Intraspecies Genetic Relatedness among Strains of *Acholeplasma laidlawii* and of *Acholeplasma axanthum* by Nucleic Acid Hybridization

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(Received 1 September 1982; revised 21 October 1982)

This study compares the intraspecies genetic relatedness among strains of two established species of *Acholeplasma*. Radiolabelled DNA probes were prepared from three strains of *Acholeplasma laidlawii* and two strains of *Acholeplasma axanthum*, by using the nick translation method. The labelled DNA probes of these two strains were hybridized to an excess of unlabelled DNA from 12 strains of *Acholeplasma laidlawii* and from six strains of *Acholeplasma axanthum*, respectively. Nucleic acid hybridization analyses showed a wide variation among strains within each of the two established species, ranging from 48 to 100% homology. The results demonstrate that strains isolated from diverse hosts and habitats within a given species of *Acholeplasma* exhibit extensive genotypic variations.

INTRODUCTION

The acholeplasmas are a group of cell wall free prokaryotes distinguished from other members of the *Mollicutes* by their ability to grow in artificial medium without the addition of animal serum, cholesterol or sterols. With the possible exception of *Mycoplasma arginini* (Orning *et al.*, 1978), most *Mycoplasma* species have a restricted host range. However, the acholeplasmas have been found widely distributed in nature, having been recovered from a variety of animals including birds, rodents, cats, swine, sheep, cattle, horses, goats and primates (Tully, 1979) and from contaminated cell cultures (Barile, 1979). In addition, acholeplasmas have been isolated from soil (Seiffert, 1937), plants and flowers (Daniels & Meddins, 1972; Eden-Green, 1977, 1978; Eden-Green & Tully, 1979; Kleinhempel *et al.*, 1972; McCoy *et al.*, 1980; Whitcomb *et al.*, 1982) and vegetables (Somerson *et al.*, 1982).

The current classification scheme for acholeplasmas now recognizes eight distinct species. Until recently, differentiation of each species was based on conventional serological and a limited number of biochemical tests (26). However, recent findings (Aulakh *et al.*, 1983) have demonstrated that the eight species of *Acholeplasma* can be differentiated readily by nucleic acid hybridization analysis, and that these techniques can be used to examine candidate species of *Acholeplasma* (Aulakh *et al.*, 1979; Rose *et al.*, 1980; Stephens *et al.*, 1981). Most of the strains examined in these studies were isolated from animal tissues. In order to determine genetic relatedness among strains within a given species and isolated from diverse plant and animal tissues, a comparative study was undertaken to examine a selected series of *Acholeplasma axanthum* and *Acholeplasma laidlawii* strains. This report presents the results of this study.

Table 1. *Strains of Acholeplasma laidlawii and Acholeplasma axanthum used in the study*

Species and strain of acholeplasma	Isolated from:	Isolated by:	Received from:
<i>Acholeplasma laidlawii</i>			
MIST	Compost	G. Seiffert	E. A. Freundt
ALGEN	Soil	G. Seiffert	E. A. Freundt
L	Soil	G. Seiffert	E. A. Freundt
PG-9	Sewage	P. P. Laidlaw	D. G. ff. Edward
PG-10(B-15)	Bovine, urogenital	D. G. ff. Edward	D. G. ff. Edward
J-18S	Bovine, urogenital	N. O. Olson	D. G. ff. Edward
643N	Bovine, nasal	N. O. Olson	D. G. ff. Edward
PG-5	Rat lung	E. Kleineberger	D. G. ff. Edward
STR	Rat	L. Dienes	R. Lemcke
H3-10	Chicken, sinus	H. Alder	H. Adler
KHS	Goat	M. Shifrine	P. F. Smith
OR	Human, oral	S. Razin	S. Razin
<i>Acholeplasma axanthum</i>			
S-743	Cell culture	C. Friend	J. G. Tully
Swine D1	Swine	D. Schimmel	L. Stipkovits
H86N	Bovine, nasal	N. O. Olson	J. G. Tully
1190	Sewage, lagoon	R. Ross	R. Ross
J248	Coconut palm	S. Eden-Green	J. G. Tully
501	Broccoli	N. Somerson	
<i>Mycoplasma capricolum</i>			
Calif. kid	Goat (kid)	M. Tourtellotte	ATCC 27343
Avian DNA	Duck liver	Prepared by one of us (G.A.)	

METHODS

Organisms employed and cultivation techniques. The origin of the *Acholeplasma* species and the strains used in this study are listed in Table 1. Each strain was purified by the filtration cloning procedure as described previously (Subcommittee on the Taxonomy of Mollicutes, 1979). All acholeplasmas were grown in 4 l quantities of 1% (w/v) bovine serum fraction broth medium as described elsewhere (Aulakh *et al.*, 1979). Organisms were grown at 37 °C for 24 h and collected by centrifugation (16000 *g* for 1 h in GSA rotor, Sorvall) at 4 °C. The acholeplasma cell pellets were resuspended in 50 ml Hanks' balanced salt solution and centrifuged at 27000 *g* for 30 min in an SS-34 rotor (Sorvall) at 4 °C. The pellets were immediately used for the purification of cell DNA. *Mycoplasma capricolum* (ATCC 27343) was used as a negative control organism in the hybridization studies. Avian (duck) DNA was also used as a control.

DNA purification. The pellet from each strain was lysed in a solution containing 8 M-urea, 1% SDS, 1 M-NaClO₄, 1 mM-EDTA and 0.24 M-NaH₂PO₄/Na₂PO₄ (PB). The DNA was purified using the hydroxyapatite batch elution method as described elsewhere (Aulakh *et al.*, 1979; Britten *et al.*, 1968, 1974). The purified DNA was pelleted in a Ti 60 rotor at 40000 r.p.m. for 18 h at 20 °C. The pellet was resuspended in TNE buffer (0.01 M-Tris, pH 7.8, 0.1 M-NaCl, 0.001 M-EDTA) and stored at 4 °C.

[³H]DNA probe synthesis. Purified native DNA was labelled *in vitro* with all four labelled nucleotide triphosphates by using the nick-translation method (Kelly *et al.*, 1970; Maniatis *et al.*, 1975) and processed for hybridization as previously described (Aulakh *et al.*, 1979, 1983; Britten *et al.*, 1974). The specific activity of the [³H]DNA probes was approximately 1.5–2.0 × 10⁷ c.p.m. µg⁻¹.

Nucleic acid hybridization procedure. The hybridization procedures used were the same as those described previously (Aulakh & Gallo, 1977; Aulakh *et al.*, 1979, 1983; Stephens *et al.*, 1981). The hybridization mixture contained 1 mg of unlabelled sheared DNA ml⁻¹, 150000 c.p.m. [³H]DNA probe ml⁻¹ and final concentrations of 0.4% 0.001 M and 0.48 M SDS, EDTA and PB, respectively. Reaction mixtures were denatured at 105 °C for 5 min and incubated at 65 °C overnight to a C₀t value of > 300 (Britten *et al.*, 1974). Hybridized DNA was separated from single stranded DNA using a hydroxyapatite column equilibrated at 60 °C with 0.12 M-PB containing 0.2% SDS. Radioactive material not adsorbing to the hydroxyapatite column was considered to be single stranded DNA. The hybridized double stranded DNA was then eluted from the column with 0.48 M-PB containing 0.2% SDS.

Thermal elution midpoint (t_{0.50}) determinations. Thermal elution midpoints (t_{0.50}) were performed by preparing hydroxyapatite columns as described above. The hybridized DNA was adsorbed on to the column at 60 °C and washed thoroughly with 0.12 M-PB containing 0.2% SDS at 4 °C increments up to 100 °C. All measurements of

radioactivity were performed by adding 12 ml Aquasol to 4 ml of eluate and counting in a Packard Tricarb liquid scintillation counter.

Serological tests. All of the *A. laidlawii* strains had previously been shown to be related to the type strain PG-8 of this organism by growth inhibition (Clyde, 1964; Orning *et al.*, 1978) and immunofluorescence (DelGiudice *et al.*, 1967) serological techniques (Tully 1973; Tully & Razin, 1968). Several of the *A. axanthum* strains had also been documented previously as being serologically related to the type strain S743 of this species (Tully 1973). Those not included and representing more recent isolates, were identified by growth inhibition and immunofluorescence tests with antiserum prepared to the H86N strain of *A. axanthum*, using procedures described earlier (Tully 1973; Tully & Razin, 1968).

RESULTS

Characterization of [³H]DNA probes

The [³H]DNA probes prepared to the PG-9, L, and MIST strains of *Acholeplasma laidlawii* hybridized 82.3, 91.9 and 82.0% to their homologous DNA, respectively. Similarly, the [³H]DNA probes derived from the S-743 and Swine D1 strains of *A. axanthum* hybridized 78.9% and 86.8% to their homologous DNA. The value obtained with homologous DNA was normalized to 100% for comparison purposes. These five [³H]DNA probes did not hybridize to the unrelated DNA derived from *M. capricolum* and a background sticking between 1.1 and 2.9% was observed with the negative control avian (duck) DNA to hydroxyapatite columns. These results demonstrate the specificity of the probes used in this study (Tables 2 and 3).

Nucleic acid homology tests with A. laidlawii

The results of the hybridization with [³H]DNA probes derived from the PG-9, L, and MIST strains of *A. laidlawii* are presented in Table 2. A wide variation of hybridization values were seen among the various strains of *A. laidlawii* used in this study. The hybridization values for ³H-MIST, ³H-L and ³H-PG-9 DNA probes ranged from 63 to 93% for the 12 strains examined. Thermal elution midpoints (*t*_c50) were determined for most of the homoduplexes and heteroduplexes. The *t*_c50 values for the three homoduplexes ranged from 82.5 to 84.1 °C and the values for the heteroduplexes ranged from 77.6 to 83.5 °C when tested against the two DNA probes.

Nucleic acid homology tests with A. axanthum

The results of hybridization using two [³H]DNA probes of *A. axanthum* are presented in Table 3. As seen with *A. laidlawii*, *A. axanthum* also demonstrated a wide range of nucleotide diversity among the six strains used in the study, and ranged from 48.3 to 81.7% homology when tested against the strain S743 and Swine D1 [³H]DNA probes. The *t*_c50 values for the two homoduplexes of the S743 and Swine D1 probes were 82.0 and 81.0 °C, respectively (Table 3). The *t*_c50 values of heteroduplexes with these two probes ranged from 76.0 to 80.0 °C among the six strains examined.

DISCUSSION

We have attempted to establish here the genetic interrelationships among a number of strains isolated from diverse sources and previously identified either as *A. laidlawii* or *A. axanthum* by a number of conventional biochemical and serological tests (Tully & Razin, 1969). We have shown previously that nucleic acid hybridization can provide a critical indicator in determining the genetic interrelationship among recognized species of *Acholeplasma* (Aulakh *et al.*, 1979, 1983; Stephens *et al.*, 1981) and *Mycoplasma pneumoniae* (Chandler *et al.*, 1982). Studies on the genetic relatedness among the eight established species of *Acholeplasma* showed that there was very little homology among the *Acholeplasma* species (generally less than 10%) although a cluster of *A. laidlawii*, *A. granularum*, *A. oculi* and *A. hippikon* type strains showed homologies of from 10 to 20%. Prototype strains of *A. laidlawii* and *A. granularum* showed the most relatedness and hybridized at the level of 20%. Thus, the distinctions observed among species of *Acholeplasma*

Table 2. *Degree of DNA-DNA hybridization using [³H]DNA probes derived from A. laidlawii strains L and MIST with excess unlabelled DNA from other A. laidlawii strains*

Strain	Percentage hybridization of [³ H]DNA probe to <i>A. laidlawii</i> strains*		
	Pg-9	L	MIST
MIST	68.4 (83.1)	82.7 (90.0) [83.5]	82.0 (100) [83.2]
ALGEN	58.1 (70.6)	68.9 (75.0) [81.0]	65.2 (79.5) [82.0]
L	64.0 (77.8)	91.9 (100) [84.1]	76.2 (92.9) [82.0]
PG-9	82.3 (100) [82.5]	72.0 (78.0) [79.5]	68.4 (83.0) [80.0]
PG-10	59.1 (71.8)	73.2 (80.0) [81.0]	70.4 (85.8) [81.5]
J18S	65.1 (79.1)	68.4 (74.4) [79.0]	64.7 (78.9) [81.0]
643N	73.1 (88.9)	74.4 (81.0) [80.0]	70.4 (85.8) [79.5]
PG-5	71.7 (87.1)	63.8 (69.5) [78.5]	61.1 (74.5) [77.6]
STR	58.7 (71.3)	60.3 (66.7) [78.0]	51.6 (62.9) [78.4]
H3-10	65.1 (79.1)	55.5 (64.5) [78.0]	57.4 (70.0) [78.0]
KHS	68.8 (83.6)	62.0 (67.4) [78.3]	59.7 (72.8) [78.4]
OR	71.8 (87.2)	61.6 (67.0) [78.0]	56.8 (69.2) [78.4]
<i>M. capricolum</i>	2.0	2.1	2.5
Duck DNA	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%. The values in parentheses represent normalized values; results for homologous DNA are shown in *italic*. The figures in square brackets show the thermal elution midpoint of the DNA-DNA duplexes.

Table 3. *Degree of DNA-DNA hybridization using [³H]DNA probes derived from A. axanthum strains S743 and Swine D1 with excess unlabelled DNA from other A. axanthum strains*

Strain	Percentage hybridization of [³ H]DNA probe*	
	S743	Swine D1
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]
<i>M. capricolum</i>	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%. The values in parentheses represent normalized values; results for homologous DNA are shown in *italic*. The figures in square brackets show the thermal elution midpoint of the DNA-DNA duplexes.

attained by conventional biochemical and serological tests correlated extremely well with data obtained by nucleic acid hybridization (Aulakh *et al.*, 1983). The availability of strains of *A. axanthum* and *A. laidlawii* derived from a number of diverse origins (i.e. various mammalian hosts, plants, soil, compost, sewage) provided an opportunity to study the genetic relatedness among these various strains by nucleic acid hybridization.

The results presented indicate that strains of *A. axanthum* and *A. laidlawii* (classified by serologic and biochemical procedures) showed a considerable amount of variation in nucleic acid sequence homology. The DNA probe of the Swine D1 strain of *A. axanthum* hybridized 86.8% to its homologous DNA and from 42.0% to 71.0% with DNA from five other strains of *A. axanthum*. The maximum amount of homology was observed between the Swine D1 probe and strain 1190 DNA. The close relatedness between these two strains was also reflected in the t_{c50} value for the heteroduplex which was only 1 °C lower than the Swine D1 homoduplexes and equivalent to only 1.5% mismatching in base pairing (Laird *et al.*, 1969). Larger differences (4 to 5 °C) in t_{c50} were observed for the heteroduplexes of *A. axanthum* strains isolated from different

hosts (bovine, cell culture and plants) with the Swine D1 strain. Hybridizations were also performed using a probe derived from a cell culture strain S743. Again, there was a large difference (3 to 6 °C) in the t_{e50} values for heteroduplexes of strains isolated from diverse sources. The hybridization values of these diverse strains from different origins ranged from 52.5 to 70.1% homology with the cell culture strain S743 probe. The considerable genotypic heterogeneity among *A. axanthum* strains was also reflected by marked differences in the electrophoretic patterns of the digestion products of their DNAs by restriction endonucleases (Razin *et al.*, 1983). It is tempting to suggest that the amount of homology or relatedness among strains may be influenced by the residing host.

Probes prepared to the PG-9, L and MIST strains of *A. laidlawii* showed results similar to that observed with *A. axanthum* strains. DNA probes of the PG-9, L and MIST strains of *A. laidlawii* hybridized 82.3, 91.9, and 82.0% to their homologous DNA. The amount of homology with these three probes (PG-9, L and MIST) and the other 11 heterologous DNA ranged from 62.9 to 92.9%. The t_{e50} values ranged accordingly and varied from 77.6 °C for ³H-MIST and PG-5 to 83.5 °C for the ³H-L strain and MIST indicating 8.5 and 0.9% mismatching of base pairing for the heteroduplexes, respectively. Reciprocal hybridizations using the MIST probe confirmed the high level of nucleotide homology between the L and MIST strains (≥90%). The t_{e50} values for these heteroduplexes were only 0.6 to 0.8 °C lower than the homoduplexes indicating less than 1.2% mismatching in base pairing. The results obtained for the *A. laidlawii* strains also suggest that the amount of relatedness or homology may be influenced by the host from which the strain was isolated.

Although the basis for the large variation among these strains of *A. laidlawii* and *A. axanthum* is unknown, these strains were recovered from diverse host and habitats. Whether the host or habitat influences the genotypic expression of the organisms remains to be determined. Such studies are under investigation. Nonetheless, five strains of *M. pneumoniae* isolated from different patients (but only one host) with primary atypical pneumonia showed from 85 to 100% homology. These data indicate that strains of *M. pneumoniae* are very similar and that the species *M. pneumoniae* is remarkably homogeneous (Chandler *et al.*, 1982).

This and earlier studies (Aulakh *et al.*, 1979, 1983; Stephens *et al.*, 1981) show that the homology among strains of a given *Acholeplasma* species is approximately 50% or more, showing no more than 12% mismatching in base pairing among the common sequences. In addition, the prototype strains representing eight different *Acholeplasma* species show less than 25% homology and more than 12% mismatching of base pairing. These data may provide useful information to be used in guidelines to define properties required to establish a new species of *Acholeplasma*.

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