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Interspecies and Intraspecies DNA Homology Among Established Species of *Acholeplasma:* A Review

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Radiolabeled DNA probes prepared *in vitro* by the nick translation method were used to determine the nucleotide sequence homology among the eight established and one unclassified species of *Acholeplasma.* Very little DNA homology (2 to 21 percent) was found among these nine distinct species and the heteroduplexes showed at least 15 percent mismatching as determined by thermal elution endpoints. The data obtained by hybridization analyses paralleled the results obtained by the growth inhibition and epi-immunofluorescence serologic procedures. The small amount of nucleotide sequence homology among the nine distinct species indicate that the *Acholeplasma* species are quite distinct and unrelated to each other genomically, findings which should provide useful insight on the molecular biology and evolutionary pathways of these organisms. Labeled ^{3}H-DNA probes to five strains of either *A. laidlawii* or *A. axanthum* hybridized to a varying degree to excess amounts of unlabeled DNAs from 12 strains of *A. laidlawii* and six strains of *A. axanthum,* respectively. Nucleic acid hybridization analyses showed a wide variation (48 to 100 percent) in DNA homologies among different strains of the two species. The results demonstrate that strains of *A. laidlawii* and/or *A. axanthum* isolated from diverse hosts and habitats (birds, rodents, cats, swine, sheep, cattle, horses, goats, primates, and plants) exhibit extensive genotypic variations. ^{3}H-DNA-DNA hybridization procedures were found to be extremely useful in establishing or confirming the existence of distinct species within the genus *Acholeplasma.*

INTRODUCTION

The genus *Acholeplasma* comprises a group of cell wall-less prokaryotes that can be distinguished from other members of the class Mollicutes by their ability to grow in artificial medium without the addition of animal serum, cholesterol, or sterols [1]. The *Acholeplasma* species have been differentiated by a limited number of conventional biochemical and serological tests [1]. Although these conventional procedures have proven useful in the separation of species within the genus *Mycoplasma,* the application of these techniques to the *Acholeplasma* species has presented several problems. These problems include: (*a*) the presence of low levels of anti-acholeplasma antibody in rabbit pre-immunization sera, (*b*) antisera of low potency, especially growth inhibition antibodies, and (*c*) the ability of the

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acholeplasma membrane to adsorb serum proteins, resulting in serologic cross-reactions with different organisms grown in the same serum-containing medium [1]. Thus, the separation of the acholeplasmas by genomic markers such as that provided by the ^{3}H-DNA-DNA hybridization techniques could furnish critical information in determining interrelationships among the *Acholeplasma.* These DNA-DNA hybridization techniques have been used recently to demonstrate a lack of genetic relatedness between new unclassified strains of *Acholeplasma* isolated from tissue cultures [2] and from plant tissues [3]. In this report, data is provided to show a lack of genomic relatedness among all eight established and one distinct unclassified species of *Acholeplasma.* Data is also provided to show that there is wide variation in DNA homologies among strains of *A. laidlawii* and *A. axanthum* isolated from a diverse and wide variety of host and habitats.

RESULTS

DNA Homology Test Among Acholeplasma Species

The procedures used for the ^{3}H-DNA-DNA hybridization studies were described elsewhere [2,3,4,5,6,7,8,9,10,11]. The hybridization data for the eight species of *Acholeplasma,* for one unclassified, distinct new *Acholeplasma* species (strain lemon), and one species of *Mycoplasma* are listed in Table 1. Each ^{3}H-DNA probe hybridized extensively with its homologous DNA, giving values ranging from 75.1 percent for *Acholeplasma* sp. strain Lemon to 90.4 percent for *A. granularum.* Each *Acholeplasma* species tested did not hybridize significantly to DNA from duck (0.2 to 2.9 percent) or *M. capricolum* (2.0 to 4.3 percent), unrelated DNAs which served as negative controls. Hybridization values obtained with the homologous DNA were normalized to 100 percent homology for purposes of comparison.

The results of study among the nine distinct *Acholeplasma* species indicate that each species had very little homology with any of the other species (Table 1). The *A. axanthum* probe hybridized less than 7 percent with DNAs of other *Acholeplasma* species or with *M. capricolum.* Similar results were obtained with the *A. morum* probe, which produced a maximum of 6.5 percent homology with DNA of *A. laidlawii.* The DNA probe of *A. equifetale* showed little homology (9.4 percent) with unlabeled DNAs of *A. granularum, A. laidlawii,* and *A. hippikon.* On the other hand, *A. granularum* showed the greatest amount of homology with DNAs of several species, ranging from a maximum of 20.2 percent homology with *A. laidlawii,* 15.4 percent with *A. oculi,* and approximately 9–10 percent with *A. hippikon, A. equifetale,* and *A. modicum.* In reciprocal tests, the *A. laidlawii* probe produced 21 percent homology with unlabeled DNA of *A. granularum,* 15 percent with *A. oculi,* 11 percent with *A. hippikon,* and to a lesser amount (>7 percent) with other *Acholeplasma* species. The *A. hippikon* probe showed 14 to 15 percent homology with unlabeled DNAs of *A. oculi, A. laidlawii,* and *A. granularum,* and about 3 to 7 percent with the other *Acholeplasma* species. The *A. oculi* probe hybridized 13 to 14 percent with DNAs from *A. laidlawii, A. granularum,* and *A. hippikon* and to a lesser extent with the other *Acholeplasma* species. The most recently recognized species, *Acholeplasma* sp. strain Lemon (no epithet has been given as yet) hybridized less than 5.8 percent with DNAs of all other *Acholeplasma* species, a finding which indicates that it represents a new *Acholeplasma* species. The Lemon strain has been shown to be an acholeplasma by conventional procedures [1].

Thermal elution midpoints (t_e50) were determined for the double-stranded DNA fractions eluted off hydroxyapatite columns for each homoduplex and for a few

TABLE 1
Degree of Hybridization Using ^{3}H-DNA Probes of the Eight Species of *Acholeplasma* and *M. capricolum* with DNAs from Different Species of *Acholeplasma, Mycoplasma capricolum,* and *Duck*

Source of the ^{3}H-DNA probe species (strain)	% hybridization with excess unlabeled DNA										
	M. capricolum	*A. axanthum*	*A. morum*	*A. modicum*	*A. equifetale*	*A. granularum*	*A. laidlawii*	*A. hippikon*	*A. oculi*	*A.* sp. (*lemon*)	*Duck*
Mycoplasma capricolum (California kid)	87.4[a](100, 81.3)[c]	2.0(2.3)	2.5(2.9)	3.0(3.4)	2.8(3.2)	4.3(4.9)	2.5(2.9)	3.5(4.0)	2.0(2.3)	ND	2.3(2.6)
A. axanthum (S743)	5.0(6.3)[b]	78.9(100, 82.0)	4.0(5.1)	2.3(2.9)	4.4(5.8)	3.8(4.8)	5.1(6.5)	4.2(5.3)	3.7(4.7)	ND	2.9(3.8)
A. morum (72-043)	2.4(2.9)	4.4(5.3)	83.7(100, 84.3)	3.6(4.3)	4.0(4.8)	3.2(3.8)	5.4(6.5)	5.0(6.0)	3.3(3.9)	ND	1.4(1.7)
A. modicum Squire (PG-49)	4.1(4.6)	5.5(6.2)	5.4(6.1)	89.2(100, 84.1)	4.0(4.5)	7.7(8.6)	5.3(5.9)	5.4(6.1)	3.8(4.3)	ND	2.1(2.4)
A. equifetale (C112)	2.0(2.6)	4.0(5.1)	3.1(4.0)	3.4(4.3)	78.4(100, 83.4)	7.4(9.4)	7.4(9.4)	7.4(9.4)	1.6(2.0)	ND	1.9(2.4)
A. granularum (BTS39)	2.9(3.2)	5.9(6.5)	3.9(4.3)	8.3(9.2)	9.2(10.2)	90.4(100, 84.3)	18.3(20.2, 74.5)	9.6(10.6)	13.9(15.4, 71.0)	ND	1.4(1.5)
A. laidlawii (PG-9)	2.6(3.0)	5.7(6.6)	5.0(6.8)	5.3(6.1)	7.4(8.5)	18.3(21.1, 74.5)	86.9(100, 82.5)	9.6(11.0)	13.0(15.0)	ND	1.8(2.1)
A. hippikon (C1)	2.9(3.2)	4.9(5.5)	7.0(7.8)	5.8(6.5)	6.2(6.9)	12.4(13.8, 75.0)	12.5(13.9, 74.0)	89.8(100, 85.0)	13.8(15.4, 73.5)	ND	2.6(2.9)
A. oculi (19L)	3.1(3.9)	3.3(4.2)	2.8(3.6)	4.7(6.0)	7.0(8.9)	10.2(13.0)	11.6(14.8)	10.2(13.0)	78.6(100, 83.6)	ND	0.2(0.3)
Acholeplasma sp. (lemon)	3.4(4.5)	2.5(3.3)	0.8(1.1)	5.8(7.7)	1.3(1.7)	0.8(1.1)	1.7(2.3)	1.0(1.3)	1.7(2.3)	75.1(100, 82.0)	0.2(0.3)

[a] The percentage hybridization with the ^{3}H-DNA probe; data taken from reference [4]
[b] The first value in parentheses represents the normalized values.
[c] The second value in parentheses shows the thermal elution midpoint (t.50) of the DNA-DNA duplexes.

selected heteroduplexes. The t_e50 values of the homoduplexes for the nine Acholeplasma species are shown in Table 1. The *A. axanthum* and *Acholeplasma* sp. strain Lemon homoduplexes gave the lowest value of 82.0°C and *A. hippikon* homoduplex the highest value of 85.0°C. The t_e50 values of all heteroduplexes were 8° to 13°C lower than the values for each of the homoduplexes. *A. granularum* had a t_e50 value of 84.3°C for its homoduplex and 74.5°C and 71.0°C for heteroduplexes with *A. granularum* and *A. oculi,* respectively. The t_e50 value for the *A. laidlawii* homoduplex was 82.5°C, and the heteroduplex value with *A. granularum* was 74.5°C. A t_e50 value of 85.0°C was obtained for the *A. hippikon* homoduplex and 75.0°C, 74.0°C, and 73.5°C t_e50 for heteroduplexes with *A. granularum, A. laidlawii,* and *A. oculi,* respectively. The t_e50 value of the *A. oculi* homoduplex was 83.6°C.

DNA Homology Among Strains of A. axanthum *and* A. laidlawii

Using a ^{3}H-DNA probe derived from strain S743 of *A. axanthum,* values of from 52.5 to 100 percent homology were obtained with unlabeled DNAs derived from the five other strains (Table 2). Similarly, the Swine D1 probe gave homology values from 48.3 to 100 percent with the five other strains of *A. axanthum.* The thermal elution midpoints (t_e50) for the heteroduplexes ranged from 1.0 to 5.0°C lower than the t_e50 values of the corresponding homoduplex, indicating from 1.5 to 7.5 percent mismatching of the heteroduplexes (Table 2). Similar results were obtained with the t_e50 values of different *Acholeplasma laidlawii* strains (Table 2). The ^{3}H-DNA probe prepared against the PG-9 strain hybridized from 70.7 to 100 percent with unlabeled DNAs of the eleven other strains. The DNA probes to strains L, MIST, and PG-9 hybridized from 64 to 100 percent, 62.9 to 100 percent, and 71.3 to 100 percent, respectively, to the other eleven corresponding strains of *A. laidlawii.* The t_e50 values of the *A. laidlawii* strains varied from 0.6 to 6.0°C below the homoduplex t_e50 values (Table 3).

TABLE 2
Degree of DNA-DNA Hybridization Using ^{3}H-DNA Probes Derived from *A. axanthum* Strains S743 and Swine D1 with Excess Unlabeled DNA from Other *A. axanthum* Strains

Source and Strain of *A. axanthum* used as excess unlabeled DNA		% hybridization of ^{3}H-DNA probe	
Strain	Source	S743	Swine D1
S743	cell culture	*78.9[a](100)[b][82.0][c]*	52.5(60.8)[76.0]
Swine D1	swine, lung	48.0(60.8)[76.5]	*86.8(100)[81.0]*
H86N	bovine, nasal	55.3(70.1)[78.3)	57.9(66.7)[77.5]
1190	lagoon[d]	41.8(52.9)[76.7]	71.0(81.7)[80.0]
J248	coconut	51.3(65.0)[77.0]	61.8(71.2)[76.5]
501	broccoli	41.5(52.5)[77.0]	42.0(48.3)[76.0]
M. capricolum		2.1	1.8
Duck DNA		2.9	1.1

[a] Each value represents the average from two separate experiments. Values from each experiment did not differ by more than 4 percent. Data taken from reference [4].

[b] The values in parentheses represent normalized values.

[c] The figures in brackets show the thermal elution midpoint of the DNA-DNA duplexes.

[d] This lagoon was used to service swine and cattle.

TABLE 3
Degree of DNA-DNA Hybridization Using ^{3}H-DNA Probes Derived from *A. laidlawii* Strains L and MIST with Excess Unlabeled DNA from Other *A. laidlawii* Strains

Source and Strain of *A. laidlawii* used as excess unlabeled DNA		% hybridization of ^{3}H-DNA probe to *A. laidlawii* strains[a]		
Strain	Source	PG-9	L	MIST
MIST	compost	68.4(83.1)[b]	82.7(90.0)[83.5]	*82.0(100)[83.2]*
ALGEN	soil	58.1(70.6)	68.9(75.0)[81.0]	65.2(79.5)[82.0]
L	soil	64.0(77.8)	*91.9(100)[84.1]*	76.2(92.9)[82.0]
PG-9	sewage	*82.3(100)[82.5]*[c]	72.0(78.0)[79.5]	68.4(83.0)[80.0]
PG-10	bovine, urogenital	59.1(71.8)	73.2(80.0)[81.0]	70.4(85.8)[81.5]
J18S	bovine, urogenital	65.1(79.1)	68.4(74.4)[79.0]	64.7(78.9)[81.0]
643N	bovine, nasal	73.1(88.9)	74.4(81.0)[80.0]	70.4(85.8)[79.5]
PG-5	rat, lung	71.7(87.1)	63.8(69.5)[78.5]	61.1(74.5)[77.6]
STR	rat	58.7(71.3)	60.3(66.7)[78.0]	51.6(62.9)[78.4]
H3-10	chicken, sinus	65.1(79.1)	55.5(64.5)[78.0]	57.4(70.0)[78.0]
KHS	goat	68.8(83.6)	62.0(67.4)[78.3]	59.7(72.8)[78.4]
OR	human, oral	71.8(87.2)	61.6(67.0)[78.0]	56.8(69.2)[78.4]
M. capricolum	goat	2.0	2.1	2.5
Duck		1.2	1.3	1.9

[a] Each value represents the average from two separate experiments. Values from each experiment did not differ by more than 4 percent. Data taken from reference [4].

[b] The values in parentheses represent normalized values.

[c] The figures in brackets show the thermal elution midpoint of the DNA-DNA duplexes.

DISCUSSION

Although several investigators have examined mycoplasmas by use of nucleic acid hybridization procedures [2,3,7,12,13,14,15,16,17] our studies represent the first attempt to establish the genomic interrelationships among all recognized species of mycoplasmas within one genus (*Acholeplasma*) in the class *Mollicutes* [4]. There are several advantages in using the genus *Acholeplasma:* the organisms grow without difficulty and yield large quantities of DNA, and the genus is composed of only eight established species. The ^{3}H-DNA probes used in this study were labeled to a high specific activity of 1–2 $\times$ 10^7 cpm/μg using nick translation techniques [8,9]. The ^{3}H-probes were hybridized under conditions of 0.48 M phosphate buffer, pH 6.8 (PB), 1 mM EDTA, 0.2% SDS, 1 mg/ml of sheared unlabeled DNA, and 15,000 cpm of ^{3}H-probe in a total reaction volume of 100 μl. The DNA in the reaction mixture was denatured at 100°C for five minutes and then allowed to reassociate at 65°C to a C_o + value of 300 when $\geq$ 99 percent of the DNA sequences have reassociated [2,3,5].

Hydroxyapatite chromatography was then utilized to separate hybridized from single-stranded DNA. Samples were diluted in 0.12 M PB and applied to hydroxyapatite columns (1 cm^3) heated to 60°C. Material eluting from the column under these conditions was considered to be single-stranded DNA. The hybridized or double-stranded DNA was then eluted from the column with 0.48 M PB [5]. Hydroxyapatite chromatography was utilized in this study because it can easily and accurately separate reassociated and non-reassociated DNA, can be used to process large or small quantities of DNA, and does not suffer from the problems that plague filter binding assays such as leaching of immobilized DNA from the filter and variability in binding of DNA to the filter.

The results presented indicate that the eight established species and the new unclassified species (strain Lemon) of *Acholeplasma* have very little interspecies nucleotide sequence homology. The DNA probes to *A. granularum, A. oculi, A. laidlawii,* and *A. hippikon* showed the largest amount of cross-reactions, but the values were only 10.6 to 21.2 percent. The greatest amount of interspecies homology observed was between *A. granularum* and *A. laidlawii* (21.2 percent). Very little hybridization (2.0 to 9.4 percent) was observed with DNA probes of *A. axanthum, A. morum, A. modicum, A. equifetale,* and *Acholeplasma* sp. strain Lemon. Thermal elution midpoint (t_e50) determinations were performed on the double-stranded DNA fractions eluted from hydroxyapatite columns for all homoduplexes and for a few selected heteroduplexes to measure the quality of the DNA-DNA duplex. A 1°C difference in t_e50 value is the equivalent to approximately 1.5 percent mismatching of bases [18]. There was 15 percent or greater mismatching in the base pairing among heteroduplexes of different *Acholeplasma* species.

Unlike most mycoplasmas belonging to the class *Mollicutes* (with the possible exception of *M. arginini*), acholeplasmas have been recovered from a wide variety of sources. Because the currently available serological methods differentiate between species but do not establish accurately the interrelatedness among strains within a species, it was of interest to determine the relationship among acholeplasma strains isolated from different hosts and habitats (birds, rodents, cats, swine, sheep, cattle, horses, goats, primates, and plants) by their nucleotide sequence homology. Twelve different strains of *A. laidlawii* and six strains of *A. axanthum* were examined by nucleic acid hybridization. Probes derived from the S743 and Swine Dl strains of *A. axanthum* gave homology values of 52.5 to 100 percent and 48.3 to 100 percent to the other strains, respectively. The probe to Swine Dl hybridized at a value of 81.7 percent to the 1190 strain, with corresponding similar t_e50 values (81°C and 80°C). The thermal midpoints (t_e50) values determined for the heteroduplexes ranged from 1.0 to 5.0°C lower than the t_e50 values of the corresponding homoduplex, indicating approximately 1.5 to 7.5 percent mismatching of base pairs among different strains. In general, the amount of mismatching of base pairs in the heteroduplex correlated directly with the amount of percentage homology; i.e., the greater the hybridization, the more similar were the t_e50 values. Similarly, the ^{3}H-DNA probes prepared to the PG-9, MIST, and L strains gave homology values from 62 to 100 percent with the other *A. laidlawii* strains.

These intraspecies homology values obtained with *A. laidlawii* and *A. axanthum* were quite different from those obtained among strains of *Mycoplasma pneumoniae.* Six human strains of *M. pneumoniae* were shown to be very similar. Five of the strains had greater than 92 percent homology, and the thermal elution midpoint values were also very similar [7]. These findings suggest a possible host selection pressure on genotypic characteristics.

The data presented indicate that approximately 50 percent or greater DNA homology is present among different strains of two species of *Acholeplasma* and that approximately 25 percent or less DNA homology was present among different established species of *Acholeplasma.* It is hoped that these DNA hybridization studies will provide insight into the molecular biology and evolution of these organisms and provide a model system for examining relationships between different species and strains of a given species among different genera in the class *Mollicutes.*

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