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Properties of the Nucleases of Mollicutes

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Extracts of the Mollicutes *Acholeplasma equifetale*, *Acholeplasma laidlawii* B, *Mycoplasma arthritidis*, *Mycoplasma pulmonis*, and *Mycoplasma pneumoniae* had DNase and endonuclease activity. *A. laidlawii* B had at least two peaks of DNase activity in sucrose gradients with sedimentation coefficients of 3.1S and 4.3S. These fractions also had endonuclease activity with different substrate specificities. *A. laidlawii* B may have more than two peaks of endonuclease activity in sucrose gradients.

The presence of nucleases in Mollicutes has been reported (2–4, 17, 18, 20, 25, 30). Only one study of Mollicutes endonuclease activity has been published (29). We studied Mollicutes DNA nucleases, particularly the endonuclease activity, to determine their properties and whether species could be distinguished from each other based on these enzymatic activities, because of their role in the induction of pathology (6, 22, 31), and to isolate unhydrolyzed Mollicutes DNA.

Nucleic acid precursors are growth factors of Mollicutes (11, 15, 16, 23, 24, 26, 28). This growth requirement can be met by nucleosides or undegraded DNA or RNA (24). Stanbridge et al. (27) observed karyological and morphological changes in human diploid cells infected with *Mycoplasma pulmonis* and associated these changes with competitive mycoplasmal interference of host DNA synthesis. Gabridge and Polinsky have shown that the human pathogen *Mycoplasma pneumoniae* in tracheal explants induces ciliostasis and decreases cellular respiration, dehydrogenase activity, rate of oxygen uptake, and ATP content and have related all of these aberrations to ATP and adenine levels (5). Hu et al. (10) showed that in vitro infection with *M. pneumoniae* causes a diminution of host protein and RNA synthesis. The concept of Mollicutes interference in host nucleic acid metabolism is supported by the findings that most Mollicutes can produce adenine from adenosine but that the human pathogen *M. pneumoniae* cannot, and further, that adenine spares the pathological effect of *M. pneumoniae* infection in hamster tracheal explants (6, 7). The nuclease of parasitic Mollicutes may contribute to host pathology by catalyzing reactions which produce Mollicutes nucleic acid growth factors from host nucleic acid precursors or DNA.

Acholeplasma equifetale N93, *Acholeplasma laidlawii* B-PG9, *Mycoplasma arthritidis* 07, and *M. pulmonis* KON were received from J. Tully, National Institutes of Health, Bethesda, Md., and were grown in Edward medium (1) with 0.5 to 3% (vol/vol) heat-reinactivated horse serum (control lot 268095 or 200011H; K. C. Biologicals, Lenexa, Kans.). *M. pneumoniae* FH was obtained from N. L. Somerson, The Ohio State University, Columbus, and was grown in SSR2 medium (21) with 2% (vol/vol) heat-reinactivated (56°C for 1 h) horse serum replacing the PPLO serum fraction.

Organisms were harvested and washed in kappa buffer by centrifugation (19). Cell pellets (about 0.5 ml) were washed four times in about 300 volumes of kappa buffer to remove contaminating, strongly interfering traces of horse serum DNase and endonuclease activities which are not completely inactivated by heating at 57°C for 1 h. The washed cell pellets were disrupted by hypotonic shock by suspension in 200 volumes of diluted kappa buffer (diluted 1:40 with deionized water) (19). The crude lysate was centrifuged at 39,080 × *g* for 40 min at 4°C. The supernatant fluid was adjusted to 50 mM Tris-hydrochloride (pH 7.4)–7.5 mM $MgCl_2$–1.5 mM $CaCl_2$. The mixture was incubated for 1 h at 37°C to hydrolyze released Mollicutes DNA. After incubation, the mixture was centrifuged at 144,880 × *g* for 1 h or at 303,500 × *g* for 0.7 h at 4°C. The supernatant fluids were stored at −40°C until assayed.

The combination of Mg^{2+} and Ca^{2+} tested over the range of 0 to 10 mM at pH 8.0 which gave optimum DNase activity was 7.5 mM Mg^{2+} with 1.5 mM Ca^{2+}. Except for *A. laidlawii* B

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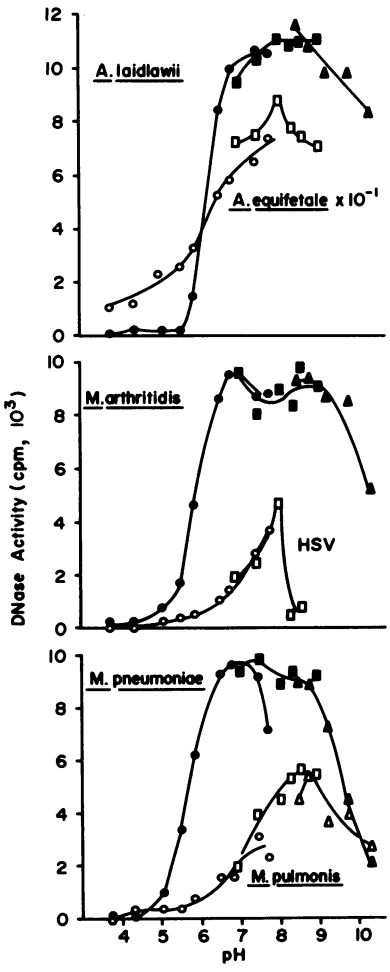

FIG. 1. Effect of pH on DNase activity of Mollicutes extracts. DNase activity was measured as the non-precipitable, acid-soluble radioactivity in trichloroacetic acid reaction mixtures containing *Escherichia coli* [^{14}C]DNA and Mollicutes extracts (8). The standard reaction mixture contained Tris-hydrochloride (○, ●), dimethylglutaric acid (□, ■), or glycine (△, ▲), 25 to 32 mM; $MgCl_2$, 7.5 to 9.3 mM; $CaCl_2$, 1.5 to 1.9 mM; albumin, 100 μg; β-mercaptoethanol, 5 mM; *E. coli* [^{14}C]DNA, 3 μg (approximately 20,000 cpm); 2 to 50 μl of Mollicutes extract (0.2 to 15.5 μg of protein); and distilled water in a final volume of 0.200 ml. The Mollicutes extract was added to start the reaction, and the mixture was incubated at 37°C for 30 min. The reaction was stopped by adding sheared calf thymus DNA (type I, 2 mg/ml of distilled water; Sigma Chemical Co., St. Louis, Mo.) with mixing and then 25 μl of cold 50% (wt/vol) aqueous trichloroacetic acid. After standing for 10 min in ice, the mixture was centrifuged at 1,000 × *g* for 10 min, neutralized with 50 μl of 1.2 M KOH, and assayed for radioactivity in 7.5 ml of Budget-Solve scintillator fluid (RPI Corp., Mount Prospect, Ill.) with a 7000 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Herpes simplex virus type 1 DNase from infected KB cells was used as a control and prepared as previously described (8). Protein was determined by the procedure of Lowry et al. (12).

extracts, no activity was observed in the presence of 5 mM EDTA. At pH 5.0 or 8.0, 1 to 5 mM Zn^{2+} completely inhibited the DNase activity in all extracts. This observation may be useful in the isolation of Mollicutes DNA.

DNase activity from *A. laidlawii* B, *A. equifetale*, *M. arthriditis*, and *M. pneumoniae* displayed a pH optimum from pH 6.5 to 9.0 (Fig. 1). DNase activity from *M. pulmonis* showed a narrower optimum at pH 8.5, and with the control herpes simplex virus DNase preparation, the optimum pH was 8.0 (8).

DNase activity decreased as the ionic strength was increased. Inhibition of *M. arthriditis*, *M. pneumoniae*, and *M. pulmonis* DNases followed a sigmoidal curve. Inhibition of *A. laidlawii* B DNase by NaCl followed a bimodal curve. At 0.45 M NaCl, the *A. laidlawii* B DNase activity was inhibited 50% and the activities of *M. pulmonis*, *M. arthritidis*, and *M. pneumoniae* were completely inhibited. *A. laidlawii* B preparations had some DNase activity at 1.0 M NaCl.

Endonuclease activity was present in all Mollicutes, and all extracts completely degraded all PM2 form I DNA. At 20 mM EDTA, all endonuclease activity was abolished, with the exception of some activity present in the *A. laidlawii* B extract.

The data shown in Fig. 2A show six or seven peaks of DNase activity, three major peaks at 3.1S, 4.3S, and 8.3S. There are five prominent regions of endonuclease activity seen in Fig. 2B: fractions 20 through 26, 30 through 36, 52 through 54, and 58 through 60; some activity is also evident in fractions 40 through 46. These five regions of endonuclease activity represent about 10, 6, 21, 3, and 15%, respectively, of the total protein recovered from the gradient. DNase peaks at 3.1S and 4.3S correspond to the regions 20 through 26 and 30 through 36 of endonuclease activity. Differences in the substrate specificity of the putative endonucleases are apparent (Fig. 2B). Although fractions 24 and 40 have about the same amount of DNase activity (Fig. 2A), in the endonuclease assay their reaction products differ. Analysis of the endonuclease reaction mixture with fraction 24 showed that the open circular form was the predominant product, whereas the linear form predominated when fraction 40 was used.

Chen and Hung suggested the presence of two DNases in *M. pneumoniae* grown with unheated horse serum (3). We concluded with more certainty that extracts of *A. laidlawii* B have at least two and perhaps as many as five nucleases (Fig.

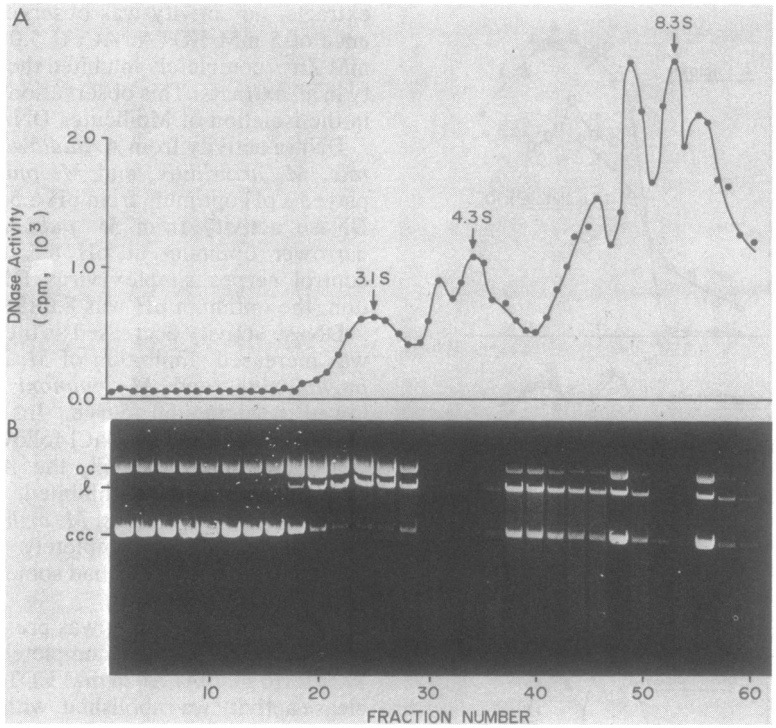

FIG. 2. Sedimentation of nucleases in an extract of *A. laidlawii* B in a sucrose density gradient. Sedimentation was from left to right, with fraction 60 being at the bottom of the tube. Centrifugation in 10 to 40% (wt/vol) sucrose density gradients was performed by the method of Martin and Ames (13). Samples of 0.15 ml containing 93 μg of protein were layered on an 11-ml gradient which contained, in addition to sucrose, 50 mM Tris-hydrochloride (pH 7.4), 0.2 M KCl, 2 mM $MgCl_2$ and 5 mM β-mercaptoethanol and centrifuged for 68.25 h at 272,700 × *g* (SW41 Ti rotor; Beckman Instruments) at 4°C. Upon completion of the centrifugation, each gradient was removed from the top of the tube with an Auto Densi-Flow IIC (model 2-5170; Buchler Instruments, Fort Lee, N.J.) and distributed into about 60 equal fractions. Each fraction was assayed by the DNase test, and alternate fractions were assayed by the endonuclease tests. (A) DNase activity (●) was measured as described in the legend to Fig. 1, but the reaction mixtures contained 20 μl of each fraction and were 25 mM in Tris-hydrochloride (pH 7.4), and the incubation time was 3 h. Estimation of the sedimentation coefficient of several peaks of DNase activity along the gradient was calculated by the method of McEwen (14). (B) Endonuclease activity was qualitatively assayed by observing the loss of bacteriophage PM2 form I DNA after electrophoresis of the reaction mixture in 0.7% (wt/vol) agarose in Tris-acetate-EDTA buffer (9). The standard reaction mixture contained the same concentration of reagents as that used for the DNase assay described in the legend to Fig. 1, except 25 mM Tris-hydrochloride (pH 7.4) was used and *E. coli* [^{14}C]DNA was replaced with 1 μg of superhelical, covalently closed circular PM2 form I DNA prepared as previously described (8, 9). The reaction was initiated by the addition of 5 μl of the sucrose gradient fractions (0.4 to 1.6 μg of protein), and the total volume of the reaction mixture was 0.025 ml. The reaction was terminated after 30 min by the addition of 5 μl of 5% (wt/vol) sodium dodecyl sulfate–0.14 M EDTA–0.1% (wt/vol) bromophenol blue, followed by 5 μl of 70% (wt/vol) sucrose. After heating at 60°C for 2 min, 25 μl of the mixture was loaded into a slot in the gel. Electrophoresis was conducted at 200 V for approximately 5 h. The positions of open circular (oc), linear (l), and covalently closed circular (ccc) PM2 form I DNA are indicated.

2). Higher molecular-weight activity (8.3S) may be a polymeric or aggregated form of the 3.1S activity or 4.3S activity or both.

We suggest that, in pure culture, Mollicutes nucleases present as inoculum contaminants degrade medium DNA or oligonucleotides to more assimilable precursors necessary for Mollicutes growth. Nucleases released from the Mollicutes cells during culture (4) or present in horse serum may similarly serve to alter nucleic acids in the medium to nutritionally usable forms and act as modulators of in vitro Mollicutes growth.

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