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Nucleic Acid Relationships Among *Acholeplasma* Species

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^{3}H-labeled *Acholeplasma* DNA probes were generated in vitro by the nick-translation method and used to determine the nucleotide sequence homology among the type strains of the eight currently recognized species of *Acholeplasma*. Very little nucleotide sequence homology (≤18%) was found among the eight species, with heteroduplexes showing at least 12% or more mismatching as determined by thermal elution midpoints. The small amount of nucleotide sequence homology among the eight species indicates that these species are quite distinct and are not closely related to each other genomically.

The genus *Acholeplasma* is a group of wall-free procaryotes classified with other mycoplasmas in the class *Mollicutes*. They are distinguished from *Mycoplasma* species and other genera in the class by their ability to grow in culture medium free of animal sera, cholesterol, or other sterols. For the most part, the *Acholeplasma* species have been differentiated by a limited number of biochemical properties (including lack of a sterol requirement) and by serological techniques (28). Although serological procedures have proved very useful for separation of species in the genus *Mycoplasma*, their application to *Acholeplasma* species has presented several problems. Rabbit preimmunization sera frequently contain low but measurable levels of anti-acholeplasmal activity, and antisera prepared to acholeplasmas are generally of low potency. Although the specificity of serological tests for acholeplasmas has been improved (29), separation by using nucleic acid hybridization would offer considerable information on the genomic relatedness of these organisms. In fact, nucleic acid hybridization techniques and thermal stability studies have proved to be valuable tools for the genomic and genotypic analysis of various procaryotic and eucaryotic organisms (2, 3, 6, 8, 10–12, 14, 16, 18, 20–23, 25, 27). Therefore, it is important to establish the DNA relationship among these species. DNA hybridization comparisons have been made of strains in the first two species (*Acholeplasma laidlawii* and *Acholeplasma granularum*) in the genus. Neimark (21) immobilized DNA on nitrocellulose filters and showed that strain S-743 (the type strain) of *Acholeplasma axanthum* was unrelated to strains of *A. laidlawii* and *A. granularum*. Using labeled RNA-DNA hybridization techniques, he reported 32 to 34% homology between *A. granularum* and *A. laidlawii*. Recently, we have used nick-translation techniques to show a lack of DNA homology between the established species of *Acholeplasma* and new strains of acholeplasmas isolated from tissue cultures (2, 24) and from plants (26). We report here the relationships among the eight currently recognized *Acholeplasma* species as determined by DNA-DNA hybridization and serological procedures.

MATERIALS AND METHODS

Cultures. The *Acholeplasma* type strains were used, except that PG 9 replaced PG 8, and are listed in Table 1. Each strain had been filter cloned three times (13) and each had been grown for at least 10 consecutive passages in a serum-free culture medium (28). *Mycoplasma capricolum* California kid (the type strain) was used as the control organism representing the sterol-requiring mycoplasmas. The medium contained mycoplasma broth base (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 10% fresh yeast extract and either 0.5% (vol/vol) bovine serum albumin (fraction V) (Difco Laboratories, Detroit, Mich.), 10 mg of palmitic acid per liter, and 0.01% (vol/vol) Tween 80 (29) or 1% (vol/vol) bovine serum fraction (Difco). Each *Acholeplasma* species was grown in 2-liter quantities of broth medium described previously (2, 26) at 37°C for 48 h and harvested by centrifugation at 15,000 × *g* for 1 h at 4°C. Pellets were suspended in Hanks balanced salt solution and then sedimented again by centrifugation at 27,000 × *g* for 30 min at 4°C. The cell pellets of each species were frozen at −70°C until the DNA was extracted and purified.

DNA purification. DNA was purified by the hydroxyapatite batch elution technique (5). Cells were lysed in a solution containing 8 M urea, 1% sodium dodecyl sulfate (SDS), 1 M $NaClO_4$, 0.24 M NaH_2PO_4-Na_2HPO_4 (phosphate buffer), and 1 mM EDTA. The

cell lysate was homogenized for 15 min in a tissue homogenizer and then vigorously shaken for 15 min with an equal volume of a 24:1 (vol/vol) mixture of choloroform and octanol. The liquid phases were separated by centrifugation at 3,000 × *g* for 30 min. DNA in the aqueous phase was adsorbed onto 10 g of hydroxyapatite. The hydroxyapatite was washed five times with 40 ml of MUP buffer (8 M urea, 0.24 M phosphate buffer) to remove proteins and RNA and three times with 0.03 M phosphate buffer to remove the urea. The DNA was then eluted with 0.48 M phosphate buffer, sedimented by centrifugation at 144,000 × *g* for 24 h at 15°C (Beckman L-5-75 centrifuge, Ti 50 rotor), suspended in buffer containing 0.1M NaCl and 0.01 M Tris-hydrochloride (pH 8.3), sheared to a piece the size of approximately 400 nucleotide fragments, and further purified (1).

[^{3}H]DNA probe synthesis and processing. DNA was labeled in vitro by a modification of methods reported previously (15, 19). Two micrograms of native DNA was labeled in a reaction mixture containing 0.027 mM concentrations of each of the four ^{3}H-labeled nucleoside triphosphates, 50 mM Tris-hydrochloride (pH 7.8), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 50 μg of bovine serum albumin per ml, 6 U of *Escherichia coli* DNA polymerase I (Boehringer-Mannheim Corp., Indianapolis, Inc.), and 2 μl of 0.1 μg of DNase I per ml. The reaction was terminated between 1 and 2 h by the addition of 0.4 M NaCl and 0.4% SDS when 1.0×10^7 to 2.0×10^7 cpm of labeled nucleotides was incorporated per μg of DNA as determined by trichloroacetic acid precipitation.

The ^{3}H-labeled DNA was separated from unincorporated labeled nucleoside triphosphates by adsorption onto hydroxyapatite (60°C, 0.12 M phosphate buffer, 0.2% SDS) and washed with 0.12 M phosphate buffer until background radioactivity was obtained. The probe was eluted with 0.48 M phosphate buffer and then sheared into about 400-nucleotide fragments by passing the DNA solution through a French press cell (American Instrument Company, Silver Spring, Md.) at 50,000 lb/in^2 (340 MPa). The [^{3}H]DNA probe was extracted with chloroform and then with phenol-cresol (8:1) and dialyzed overnight against 0.1 M Tris-hydrochloride–0.01 M NaCl–0.001 M EDTA (pH 7.5; TNE buffer). After concentration by lyophilization, the probe was reconstituted in 1 ml of 0.12 M phosphate buffer and denatured at 105°C for 5 min. The probe was then incubated at 65°C for 30 min, the cross-linked and foldback DNA was removed by adsorption onto hydroxyapatite (60°C, 0.12 M phosphate buffer, 0.2% SDS), and the purified, radioactive single-stranded DNA preparation was used for the hybridization studies.

Nucleic acid hybridization. The hybridization procedure used was described previously (1, 2, 26). The 100 μl of hybridization mixture contained 100 μg of unlabeled sheared DNA, 15,000 cpm of the [^{3}H]DNA probe, and final concentrations of 0.2%, 0.001 M, and 0.48 M SDS, EDTA, and phosphate buffer, respectively. Reaction mixtures were overlaid with mineral oil to prevent evaporation. Reaction mixtures were heated to 105°C for 5 min and incubated at 65°C overnight. Unhybridized, single-stranded DNA was separated on a hydroxyapatite column equilibrated at 60°C with 0.12 M phosphate buffer containing 0.2% SDS. Hybrid DNA was eluted with 0.48 M phosphate buffer containing 0.2% SDS, and radioactivity was monitored by adding 12 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) to 4 ml of eluate in a scintillation counter.

T_e50 determinations. The thermal elution midpoint (T_e50) determinations were performed in a hydroxyapatite column (60°C, 0.12 M phosphate buffer, 0.2% SDS). The adsorbed hybridization mixture was washed 5 times with 4 ml of 0.12 M phosphate buffer containing 0.2% SDS to remove single-stranded DNA, and then the temperature was raised in 4°C increments to 100°C. The column was washed after each increment, and radioactivity was measured as described above.

Serological procedures. The *Acholeplasma* species were examined by the growth inhibition (7) and epi-immunofluorescence (9) procedures. Each antiserum was prepared against the type strain, with the exception that strain H86N of *A. axanthum* and strain N93 of *Acholeplasma equifetale* were used instead. A satisfactory growth-inhibiting antiserum to *Acholeplasma hippikon* was not available. For the growth inhibition test, undiluted antiserum was tested against young, actively growing broth cultures. The cultures were diluted 1:1,000 in broth and inoculated onto agar plates which were incubated at room temperature (25°C) for 48 h and at 37°C for an additional 24 h before zones of inhibition were measured.

For the direct epi-immunofluorescence tests, fluorescein-conjugated antiserum was serially diluted (twofold) in phosphate-buffered saline (pH 7.8), and each dilution was tested against homologous and heterologous *Acholeplasma* colonies grown for 24 to 48 h. Titers were recorded as the last dilution that produced a positive staining reaction.

RESULTS

Hybridization among *Acholeplasma* species. The hybridization data for the eight *Acholeplasma* species and the one *Mycoplasma* species are listed in Table 1. Each DNA probe hybridized extensively with its homologous DNA but not with the DNAs derived from duck tissues (≤3%) or from *M. capricolum* (≤5%), which served as negative DNA controls. The results of cross-hybridization among the eight established *Acholeplasma* species and *M. capricolum* indicate that each species tested has very little homology (≤8%) with the other species (Table 1). *A. granularum* and *A. laidlawii* DNAs showed a very small amount of homology with DNAs of several species. The values of *A. granularum* ranged from a maximum of 18% hybridization with *A. laidlawii* to 14% with *Acholeplasma oculi* and approximately 8 to 10% with *A. hippikon*, *A. equifetale*, and *Acholeplasma modicum*. In reciprocal tests, the *A. laidlawii* probe produced about 18% hybridization with unlabeled DNAs of *A. granularum* and 13% hybridization with *A. oculi*.

T_e50 values of duplexes. T_e50 values were determined for each homoduplex and for a selected few heteroduplexes. The *A. axanthum* homoduplex gave the lowest value, 82°C, and

TABLE 1. Degree of hybridization with [^{3}H]DNA probes of eight species of *Acholeplasma* and *M. capricolum*

Source of the [^{3}H]DNA probe	% Hybridization with excess unlabeled DNA from:								
	M. capricolum	*A. axanthum*	*A. morum*	*A. modicum*	*A. equifetale*	*A. granularum*	*A. laidlawii*	*A. hippikon*	*A. oculi*
M. capricolum (California kid)	87	2	3	3	3	4	3	4	2
A. axanthum (S-743)	5	79	4	2	4	4	5	4	4
A. morum (72-043)	2	4	84	4	4	3	5	5	3
A. modicum Squire (PG-49)	4	6	5	89	4	8	5	5	4
A. equifetale (C112)	2	4	3	3	78	7	7	7	2
A. granularum (BTS-39)	3	6	4	8	9	90	18	10	14
A. laidlawii (PG 9)	3	6	5	5	7	18	87	10	13
A. hippikon (C1)	3	5	7	6	6	12	13	90	14
A. oculi (19-L)	3	3	3	5	7	10	12	10	79

the *A. hippikon* homoduplex gave the highest value, 85°C. The T_e50 values of all heteroduplexes studied were 8 to 13°C lower than the values for each of the homoduplexes. *A. granularum* had T_e50s of 84°C for its homoduplex and 75 and 71°C for heteroduplexes with *A. laidlawii* and *A. oculi*, respectively. The T_e50 value for the *A. laidlawii* homoduplex was 83°C, and the heteroduplex value with *A. granularum* was 75°C. Thus, the T_e50 values indicate that the heteroduplexes were thermally less stable than the homoduplexes, indicating a considerable amount of mismatching among the base pairings of heteroduplexes.

Serological relationships. Using optimal conditions for the preparation of antiserum and for the performance of serological tests (28), we observed no cross-reactivity by growth inhibition assay and only a few one-way cross-reactions were noted by epi-immunofluorescence between the *A. oculi* conjugate and *A. laidlawii*, the *A. axanthum* conjugate and *A. modicum*, and the *A. modicum* conjugate and *A. oculi*.

DISCUSSION

This report examines the amount of nucleotide sequence homology among the eight established *Acholeplasma* species. The genus *Acholeplasma* currently comprises a limited number of recognized species. The results presented indicate that the eight species of *Acholeplasma* (classified by serological and biochemical procedures) are distinct and readily differentiated by DNA-DNA hybridization techniques. Labeled DNA probes from each *Acholeplasma* species hybridized between 78 and 90% to their homologous unlabeled DNA, and very little hybridization was observed among heterologous species. However, a small amount of cross-hybridization was seen between *A. granularum*, *A. oculi*, *A. laidlawii*, and *A. hippikon*. *A. laidlawii* and *A. granularum* showed the highest amount (≈18%) of homology.

T_e50 values were used to determine the quality of each DNA-DNA homoduplex and also of a few selected heteroduplexes formed by a cluster of *Acholeplasma* species producing 10% or more hybridization. A 1°C difference in the T_e50 value is equivalent to approximately 1.5% mismatching of base pairing (17). Thus, the 8 to 13°C lower T_e50 values produced among the *A. laidlawii*, *A. granularum*, *A. oculi*, and *A. hippikon* cluster corresponded to 12 to 20% mismatching in the base pairing of these heteroduplexes.

Recently, the genetic diversity among strains of *A. laidlawii* and *A. axanthum* was examined by [^{3}H]DNA-DNA hybridization (E. B. Stephens, G. S. Aulakh, D. L. Rose, J. G. Tully, and M. F. Barile, J. Gen. Microbiol., in press). The values obtained with three different *A. laidlawii* probes ranged from 63 to 93% against 12 strains of *A. laidlawii* examined and from 48 to 82% against 6 strains of *A. axanthum* examined, using two *A. axanthum* probes.

The *Acholeplasma* species share common nutritional, biochemical, physiological, and genetic characteristics. They do not require sterols for growth, have a genome size of 10^9 daltons, have similar enzyme expressions (e.g., superoxide dismutase), can synthesize neutral lipids and phospholipids from acetate, and can be grown in a defined synthetic medium. Nonetheless, the data presented indicate that the eight established

species of *Acholeplasma* have very little interspecies nucleotide sequence homology. Each of the eight species classified earlier by conventional serological and biochemical procedures was shown to be distinct by hybridization and T_e50 techniques.

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