (McCarthy and Bolton, 1963). Other investigators have also shown that the degree of genetic compatibility and molecular hybridization in solution became less with increasing differences in the DNA base composition of heterologous organisms (Schildkraut, Marmur, and Doty, 1961; Marmur, Schildkraut, and Doty, 1961; Catlin and Cunningham, 1961; Marmur et al., 1963; Catlin and Cunningham, 1964). To determine whether any regions of nucleic acid homology existed between the bacterium and mycoplasmas, the agar-column technique was utilized. As illustrated in Table 1,

no genetic homology could be demonstrated between *H. gallinarum* and the two mycoplasmas.

Another pair of organisms which have often been suspected of being genetically related are *Streptococcus* MG and *M. pneumoniae* (the Eaton agent). Subsequent experiments in our laboratories (McGee et al., 1965) demonstrated that the DNA of both strains had an almost identical composition. However, although the nucleic acids of each organism were capable of specific duplex formation in homologous reactions, there was no specific duplex formation in heterologous preparations. Thus, even though there was a similarity in gross base composition, there was no genetic homology between *Streptococcus* MG and *M. pneumoniae*.

To date, we have been unable to provide any evidence of genetic homology between mycoplasmas and bacteria. Nonetheless, this does not preclude the possibility of such a relationship. Further hybridization attempts between mycoplasmas and bacteria with similar base compositions may yet expose a genetic relatedness.

Until more information is obtained concerning phenotypic similarities, DNA base composition, genetic compatibility, or molecular hybridization, the possible classification of organisms within the genus *Mycoplasma* (Dienes, 1960; Edward, 1960; Klieneberger-Nobel, 1960) may be restated in terms of the DNA base compositions, as determined by various investigators (Lynn and Smith, 1957; Morowitz et al., 1962; Jones and Walker, 1963; Neimark and Pène, 1965). The range (23.6 to 46.6% G + C) and diversity of per cent composition suggest that (i) these organisms are derived from a variety of bacteria or (ii) they may form autonomous groups of taxo or genospecies arranged under a common nomenspecies (Ravin, 1963).

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LITERATURE CITED

- CATLIN, B. W., AND L. S. CUNNINGHAM. 1961. Transforming activities and base contents of deoxyribonucleate preparations from various neisseriae. *J. Gen. Microbiol.* **26**:303-312.
- CATLIN, B. W., AND L. S. CUNNINGHAM. 1964. Genetic transformation of *Neisseria catarrhalis* by deoxyribonucleate preparations having different average base compositions. *J. Gen. Microbiol.* **37**:341-352.
- DIENES, L. 1960. Biology of the pleuropneumonia-like organisms. *Ann. N.Y. Acad. Sci.* **79**:481.
- DOWELL, V. R., JR., J. C. LOPER, AND E. O. HILL. 1964. Constancy of deoxyribonucleic acid base composition in the transition of *Sphaerophorus necrophorus* from bacilli to large bodies. *J. Bacteriol.* **88**:1805-1807.
- EDWARD, D. G. ff. 1960. Biology of the pleuropneumonia-like organisms. *Ann. N.Y. Acad. Sci.* **79**:309-311, 481-483.
- EDWARD, D. G. ff., AND A. D. KANAREK. 1960. Organisms of the pleuropneumonia group of avian origin: their classification into species. *Ann. N.Y. Acad. Sci.* **79**:696-702.
- FREUNDT, E. A. 1958. The *Mycoplasmataceae*. Aarhus Stiftsbogtrykkerie, Copenhagen.
- GUILD, W. R. 1963. Evidence for intramolecular heterogeneity in pneumococcal DNA. *J. Mol. Biol.* **6**:214-229.
- JOHNSON, E. M., S. FALKOW, AND L. S. BARON. 1964. Recipient ability of *Salmonella typhosa* in genetic crosses with *Escherichia coli*. *J. Bacteriol.* **87**:54-60.
- JONES, A. S., AND R. T. WALKER. 1963. Isolation and analysis of the deoxyribonucleic acid of *Mycoplasma mycoides* var. *capri*. *Nature* **198**:588-589.
- KELTON, W. H., R. F. GENTRY, AND E. H. LUDWIG. 1960. Derivation of gram-positive cocci from pleuropneumonia-like organisms. *Ann. N.Y. Acad. Sci.* **79**:410-421.
- KLIENEBERGER-NOBEL, E. 1960. Biology of the pleuropneumonia-like organisms. *Ann. N.Y. Acad. Sci.* **79**:483-484.
- LYNN, R. J., AND P. F. SMITH. 1957. Nucleic acid content of pleuropneumonia-like organisms from human sources. *J. Bacteriol.* **74**:811-817.
- MCCARTHY, B. J., AND E. T. BOLTON. 1963. An approach to the measurement of genetic relatedness among organisms. *Proc. Natl. Acad. Sci. U.S.* **50**:156-164.
- MCCARTHY, B. J., AND E. T. BOLTON. 1964. Interaction of complementary RNA and DNA. *J. Mol. Biol.* **8**:184-200.
- MCGEE, Z. A., M. ROGUL, S. FALKOW, AND R. G. WITTLER. 1965. The relationship of *Mycoplasma pneumoniae* (Eaton Agent) to *Streptococcus* MG: Application of genetic tests to determine relatedness of L-forms and PPLO to bacteria. *Proc. Natl. Acad. Sci. U.S.* **54**:457-461.
- McKAY, K. A., AND J. R. E. TAYLOR. 1954. The reversion of L type cultures previously described as pleuropneumonia-like and associated with chronic respiratory disease to an organism resembling *Hemophilus gallinarum*. *Can. J. Comp. Med. Vet. Sci.* **18**:7-12.
- McKAY, K. A., AND R. B. TRUSCOTT. 1960. Reversion of avian pleuro-pneumonia-like organisms to bacteria. *Ann. N.Y. Acad. Sci.* **79**:465-480.
- MARMUR, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3**:208-218.
- MARMUR, J., AND P. DOTY. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Mol. Biol.* **5**:109-118.
- MARMUR, J., C. L. SCHILDKRAUT, AND P. DOTY.

1961. The reversible denaturation of DNA and its use in studies of nucleic acid homologies and the biological relatedness of microorganisms. *J. Chim. Phys.* **58**:945-955.
- MARMUR, J., E. SEAMAN, AND J. LEVINE. 1963. Interspecific transformation in *Bacillus*. *J. Bacteriol.* **85**:461-467.
- MOROWITZ, H. J., M. E. TOURTELLOTTE, W. R. GUILD, E. CASTRO, C. WOESE, AND R. C. CLEVERDON. 1962. The chemical composition and submicroscopic morphology of *Mycoplasma gallisepticum*, avian PPLO 5969. *J. Mol. Biol.* **4**:92-103.
- NEIMARK, H. C., AND J. J. PÈNE. 1965. Characterization of pleuropneumonia-like organisms by deoxyribonucleic acid composition. *Proc. Soc. Exptl. Biol. Med.* **118**:517-519.
- PANOS, C. 1965. Cellular physiology during logarithmic growth of a streptococcal L-form. *J. Gen. Microbiol.* **39**:131-138.
- RAVIN, A. W. 1963. Experimental approaches to the study of bacterial phylogeny. *Am. Naturalist* **97**:307-318.
- SCHILDKRAUT, C. L., J. MARMUR, AND P. DOTY. 1961. The formation of hybrid DNA molecules and their use in studies of DNA homologies. *J. Mol. Biol.* **3**:595-617.
- SMITH, P. F. 1964. Comparative physiology of pleuropneumonia-like and L-type organisms. *Bacteriol. Rev.* **28**:97-125.
- SMITH, P. F., D. M. PEOPLES, AND H. E. MORTON. 1957. Conversion of pleuropneumonia-like organisms to bacteria. *Proc. Soc. Exptl. Biol. Med.* **96**:550-553.
- SMITH, P. F., AND G. H. ROTHBLAT. 1960. Relation of PPLO to bacteria. *Ann. N.Y. Acad. Sci.* **79**:461-464.
- SUEOKA, N. 1961. Variation and heterogeneity of base composition of deoxyribonucleic acids: a compilation of old and new data. *J. Mol. Biol.* **3**:31-40.
- WITTNER, R. G., S. G. CARY, AND R. B. LINDBERG. 1956. Reversion of a pleuropneumonia-like organism to a corynebacterium during tissue culture passage. *J. Gen. Microbiol.* **14**:763-774.