JOURNAL OF BACTERIOLOGY, Aug. 1983, p. 802–805 Vol. 155, No. 2
0021-9193/83/080802-04$02.00/0
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DNases of *Acholeplasma* spp.

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Received 25 January 1983/Accepted 27 May 1983

One strain from each of seven species of *Acholeplasma* was examined for the presence of DNases. Six of the strains were found to be DNase positive when assayed with DNA–methyl-green-containing agar, indicating that this method can be used to differentiate the seventh strain, *Acholeplasma axanthum*, from the remaining *Acholeplasma* spp. Electrophoretic patterns obtained from sodium dodecyl sulfate-polyacrylamide gels containing DNA revealed DNases in the cell extracts of all seven strains and in the supernatant growth medium of five of the strains. The electrophoretic patterns were highly characteristic for each strain and can be used for the rapid identification of different strains of *Acholeplasma*.

Acholeplasma spp. lack the metabolic pathways for the synthesis of pyrimidine and purine bases and therefore require these molecules in their growth medium (7, 13). Thus, one would expect to find *Acholeplasma* spp. rich in nucleases, since these enzymes would be required for the metabolism of exogenous nucleic acids. Several investigators (4–6, 9) have found DNases and RNases in the membrane and soluble fractions of *Acholeplasma laidlawii*, where others (9) have detected DNase activity in colonies of *A. laidlawii* growing in agar medium containing DNA. Razin et al. (9) compared the nuclease content of several *Mycoplasma* species, including *A. laidlawii*, and found differences in the overall level of nuclease activity. Although these investigations focused on *A. laidlawii*, the evidence strongly suggests that nucleolytic activity may be a common occurrence in the other *Acholeplasma* species.

In this study, seven strains of *Acholeplasma* were assayed for DNases. Rapid quantitative screening for DNase activity in colonies of *Acholeplasma* growing in nutrient agar was made possible by modifying the Lacks (2) DNA-methyl green plate assay. The electrophoretic technique of Rosenthal and Lacks (10), which uses sodium dodecyl sulfate (SDS)-polyacrylamide gels containing nucleic acid, was used to qualitatively analyze extracts and cell-free growth medium obtained from each strain. The modified DNA-methyl green plate assay revealed the presence of DNase activity in six of the seven strains. The electrophoretic patterns obtained from analysis of extracts and cell-free growth medium in SDS-polyacrylamide gels containing nucleic acid likewise revealed extensive DNase activity in these microorganisms. These findings have led us to suggest the use of these two assay techniques as methods for differentiating the seven strains of *Acholeplasma* used in this study.

MATERIALS AND METHODS

***Acholeplasma* strains and media.** One strain from each of the seven known species of the genus *Acholeplasma* was used. *A. laidlawii* A, *Acholeplasma axanthum* S-743, *Acholeplasma oculi* 19L, and *Acholeplasma hippikon* C1 were gifts from Harold C. Neimark, Downstate Medical Center, Brooklyn, N.Y. *Acholeplasma equifetale* C112, *Acholeplasma granularum* BTS-39, and *Acholeplasma modicum* PG49, catalog numbers 29724, 19168, and 29102, respectively, were obtained from the American Type Culture Collection, Rockville, Md. Growth medium consisted of 3% Trypticase soy broth (BBL Microbiology Systems), pH 7.8, containing 5% horse serum (Flow Laboratories, Inc.) and 10^3 U of penicillin per ml. The serum, although not required, was included to enhance the growth of *Acholeplasma* spp.

Plate assay of DNase content. The assay was modified from that described by Lacks (2) to allow for the optimal detection of DNases of *Acholeplasma* spp. Methyl green (1 mg/ml) was prepared and sterilized as described previously (2). To prevent growth of several types of spore-forming bacilli whose spores are found as contaminants in the DNA (salmon testes; Worthington Diagnostics), the DNA was prepared by dissolving in sterile water at 4°C and then adding 0.5% Trypticase soy broth–0.1 M Tris (pH 7.6)–200 U of bacitracin per ml–2,000 U of penicillin per ml. The solution was then incubated at 37°C for 48 h after which the DNA was further sterilized by precipitation with ethyl alcohol and dissolved in sterile water at 2 mg/ml. Petri dishes containing basal layers of growth medium in 1% agar were prepared in advance. For each dish, a sample of culture was plated, and the

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plates were incubated at 37°C until colonies measured approximately 250 to 500 μm in diameter. Then, to each dish was added 10 ml of DNA-methyl green overlay prepared in advance as follows: 0.75 g of agar in 30 ml of 0.25 Tris (pH 7.6), was autoclaved, cooled to 48°C, and then added to 50 ml of 2 mg of DNA per ml and 20 ml of 1 mg of methyl green per ml at 40°C. The dishes were again incubated at 37°C and then examined at appropriate times.

For some experiments, bacitracin at 100 U/ml was added to the DNA-methyl green solution. This antibiotic, like other cyclic peptide drugs, affects the integrity and function of the cytoplasmic membrane (15), causing the membrane to become "leaky." Bacitracin, therefore, was used in several assays to promote the release of DNases which would not otherwise be available extracellularly in sufficient concentration for detection by the DNA-methyl green plate assay.

Cell fractions. *Acholeplasma* spp. were grown to log phase at 37°C in 100-ml cultures. All subsequent operations were carried out at 0 to 4°C. Cells were sedimented by centrifugation at 10,000 rpm for 40 min. To samples of supernatant fluid was added an inhibitor of trypsin-like proteolytic enzymes (12), α-toluenesulfonyl fluoride (Eastman Chemical Products, Inc.), to a final concentration of 250 μg/ml, and the solution was frozen at −20°C for future use. Cell pellets were suspended in a solution containing 0.05 M Tris-hydrochloride (pH 7.6)–0.25 M NaCl and sedimented again by centrifugation at 10,000 rpm for 20 min. Cell pellets were then dispersed in 1 ml of 0.01 M Tris-hydrochloride (pH 7.6) containing 250 μg of α-toluenesulfonyl fluoride per ml. The concentrated suspensions were lysed by sonication with a sonicator cell disrupter and then maintained at −20°C for future use.

Electrophoresis. The procedure of Rosenthal and Lacks (10) was used except for modification as follows. The source of SDS was Bio Rad Laboratories. The separating gel contained 10 μg of bovine serum albumin (Sigma Chemical Co.) per ml to enhance detection of nucleases (11). DNA was incorporated into the separating gel but not the stacking gel. The sample loading buffer contained 20% glycerol. All samples were heated for 3 min at 100°C before application.

Nuclease detection. The gels were washed and assayed for nucleases as in Lacks et al. (3), except that the buffer was changed twice in a 1-h period. The gels then were immediately assayed for nuclease activity by incubation in buffer containing divalent cations. The gels were periodically placed on a long wave UV transilluminator and photographed with a Polaroid MP4 system with type 55/PN film and Wratten no. 9 (yellow) and no. 25 (red) filters placed in front of the lens with the red filter positioned between the yellow filter and the lens.

Molecular weight determination. Bovine serum albumin, ovalbumin, myoglobin, and pancreatic DNase I were used as standard proteins as described in Rosenthal and Lacks (11).

RESULTS

DNA-methyl green plate assay. The results of the methyl green plate assays are summarized in Table 1. When nucleases are released from growing colonies and come in contact with the DNA-methyl green complex, the DNA is hydrolyzed and the complex disrupted, thereby producing a colorless halo or region around each colony. Halos formed by *Acholeplasma* spp. were similar except that the rate of halo formation was found to vary among the strains.

TABLE 1. Rate of halo formation for *Acholeplasma* spp. grown in the presence of DNA-methyl green overlay with and without bacitracin

Species	Rate (mm/h)[a]	
	With bacitracin (*n*)	Without bacitracin (*n*)
A. laidlawii A	0.074 ± 0.012 (46)	0.074 ± 0.014 (36)
A. granularum BTS-39	0.064 ± 0.019 (48)	0.063 ± 0.019 (40)
A. hippikon C1	0.056 ± 0.007 (34)	0.055 ± 0.008 (40)
A. oculi 19L	0.055 ± 0.008 (36)	0.052 ± 0.008 (32)
A. equifetale C112	0.050 ± 0.009 (46)	0.040 ± 0.007 (44)
A. modicum PG49	0.038 ± 0.004 (56)	0.038 ± 0.004 (60)
A. axanthum S-743	0.017 ± 0.003 (52)	

[a] The rate given reflects the sample mean ± the standard deviation followed by the number (*n*) of colonies sampled. Data were computed from measurements taken at 9, 21, and 43 h after overlaying with DNA-methyl green.

As indicated in Table 1, all but *A. axanthum* produced halos when assayed with DNA-methyl green. When bacitracin was included in the DNA-methyl green complex, however, halos were also observed around colonies of *A. axanthum*. The presence of bacitracin had little effect on the results obtained from the remaining six strains of *Acholeplasma*.

SDS-polyacrylamide gel assay. Detection of nucleases after electrophoresis in SDS-polyacrylamide gels cast with DNA is shown in Fig. 1. After renaturation, the nucleases hydrolyze the DNA to produce dark bands which are free of the ethidium bromide-DNA complex. The rates of appearance of bands and their sizes are proportional to the nuclease concentration.

The band of activity in lane 1 (Fig. 1) corresponds to the purified enzyme, pancreatic DNase I (M_r = 31,000). The band in Fig. 1, lane 7 (M_r = 36,000) corresponds to the nuclease present in the horse serum used in the growth medium for *Acholeplasma* spp. The band of activity given by the horse serum is also found in each odd-numbered lane beginning with lane 3 (Fig. 1), since each of these lanes contains the supernatant growth medium from one of the *Acholeplasma* strains.

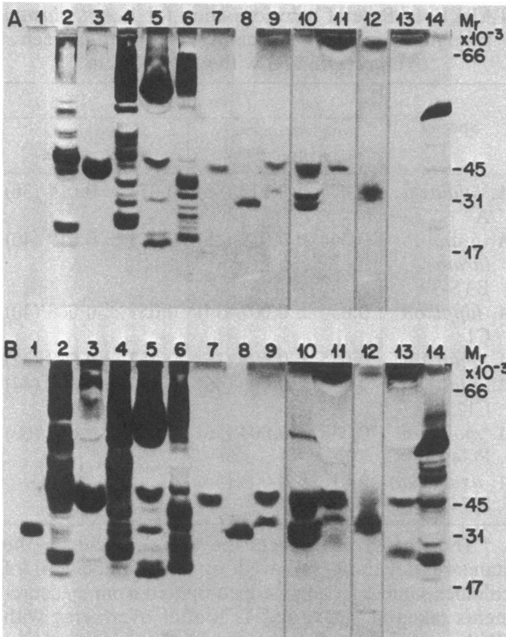

FIG. 1. DNases in *Acholeplasma* spp. detected after SDS-polyacrylamide gel electrophoresis. Photograph of gels taken (A) 12 h and (B) 45 h after electrophoresis. Wells contained: lane 1, pancreatic DNase I, 40 pg; lane 2, *A. laidlawii* A cell extract, 15 μg; lane 3, *A. laidlawii* A supernatant, 29 μg; lane 4, *A. granularum* BTS-39 cell extract, 14 μg; lane 5, *A. granularum* BTS-39 supernatant, 67 μg; lane 6, *A. equifetale* C112 cell extract, 25 μg; lane 7, 5% horse serum, 68 μg; lane 8, *A. modicum* PG-49 cell extract, 87 μg; lane 9, *A. modicum* PG-49 supernatant, 211 μg; lane 10, *A. hippikon* C1 cell extract, 6 μg; lane 11, *A. hippikon* C1 supernatant, 139 μg; lane 12, *A. axanthum* S-743 cell extract, 60 μg; lane 13, *A. oculi* 19L supernatant, 139 μg; lane 14, *A. oculi* 19L cell extract, 34 μg. Gels were stained with ethidium bromide and photographed with UV illumination. Bands that fail to fluoresce correspond to DNases. Molecular weights correspond to standard proteins run in a separate lane (data not shown) and stained with Coomassie blue.

Bands of activity, in addition to the activity at the gel origin, were found in the cell extracts of all seven strains of *Acholeplasma* and in the supernatant growth medium of five of the seven strains (Fig. 1). No bands of activity were found in the supernatant growth medium of *A. equifetale*, even though this strain was DNase positive when growing colonies were assayed with DNA-methyl green. Similarly, no bands of activity were found in the supernatant growth medium of *A. axanthum*.

All the bands of activity observed in the supernatant growth medium of *A. granularum*, *A. modicum*, and *A. oculi* have no corresponding bands in their cell extracts and therefore represent extracellular nucleases; a band of activity with no corresponding band in the cell extract was also found in the supernatant growth medium of *A. laidlawii* and *A. hippikon* at M_r = 50,500 and 34,000, respectively (Fig. 1).

Only the supernatant growth medium of *A. laidlawii* and *A. hippikon* revealed bands of activity with corresponding bands in their cell extracts (Fig. 1). It is not known, however, whether the bands with similar molecular weights represent the same nuclease. The most active band detected in the supernatant growth medium of *A. laidlawii* has a molecular weight of 36,000 and is found in the cell extract where it exhibits relatively low activity; two bands (M_r = 20,500 and 24,000) of very low activity in the supernatant growth medium have corresponding bands in the cell extract where they exhibit relatively high activity. However, the difference in activity may be due to the relative concentration of the fractions applied to the gel; for example, nucleases found in the supernatant growth medium were not concentrated, whereas those found in the cell extract were concentrated 100 times. The one band given by the supernatant growth medium of *A. hippikon*, which has a band of the same molecular weight in the cell extract (M_r = 37,500), exhibits nearly equal activity in the supernatant growth medium and cell extract.

DISCUSSION

The seven *Acholeplasma* strains examined in this study were found to contain DNases. *A. laidlawii, A. granularum, A. equifetale*, and *A. oculi* showed the greatest number of nucleases. Fewer nucleases were found in *A. hippikon* and *A. modicum*, whereas only one nuclease was detected in *A. axanthum*. The seven strains are quantitatively and qualitatively different with regard to nuclease content. No one nuclease was found to be common in all seven strains.

The nucleolytic activity present in the growth medium of *Acholeplasma* spp., as detected by the DNA-methyl green plate assay, may be due to (i) specific extracellular nucleases, (ii) intracellular nucleases released outside the cell after cell death, or (iii) bacitracin-induced leakage of cytosol or membrane nucleases or both. With *A. axanthum*, the detection of DNase activity in the medium surrounding growing colonies was dependent on the presence of bacitracin in the DNA-methyl green overlay. The remaining six strains of *Acholeplasma*, however, released substantial amounts of nucleases without the aid of bacitracin. Whether these extracellular nucleases were due to cell death or whether some may specifically be released for activity outside the cell cannot be determined from the results of the DNA-methyl green assay.

A. axanthum was DNase negative when assayed without bacitracin in the DNA-methyl green overlay. This suggests a method for differentiating this strain from the remaining six *Acholeplasma* strains. Presently, only a limited number of biochemical or physiological differences in *Acholeplasma* spp. are known (14), with no single biochemical test available that clearly separates these species. The DNA-methyl green method reported here, however, not only provides for a rapid and sensitive assay for nucleolytic activity in *Acholeplasma* spp., but also serves as an accurate screening test for the identification of *A. axanthum* from the other *Acholeplasma* strains used in this study.

The electrophoretic patterns of nucleolytic activity obtained from the SDS-polyacrylamide gel assay of cell extracts and supernatant growth medium of *Acholeplasma* spp. are highly reproducible and differ for the different strains examined and therefore can serve as "fingerprints" for the identification of these strains. Although these strains can be differentiated by their serological behavior (1) or DNA base composition and by nucleic acid hybridization techniques, the SDS-polyacrylamide gel technique has the obvious advantage of being easy to perform and it is relatively rapid.

Nucleases have been shown to function in a number of biological roles, which include genetic recombination, DNA repair, restriction of foreign DNA, and transport of transforming DNA. One explanation for the several nucleases in *Acholeplasma* spp. is that these enzymes are needed for exogenous nucleic acid breakdown to enable nucleotide transport into the cell. Studies on nutritional requirements for nucleic acid precursors and synthetic capabilities in *Acholeplasma* spp. (7, 8) have shown that these microorganisms lack the orotic pathway for pyrimidine synthesis and the enzymatic pathways for de novo synthesis of purine bases. Therefore, supplementation of at least one purine and one pyrimidine base is essential for *Acholeplasma* spp. growth. The extracellular nucleases found in *Acholeplasma* spp. no doubt degrade these large molecules, thereby making available the individual nucleotides.

Even though DNases were not detected in the supernatant growth medium of *A. equifetale*, this strain is capable of degrading exogenous DNA, as seen by the formation of halos when assayed with DNA-methyl green. Large amounts of DNases are found in the cell fraction of this strain, and therefore, degradation of exogenous DNA may be due to membrane-bound DNases or leakage of cytosol DNases after cell death or both. In addition, this strain, as well as the others examined here, may contain extracellular and intracellular nucleases that are not detectable in the SDS-polyacrylamide gel technique (10) but which function in the metabolism of exogenous DNA.

Of the seven strains of *Acholeplasma*, only *A. axanthum* appeared to lack DNases for the breakdown of exogenous DNA, as measured by the DNA-methyl green assay. However, other types of nucleases may be present in this strain, as well as in the remaining six strains. Nucleases with different optimal conditions for activity may be detected by varying pH, divalent cations, salt concentration, and other cofactors such as reducing agents and ATP in the incubation buffer (10). Thus, the nucleases shown to be present in *Acholeplasma* spp. in this study may just be a sample of a large family of nucleases to be found in these microorganisms.

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