Nucleic Acid Relationships among the Anaerobic Mycoplasmas

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(Received 14 August 1984)

The genetic relatedness between twelve selected strains among four distinct serovars of anaerobic mycoplasmas was studied using [³H]DNA–DNA hybridization, and the results were compared with data obtained from biochemical and serological tests. Radiolabelled DNA probes were prepared from five strains representing four serovars. Based on the homology results, the anaerobic mycoplasmas can be divided into five distinct groups representing five distinct species and two distinct genera. There are two species in the *Anaeroplasma* *bactoclasticum* serovar 1 group represented by strains JR and A-2, one species in serovar 2, one species in *A. abactoclasticum* serovar 3 and one among the unclassified serovar 4 anaerobic mycoplasmas. The probe to nonsterol-requiring strain 161 of serovar 4 showed no homology with any of the established nonsterol-requiring *Acholeplasma* species DNAs, or with *Mycoplasma hominis* DNA, or with avian DNA which served as a negative control. There was good correlation between the phenotypic and genotypic properties of the five distinct anaerobic mycoplasma species but the results indicate that phenotypic properties are not always adequate for speciation of the anaerobic mycoplasmas.

INTRODUCTION

An absolute requirement for anaerobic growth conditions and a high sensitivity to oxygen distinguish the anaerobic mycoplasmas from all other mycoplasmas. Both sterol- and nonsterol-requiring anaerobic mycoplasma strains have been isolated from the rumens of cattle and sheep (Robinson & Allison, 1975; Robinson *et al.*, 1975; Robinson & Rhoades, 1982). On the basis of antigenic properties, i.e. agglutination and gel diffusion precipitin tests, anaerobic mycoplasma strains were classified into four serovars (Robinson & Rhoades, 1982) which agreed with group separation based upon cultural, biochemical and biophysical properties (Robinson, 1979; Robinson & Rhoades, 1977; Robinson *et al.*, 1975). Two species have been described and assigned to the genus *Anaeroplasma*. Both are sterol-dependent; *A. bactoclasticum* possesses bacteriolytic and proteolytic enzymes whereas *A. abactoclasticum* lacks these enzymes. The taxonomic position of a third group, which consists of nonsterol-requiring organisms, has not been determined, and this group is considered unclassified (Robinson, 1984).

Because the taxonomic classification of anaerobic mycoplasmas has been based solely on selected phenotypic properties, the degrees of relatedness among strains based on DNA homology should provide useful information. The amount of genetic relatedness among various mycoplasma strains and species has been determined using nucleotide sequence homology of the chromosomal DNA and these studies have provided very useful information for the taxonomic characterization of organisms belonging to the class Mollicutes (Aulakh *et al.*, 1979, 1983; Bove *et al.*, 1982; Chandler *et al.*, 1982; Christiansen *et al.*, 1981; Stephens *et al.*, 1981, 1983). DNA hybridization data were especially useful for examining *acholeplasmas* and other mycoplasmas for which immunological information was somewhat lacking (Tully, 1979), and also confirmed

the genomic relationships among strains and species classified on the basis of their phenotypic properties (Stephens *et al.*, 1983). The purpose of the present study was to determine the relationship between the phenotypic and genomic properties of related strains among the anaerobic mycoplasmas, and to evaluate the usefulness of presently accepted procedures for the classification of anaerobic mycoplasmas.

METHODS

Organisms. Twelve anaerobic mycoplasma strains representative of four serovars classified on the basis of cultural, biophysical and metabolic properties were used (Robinson, 1979; Robinson & Allison, 1975; Robinson *et al.*, 1975). Strains were isolated from the following rumen contents: cow, strain JF (Robinson & Hungate, 1973); steers, strains 10A and A-2; all other strains were isolated from rumen contents of sheep (Robinson & Allison, 1975; Robinson *et al.*, 1975). The differential characteristics of the strains used are listed in Table 1.

Cultivation techniques. Anaerobic mycoplasma strains were grown overnight at 37 °C in clarified rumen fluid broth medium (Robinson *et al.*, 1975) using the roll tube anaerobic culture technique (Robinson & Allison, 1975; Robinson *et al.*, 1975; Robinson & Hungate, 1973). Anaerobic mycoplasmas will grow only in a prereduced medium maintained in a system which completely excludes oxygen; e.g. if resazurin in a test medium becomes oxidized, these organisms will not grow. Cells were sedimented by centrifugation at 10000 *g* for 15 min, washed twice with 0.85% sodium chloride, buffered to pH 7.2 with 0.02 M-sodium phosphate (PBS), resuspended in PBS and stored at -40 °C until used.

Extraction and purification of DNA. Washed cell pellets were lysed in a solution containing 100 mM-Tris/HCl, pH 8.0, 50 mM-EDTA and 2% (w/v) Sarkosyl. The cells were homogenized for 5 min at 4 °C in a Dounce homogenizer and then 1 g solid CsCl (BRL) and 0.1 ml ethidium bromide (10 mg ml⁻¹) were added per ml of lysate. The solution was added to nitrocellulose tubes and centrifuged to equilibrium in an SW 50.1 rotor (Beckman) at 250000 *g* for 48 h at 20 °C. The band of DNA was visualized by UV light, and recovered by puncturing the side of the tube with an 18-gauge needle and collecting in a 1 ml syringe. The ethidium bromide was removed by extracting twice with an equal volume of 5 M-NaCl, 0.1 M-Tris/HCl, pH 8.6, and 5 mM-EDTA saturated with propan-2-ol. The aqueous phase was diluted with 2 vols distilled water and precipitated by addition of 2 vols ethanol. The precipitated DNA was spooled on a glass rod, resuspended in TE buffer (0.01 M-Tris/HCl, pH 8.0, 1 mM-EDTA), and treated with 100 µg RNAase A ml⁻¹ for 30 min at 37 °C. The RNAase A was removed by extracting twice with an equal volume of TE-saturated phenol and twice with chloroform/isoamyl alcohol (24:1, v/v). The aqueous phase containing the DNA was removed, precipitated with 2 vols ethanol, spooled on a glass rod and dissolved in TE buffer. DNA to be used for liquid hybridization was further processed as previously described (Stephens *et al.*, 1981).

DNA probe synthesis. Purified DNA was radioactively labelled *in vitro* using the four labelled nucleoside triphosphates by the nick translation technique previously described (Aulakh & Gallo, 1977; Aulakh *et al.*, 1979, 1983; Kelly *et al.*, 1970; Maniatis *et al.*, 1975). In brief, the reaction mixture in a final volume of 50 µl contained 1 µg purified DNA, 50 mM-Tris/HCl, pH 7.5, 10 mM-MgCl₂, 1 mM-2-mercaptoethanol, 6 units DNA polymerase I (New England Nuclear), 1 µl DNAase I (10⁻⁷ µg ml⁻¹) (Worthington Biochemicals), 27 µM of each of the four labelled nucleoside triphosphates (New England Nuclear), and 50 µg bovine serum albumin (Cohn fraction V) ml⁻¹. The reactions were stopped with 5 µl 5% (w/v) SDS and 5 µl 0.1 M-EDTA when the specific activity reached approximately 2.0 × 10⁷ c.p.m. per µg of DNA. The probes for hybridization reactions were further processed as described by Stephens *et al.* (1981).

Nucleic acid hybridization reactions. Hybridization reaction mixtures contained 1 mg sheared unlabelled DNA ml⁻¹, 0.2% SDS, 1 mM-EDTA, 0.48 M-phosphate buffer (PB), and 15000 c.p.m. of the sheared [³H]DNA probe in a 100 µl reaction volume (Aulakh *et al.*, 1979; Stephens *et al.*, 1981). The reaction mixture was heated to 100 °C for 5 min and then incubated at 65 °C to a C₀t value > 300 (Laird *et al.*, 1969; Aulakh *et al.*, 1979). Hybrid DNA was separated from single stranded DNA on hydroxyapatite columns equilibrated at 60 °C in 0.12 M-PB and 0.2% SDS. Radioactive material not binding to the columns in 0.12 M-PB was considered to be single stranded DNA. The double stranded DNA was then eluted with 0.48 M-PB and 0.2% SDS. Radioactivity was measured by adding 12 ml Aquasol (New England Nuclear) to 4 ml sample and counting in a Beckman LS scintillation counter. Thermal elution midpoints were determined on hydroxyapatite columns, prepared as described above, by washing with 0.2% SDS in 0.12 M-PB in increments up to 100 °C. Radioactivity was counted as described above.

RESULTS

[³H]DNA-DNA hybridization studies. The DNA-DNA hybridization data for the twelve anaerobic strains are listed in Table 2. Each of the five [³H]DNA probes hybridized extensively with its homologous DNA (80%), but not with DNA from duck tissues (2%) or from *Mycoplasma hominis* (3%) which served as negative controls.

Table 1. *Differential characteristics of anaerobic mycoplasma strains*Data from Robinson & Allison (1975); Robinson *et al.* (1975).

Species and strain	Serovar	Bacteriolytic activity	Requirement for cholesterol	Mol % G + C
<i>A. bactoclasticum</i>				
JR	1	+	+	33.7
71-B1	1	+	+	ND
A-2	1	+	+	ND
10A	1	+	+	ND
5LA	2	+	+	ND
5LB	2	+	+	ND
7LA	2	+	+	32.5
<i>A. abactoclasticum</i>				
6-1	3	—	+	29.3
171	3	—	+	29.5
Unclassified				
161	4	—	—	40.2
162	4	—	—	40.3
163	4	—	—	ND

ND, Not done.

Table 2. *Percentage DNA homology using [³H]DNA probes from selected strains of anaerobic mycoplasmas with DNAs from strains of classified and unclassified Anaeroplasm species*

Source of unlabelled DNA		Percentage DNA homology of [³ H]DNA probes*				
Species and strain	Serovar	JR	A-2	5LA	6-1	161
<i>A. bactoclasticum</i>						
JR	1	100 (80) [81]	18	2	2	2
71-B1	1	60	12	5	1	2
10A	1	19	77 [80.0]	6	1	5
A-2	1	16	100 (81) [81.5]	9	2	1
5LA	2	13	10	100 (92) [83.0]	2	8
7LA	2	4	7	74 [82.0]	2	2
5LB	2	4	5	85 [82.0]	1	6
<i>A. abactoclasticum</i>						
6-1	3	1	3	1	100 (86) [82.4]	3
171	3	3	4	2	92 [82.0]	2
Unclassified						
161	4	1	1	2	1	100 (93) [82.9]
162	4	1	3	2	1	86 [81]
163	4	1	1	1	1	94 [82.5]
<i>M. hominis</i>						
PG21	—	2	1	1	1	2
Duck tissues	—	1	1	1	1	1

* Values in parentheses represent the actual hybridization percentage; values in square brackets represent the thermal elution midpoint of DNA-DNA duplexes.

The [³H]DNA probe derived from *A. bactoclasticum* serovar 1 strain JR had 80% homology to itself, and appreciable homology to *A. bactoclasticum* serovar 1 strain 71-B1 (60%), but a very low homology to the other two serovar 1 strains, 10A (19%) and A-2 (16%). There was also 13% homology to *A. bactoclasticum* serovar 2 strain 5LA and 4% or less to all the other anaerobic strains tested. The probe to *A. bactoclasticum* serovar 1 strain A-2 had 81% homology to itself, a high degree of homology to serovar 1 strain 10A but very low homology to the other two serovar 1 strains JR and 71-B1. These findings indicate that serovar 1 contains two distinct species represented by strains JR and A-2. The probe to serovar 2 strain 5LA showed high homology (74% and 85%) to the two other serovar 2 strains, 5LB and 7LA, but not to the other anaerobic mycoplasma serovars 1, 3 and 4 strains, indicating that serovar 2 represents a distinct species.

The two *A. abactoclasticum* serovar 3 strains, 6-1 and 171, had a high degree of homology (92%) to each other, but very little homology (1–2%) to the other anaerobic serovar 1, 2 and 4 strains tested, and represent a fourth distinct species. The three unclassified serovar 4 strains, 161, 162 and 163, showed a high degree of homology (86% and 94%) among each other, but 8% or less to the other serovar 1, 2 and 3 strains tested and represent the fifth distinct species. Thus, based on DNA homology, the anaerobic mycoplasmas fall into five distinct species groups.

te₅₀ values of DNA–DNA duplexes. Thermal elution midpoints (*te₅₀*) were determined for each duplex and for a few selected heteroduplexes among members of the same serovar. *A. bactoclasticum* strain JR homoduplex had the lowest *te₅₀* value measured (81 °C), whereas unclassified strain 161 and *A. bactoclasticum* strain 5LA had the higher values of 82.9 and 83.0 °C (Table 2). The *te₅₀* values of heteroduplexes between members of the same serovar were generally less than 1 °C below those of the homoduplex, indicating less than 1.5% mismatching of base pairs in the duplexes (Table 2). The *te₅₀* values support the close relationship of strains within the five species groups.

Nucleic acid homology of the unclassified anaerobic strains to acholeplasmas. Because both the unclassified anaerobic mycoplasmas and the *Acholeplasma* species do not require cholesterol for growth, studies were done to determine their genomic relatedness. There was no significant hybridization (5%) between a [³H]DNA probe prepared from the unclassified serovar 4 strain 161 and unlabelled DNA from each of the nine established *Acholeplasma* species (*A. laidlawii*, PG-9; *A. axanthum*, S743; *A. modicum*, PG-49; *A. equifetale*, C112; *A. oculi*, 19L; *A. hippikon*, C1; *A. granularum*, BTS-39; *A. morum*, S2; *A. florum*, L1), and from three unclassified *Acholeplasma* strains (PS2, J233 and 0502). Thus, the unclassified nonsterol-requiring anaeroplasmas have no genomic relatedness to the established nonsterol-requiring acholeplasmas.

DISCUSSION

The results presented indicate that there are five distinct and separate groups of anaerobic mycoplasmas based on DNA homology studies. Each group warrants species designation based on both phenotypic and genotypic properties. There are four sterol-requiring and one nonsterol-requiring species. There are two distinct species in the serovar 1 *Anaeroplasma bactoclasticum* group, the serovar 2 group represents a third *Anaeroplasma* species and the *A. abactoclasticum* serovar 3 group represents a fourth. We propose that the serovar 1 group be separated into two distinct species based on the DNA homology data.

The four sterol-requiring *Anaeroplasma* species can readily be separated from the unclassified nonsterol-requiring anaerobic mycoplasmas on the basis of the mol % G + C, which for the *Anaeroplasma* species ranges from 29% to 34% whereas in the unclassified anaerobic mycoplasma serovar 4 group it is 40%. Because of their distinctly separate genomic properties, we propose that the nonsterol-requiring anaerobic mycoplasmas be placed in a separate genus from the *Anaeroplasma* species.

The unclassified anaerobic mycoplasma serovar 4 group and the *Acholeplasma* species are both nonsterol-requiring mycoplasmas, but they are readily distinguished by their phenotypic and genotypic properties. The *Acholeplasma* species grow under aerobic conditions and the anaeroplasmas do not. The mol % G + C of the DNA is only 29–33% for the *Acholeplasma* species and 40% for the unclassified anaerobic serovar 4 mycoplasmas. Moreover, the acholeplasmas are distinct from the unclassified anaerobic serovar 4 group based on the DNA homology presented in this report. The question which remains unanswered is whether the unclassified nonsterol-requiring anaerobic mycoplasmas should be placed in the family *Acholeplasmataceae* because they are nonsterol-requiring or remain with the anaerobic mycoplasma group. We propose that the unclassified nonsterol-requiring anaerobic mycoplasmas be given genus designation and remain in the anaerobic mycoplasmas which would then consist of the genus *Anaeroplasma* containing four species, and a second genus for the unclassified serovar 4 group containing one species. For these reasons, we believe that the anaerobic mycoplasmas merit family designation within the class Mollicutes.

The results presented indicate that immunological and other phenotypic properties are not always adequate for speciation of the anaerobic mycoplasmas, as evidenced by the fact that the antigenically distinct serovar 1 group of *A. bactoclasticum* contains two distinct species based on DNA homology studies.

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