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Nuclease Activities of *Mycoplasma gallisepticum* as a Function of Culture Age in Different Media[1]

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Levels of deoxyribonuclease and ribonuclease activities in the supernatant (soluble plus ribosomes) fraction of *Mycoplasma gallisepticum* were assayed and found to be a function of strain, nutrient, and culture age. In yeast hydrolysate-enriched broth, maximal nuclease activities occurred during exponential growth.

Razin and co-workers (5) demonstrated deoxyribonuclease (EC 3.1.4.5 and 3.1.4.6) and ribonuclease (EC 2.7.7.16 and 2.7.7.17) in a number of species of mycoplasma, but, for any given species, little information is available to correlate the levels with culture age or cell constituents or to compare levels in closely related strains. This communication presents such data for *Mycoplasma gallisepticum* in two common culture media.

MATERIALS AND METHODS

Propagation and harvesting of organisms. *M. gallisepticum* strains SW (8) and Avian 5969 (11), both belonging to the A or S-6 serological type (9), were grown in two culture media: (i) PPLO (Difco) broth enriched with 1% yeast hydrolysate and 0.5% glucose, and supplemented with 10% horse serum, 0.025% phenol red, and 500 units of penicillin per ml; and (ii) PPLO broth supplemented with 2% PPLO serum fraction and 100 units of penicillin per ml.

A 250-ml Erlenmeyer flask containing 100 ml of medium was inoculated with 10 ml of a 24- or 48-hr culture and incubated statically at 37 C. At desired intervals, the total number of organisms per milliliter of suspension was determined by the electron microscopic method of Sharp and Beard (6), and the number of viable organisms was estimated by colony counts. The organisms were then harvested by centrifugation at 12,000 × *g* for 30 min, washed twice in deionized water, and suspended in 5.0 ml of deionized water. Analyses of the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) content were performed by the methods of Lynn and Smith (2).

Because horse serum proteins in the enriched broth are coagulated by acid formed during glucose fermentation and sediment during harvesting of organisms, controls were included in both chemical and enzyme assays to correct for the optical density of this material as follows. A predetermined amount of 85% lactic acid was added to uninoculated medium, thus lowering the *p*H to that of the culture being examined. The mixture was then incubated for a time corresponding to the age of the culture and harvested in the same manner as suspensions containing organisms.

Preparation of cell-free extracts. Cell-free extracts were prepared from the resuspended organisms with a French pressure cell (American Instrument Co.) at 10,000 psi and 5 C. The suspension was then centrifuged at 34,000 × *g* for 40 min (5), and the supernatant fluid was separated from the sedimented membrane fraction and stored at −20 C until assayed for enzyme activity. When desired, further centrifugation of the supernatant fluid at 150,000 × *g* for 90 min separated the soluble and ribosomal fractions.

Assay of nuclease activities. Deoxyribonuclease and ribonuclease activities were determined by the procedure of Razin et al. (5) by monitoring the release of material absorbing at 260 nm from reaction mixtures containing either highly polymerized DNA (salmon sperm) or soluble RNA (yeast) (Mann Research Laboratories, Inc., New York, N.Y.). The assay conditions were essentially those found optimal for the nucleases of *M. laidlawii* (5). Reaction mixtures were composed of 1.5 ml of 0.2 M NaCl containing 0.04 M $MgCl_2$, 2.1 ml of 0.4 M tris(hydroxymethyl)aminomethane buffer (*p*H 8.8) containing DNA or RNA (2.86 mg/ml), and 2.1 ml of cell-free supernatant fluid; 0.3 ml of thallium acetate (2.5 mg/ml) was added to inhibit microbial growth. Corrections were made for the optical densities of

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controls containing all components of the reaction mixture except cell-free supernatant fluids or the nucleic acid substrate and for the enriched broth precipitate. Corrected optical densities were converted to micromoles of acid-soluble nucleotide by assuming an optical density of 1.0 at 260 nm equivalent to 0.1 μmole of acid-soluble nucleotide per ml (1).

RESULTS

Location of nuclease activities. The membrane, ribosomal, and soluble fractions of 36-hr A5969 organisms in enriched broth were assayed. Nearly one-half (49.4%) of the ribonuclease and 28.6% of the deoxyribonuclease activity were found in the membrane fraction (Table 1). Rather small and similar proportions of the total deoxyribonuclease and ribonuclease activities were found in the ribosomal fraction (13.3 and 15.2%, respectively). The majority of deoxyribonuclease activity (58.1%) was found in the soluble fraction. It was decided to assay the total supernatant fraction (soluble plus ribosomes) for nuclease activities on a routine basis since: (i) deoxyribonuclease was of primary concern; (ii) deoxyribonuclease activity of various mycoplasmas is generally stated to reside largely in the soluble fraction (12); (iii) enzyme localization is reported unchanged in different growth media (3); and (iv) uniform resuspension of membrane fragments after explosive decompression and centrifugation was difficult. Based on the reports cited above and the results in Table 1, this procedure was estimated to yield approximately 70% of the cell deoxyribonuclease (58.1% in soluble fraction plus 13.3% in ribosomal fraction) and 50% of the ribonuclease (35.4% in soluble fraction plus 15.2% in ribosomal fraction) activities in each case.

TABLE 1. *Nuclease activities of cell fractions from 36-hr Mycoplasma gallisepticum strain A5969 organisms in enriched broth*

Fraction	Nuclease activity[a]	Per cent of total activity
Cell membranes		
Deoxyribonuclease	5.8	28.6
Ribonuclease	8.8	49.4
Soluble		
Deoxyribonuclease	11.8	58.1
Ribonuclease	6.3	35.4
Ribosomal		
Deoxyribonuclease	2.7	13.3
Ribonuclease	2.7	15.2

[a] Expressed as 10^{-15} μmoles of acid-soluble nucleotide produced per min per organism. Total activity: deoxyribonuclease, 20.3; ribonuclease, 17.8.

Nuclease activities as a function of strain, medium, and culture age. Results of deoxyribonuclease and ribonuclease assays are presented in Fig. 1 to 3. In all cases where activity was detected, maximal release of nucleotides from DNA was greater than maximal release from RNA, even when the portions of activity discarded in membrane fractions (estimated from data in Table 1) were considered.

In enriched broth (Fig. 1 and 2), both strains showed similar patterns of deoxyribonuclease and ribonuclease activities, but the times of maximal activities were different. However, in both cases peak activities preceded maximal viable counts. Maximal levels of nucleic acid occurred prior to peaks in nuclease activities and number of viable organisms in strain A5969, but in strain SW maximal contents of nucleic acids were attained subsequent to peaks in nuclease activities and number of viable organisms. Maximal activities of ribonuclease were approximately equal in both strains, but deoxyribonuclease reached a higher activity in SW organisms than in A5969. At maximal activities in both strains, deoxyribonuclease was two to three times greater than ribonuclease, but deoxyribonuclease activities decreased more sharply with age than ribonuclease. Beyond 48 hr for SW and 60 hr for A5969, activities of both enzymes were insignificant in the cell-free supernatant fluid. In SW, loss of nuclease activity roughly paralleled the sharp drop in viable organisms after 24 hr (Fig. 1), but the levels of RNA and DNA remained near the maximal levels attained at 36 hr. On the contrary, in A5969 (Fig. 2), the drop in nuclease activities beyond 30 hr was not accompanied by a sharp decrease in viable organisms, but the nucleic acid levels (both RNA and DNA) decreased markedly from the maximal values attained at 24 hr. Although deoxyribonuclease was considerably greater than ribonuclease at maximal activities, the amounts of RNA exceeded those of DNA in both organisms. In SW organisms, the differences between amounts of RNA and DNA (approximately 12 to 24%) were somewhat larger than those differences in A5969 organisms (approximately 2 to 7%) throughout the growth curve.

In PPLO broth, no deoxyribonuclease or ribonuclease activities could be demonstrated in the supernatant fraction of A5969 organisms at any age up to 90 hr, although SW organisms in this medium had activities of both nucleases considerably greater than the activities when grown in enriched broth (Fig. 3). In PPLO broth, nuclease activities in SW did not parallel the numbers of viable organisms (Fig. 3)

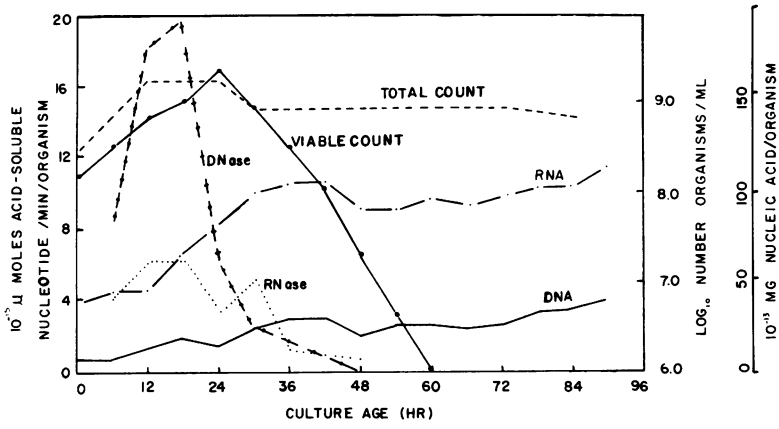

FIG. 1. *Nuclease levels, numbers of organisms, and nucleic acid contents of Mycoplasma gallisepticum strain SW in enriched broth. Organisms were ruptured by explosive decompression, and the total supernatant fraction (soluble plus ribosomes) was assayed for both nuclease activities.*

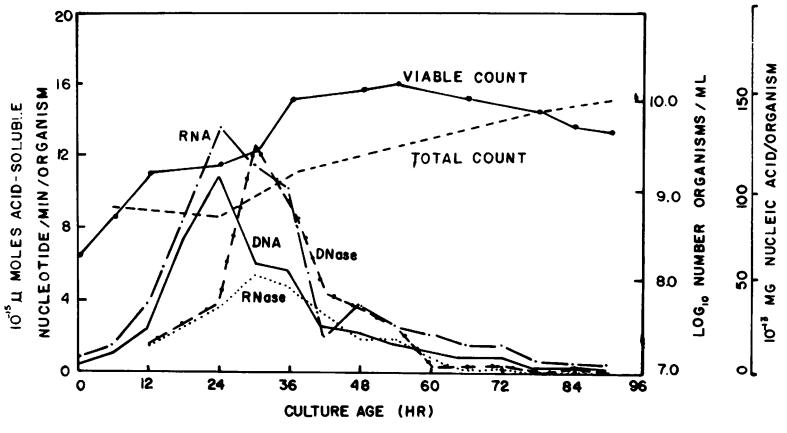

FIG. 2. *Nuclease levels, numbers of organisms, and nucleic acid contents of Mycoplasma gallisepticum strain A5969 in enriched broth. Organisms were assayed as described in Fig. 1.*

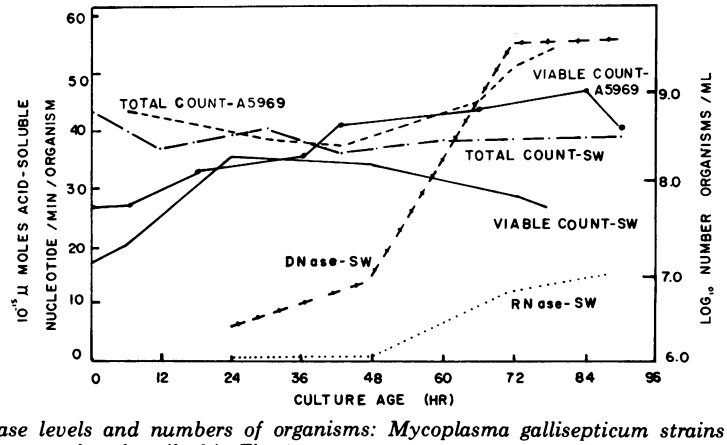

FIG. 3. *Nuclease levels and numbers of organisms: Mycoplasma gallisepticum strains in PPLO broth. Organisms were assayed as described in Fig. 1.*

but increased markedly as the number of viable organisms decreased from 48 to 72 hr. The maximal number of viable A5969 organisms occurred at 84 hr, so that detectable nuclease activity may have been beyond the time scale chosen. Analyses of nucleic acid contents were not performed on cultures grown in PPLO broth for the following reasons. No nuclease activities were found in A5969; the peaks in nuclease activities in SW were beyond the time scale chosen; and in previous studies there was no suggestion of ribosomal dissociation in this medium (8).

DISCUSSION

Levels of nuclease activities for the two strains studied differ somewhat from levels reported by others (3, 5). In those studies, however, different media were used for propagation, and the stage of growth was not specified. Furthermore, values are expressed on a milligrams of protein basis. This base was not used in the present study, as it has been shown not to correlate well with number of organisms (8).

Failure to obtain reasonable agreement between total and viable counts during the exponential and stationary phases of the growth curve for A5969 grown in enriched broth may be due to several factors. Some organisms may have been of so small a size as not to sediment during the procedure for total count determination (12,000 $\times$ *g*). Alternatively, the reproductive subunits may have been unrecognizable or nondissociated during sedimentation onto agar blocks for total counts, whereas pipetting during serial dilutions before plating for viable counts may have separated viable reproductive subunits. Since good correspondence (within 0.25 log) between total and viable counts at maximal viability was obtained for SW in both media and for A5969 in PPLO broth, the discrepancy for A5969 in enriched broth appears to be due to some unusual property of this culture rather than technical limitations of the method.

Although instability of the DNA in animal cells infected by mycoplasmas (4) has been attributed to nuclease production by the invading organism (10), the association between nucleases and pathogenicity appears more complex or subtle than a quantitative correlation between activity and degree. Cultures of SW and A5969 at 24 and 48 hr in both media were previously shown to exhibit similar pathological responses in vivo (9), but at 24 or 48 hr, A5969 in PPLO broth has no detectable deoxyribonuclease activity. It is also quite apparent that SW and A5969 strains are significantly different with respect to nucleic acid metabolism even though they are closely related serologically and pathologically.

Nuclease enzymes are believed important in autolysis of the cell, but increasing evidence suggests a role in nucleic acid synthesis during exponential growth (7, 12). Because patterns of nuclease activity similar to those reported were found in the present study, this hypothesis may also be proposed for *M. gallisepticum* propagated in yeast hydrolysate-enriched broth.

It is obvious, however, that each mycoplasma must be thoroughly characterized in each culture medium employed. Growth patterns, nuclease activities, and nucleic acid contents differ for the same culture in different media and for closely related cultures in the same medium.

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