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 genically, 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-choleplasma 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 3H-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 3H-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
Acholplasma, for one unclassified, distinct new Acholplasma species (strain
lemon), and one species of Mycoplasma are listed in Table 1. Each 3H-DNA probe
hybridized extensively with its homologous DNA, giving values ranging from 75.1
percent for Acholplasma sp. strain Lemon to 90.4 percent for A. granularum. Each
Acholplasma 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 Acholplasma 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 Acholplasma
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 unlabelled DNA of A. granularum, 15 percent
with A. oculi, 11 percent with A. hippikon, and to a lesser amount (>7 percent)
with other Acholplasma species. The A. hippikon probe showed 14 to 15 percent
homology with unlabelled DNAs of A. oculi, A. laidlawii, and A. granularum, and
about 3 to 7 percent with the other Acholplasma 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 Acholplasma species. The most
recently recognized species, Acholplasma sp. strain Lemon (no epithet has been
given as yet) hybridized less than 5.8 percent with DNAs of all other Acholplasma
species, a finding which indicates that it represents a new Acholplasma species. The
Lemon strain has been shown to be an acholplasma by conventional procedures [1].

Thermal elution midpoints (Te50) were determined for the double-stranded DNA
fractions eluted off hydroxyapatite columns for each homoduplex and for a few
| Source of the \(^3\)H-DNA probe species (strain) | \(\%\) hybridization with excess unlabeled DNA |
|----------------------------------|--------------------------------------|
| Mycoplasma capricolum (California kid) | M. capricolum | A. axanthum | A. morum | A. modicum | A. equifetale | A. granularum | A. laidlawii | A. hippikon | A. oculi | A. sp. (lemon) | Duck |
| 87.4(100, 81.3)\(^*\) | 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)\(^*\) | 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(21.1, 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) |

\(^*\)The percentage hybridization with the \(^3\)H-DNA probe; data taken from reference [4]
\(^*\)The first value in parentheses represents the normalized values.
\(^*\)The second value in parentheses shows the thermal elution midpoint (t.50) of the DNA-DNA duplexes.
selected heteroduplexes. The \( t_{50} \) values of the nine homoduplexes for the nine Acholeplasma species are shown in Table 1. The \textit{A. axanthum} and \textit{Acholeplasma} sp. strain Lemon homoduplexes gave the lowest value of 82.0°C and \textit{A. hippiklon} homoduplex the highest value of 85.0°C. The \( t_{50} \) values of all heteroduplexes were 8° to 13°C lower than the values for each of the homoduplexes. \textit{A. granularum} had a \( t_{50} \) value of 84.3°C for its homoduplex and 74.5°C and 71.0°C for heteroduplexes with \textit{A. granularum} and \textit{A. oculi}, respectively. The \( t_{50} \) value for the \textit{A. laidlawii} homoduplex was 82.5°C, and the heteroduplex value with \textit{A. granularum} was 74.5°C. A \( t_{50} \) value of 85.0°C was obtained for the \textit{A. hippiklon} homoduplex and 75.0°C, 74.0°C, and 73.5°C \( t_{50} \) for heteroduplexes with \textit{A. granularum}, \textit{A. laidlawii}, and \textit{A. oculi}, respectively. The \( t_{50} \) value of the \textit{A. oculi} homoduplex was 83.6°C.

**DNA Homology Among Strains of \textit{A. axanthum} and \textit{A. laidlawii}**

Using a \(^3\text{H}-\text{DNA} \) probe derived from strain S743 of \textit{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 \textit{A. axanthum}. The thermal elution midpoints (\( t_{50} \)) for the heteroduplexes ranged from 1.0 to 5.0°C lower than the \( t_{50} \) 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 \( t_{50} \) values of different \textit{Acholeplasma laidlawii} strains (Table 2). The \(^3\text{H}-\text{DNA} \) 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 \textit{A. laidlawii}. The \( t_{50} \) values of the \textit{A. laidlawii} strains varied from 0.6 to 6.0°C below the homoduplex \( t_{50} \) values (Table 3).

| Source and Strain of \textit{A. axanthum} used as excess unlabeled DNA | % hybridization of \(^3\text{H}-\text{DNA} \) probe |
|------------------------|---------------------|
| S743                   | 78.9(100)\(^*\) \([82.0]\) | 52.5(60.8) \([76.0]\) |
| Swine D1               | 48.0(60.8) \([76.5]\) | 86.8(100) \([81.0]\) |
| H86N                   | 55.3(70.1) \([78.3]\) | 57.9(66.7) \([77.5]\) |
| 1190                   | 41.8(52.9) \([76.7]\) | 71.0(81.7) \([80.0]\) |
| J248                   | 51.3(65.0) \([77.0]\) | 61.8(71.2) \([76.5]\) |
| 501                    | 41.5(52.5) \([77.0]\) | 42.0(48.3) \([76.0]\) |
| \textit{M. capricolum} | 2.1                 | 1.8                         |
| Duck DNA               | 2.9                 | 1.1                         |

\(^*\)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].

\(^*\)The values in parentheses represent normalized values.

\(^*\)The figures in brackets show the thermal elution midpoint of the DNA-DNA duplexes.

\(^*\)This lagoon was used to service swine and cattle.
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TABLE 3
Degree of DNA-DNA Hybridization Using 3H-DNA Probes Derived from A. laidlawii Strains L and MIST with Excess Unlabeled DNA from Other A. laidlawii Strains

| Strain | Source | % hybridization of 3H-DNA probe to A. laidlawii strains* |
|--------|--------|--------------------------------------------------------|
|        |        | PG-9 | L | MIST |
| MIST   | compost | 68.4(83.1)* | 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]* | 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 |

*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].

*The values in parentheses represent normalized values.

*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 3H-DNA probes used in this study were labeled to a high specific activity of 1–2 × 107 cpm/μg using nick translation techniques [8,9]. The 3H-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 3H-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 Cn + value of 300 when ≥ 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 cm3) 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 plaque 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 (tₑ5₀) 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 tₑ5₀ 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 tₑ5₀ values (81°C and 80°C). The thermal midpoints (tₑ5₀) values determined for the heteroduplexes ranged from 1.0 to 5.0°C lower than the tₑ5₀ 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 tₑ5₀ values. Similarly, the ³H-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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