Identification and Preliminary Characterization of External Membrane-Bound Nuclease Activities in *Mycoplasma pulmonis*

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*Mycoplasma pulmonis* has substantial DNase activity exposed on the cell surface. At least part of this activity is attributable to an endonuclease. The activity is destroyed at 56°C and inhibited by either 5 mM EDTA or 10 mM zinc chloride. It can also be eliminated by treatment of intact organisms with trypsin and is regenerated by incubation of the treated organisms in a medium that supports protein synthesis. DNase exposed at the cell surface constitutes 20% of the total DNase activity present in *M. pulmonis* extracts.

Mycoplasmas lack the biosynthetic capacity for de novo synthesis of purine bases and the orotic acid pathway for pyrimidine synthesis (12). Guanine, uracil, and thymidine are therefore required nutrients and must be obtained from the host (7, 12). These nucleic acid precursors are not freely available to pathogenic bacteria during infection. For example, *Yersinia pestis* is rendered avirulent by mutations which block purine biosynthesis even though the mutants grow well in appropriately supplemented medium in vitro (3). Thus, the mycoplasmas must have specialized mechanisms for obtaining nucleotides. Enzymatic degradation of host or microbial nucleic acids followed by uptake of the liberated nucleotides is one potential mechanism of this kind and would require a nuclease exported from the mycoplasma or exposed on its outer membrane surface. Such nucleases have not previously been described in parasitic Mycoplasma species, although nucleases present in both the soluble portion and membrane fraction of mycoplasma extracts have been identified (10, 11, 13). A nuclease clearly exposed on the cell surface has been identified in the nonpathogenic species *Acholeplasma laidlawii* (9). Here we report that *M. pulmonis*, a parasite of mucosal surfaces in the respiratory and genital tracts of mice and rats, contains external, membrane-bound nuclease activity which constitutes approximately 20% of its total DNase activity.

*M. pulmonis* UAB 6510 (8), a virulent rodent strain in its fifth in vitro passage, was grown in mycoplasma broth (5) supplemented with 10% agammar horse serum (GIBCO Laboratories, Grand Island, N.Y.)–1.25% fresh yeast extract (Difco Laboratories, Detroit, Mich.)–0.5% glucose–25 μg of Cefobid (Pfizer Pharmaceuticals, Inc., New York, N.Y.) per ml. Organisms were harvested at mid-log phase (pH 7.2), washed three times with phosphate-buffered saline (pH 7.3), and suspended in PBS or Tris-saline (TS) buffer (10 mM Tris, 140 mM NaCl; pH 7.3) to a concentration of 1 mg of protein per ml (2) (approximately 2 × 10^9 CFU/ml) (8).

Trypsin-treated *M. pulmonis* were prepared by exposing freshly washed organisms suspended in phosphate-buffered saline to the protease (100 μg of trypsin per mg of *M. pulmonis* protein) for 30 min at 37°C. This was followed by washing in phosphate-buffered saline and suspension in TS buffer. To allow protein synthesis following treatment with trypsin, a sample of the treated organisms was suspended in Hanks balanced salt solution (GIBCO) containing 10 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (Sigma Chemical Co., St. Louis, Mo.), 0.5 mg of glucose per ml, and either 1% bovine serum albumin or 10% dialyzed agammar horse serum prepared as described previously (8).

Thus, *M. pulmonis* was grown in 2 M glycerol (Sigma), incubating them for 10 min at 37°C, and injecting the suspension into distilled water (0°C) (8). Following centrifugation for 8 min at 5,000 × g to remove unlysed organisms, the membranes were collected by centrifugation at 10,000 × g for 20 min, washed twice with 30 ml of distilled water, and then suspended in TS buffer to 0.5 to 1.0 mg of protein per ml (2). Supernatant fluid from membrane suspensions was collected after centrifugation at 300,000 × g for 15 min in an airfuge (Beckman Instruments, Inc., Palo Alto, Calif.).

The assay mixture typically contained 20 to 50 μl of an *M. pulmonis* whole cell or membrane suspension and, unless otherwise indicated, 1 μg of plasmid pACYC184 DNA (4). This plasmid DNA was isolated from *Escherichia coli* K-12 LE392 (pACYC184) by the extraction procedure of Birnboim (1) followed by isopinic ultracentrifugation in CsCl-ethidium bromide (6). Plasmid DNA was labeled with tritium by growing this strain in broth containing [3H]thymidine (New England Nuclear Corp., Boston, Mass.). 32P-labeled plasmid DNA (specific activity, 10^7 cpm/μg) was prepared by nick translation (6).

Reaction mixtures were mixed by inversion and incubated at 37°C for 30 min. They were then transferred to an ice bath, and 10 mM EDTA was added to inhibit nuclease activity. DNA was precipitated from the supernatant fluid after centrifugation of the mixtures at 12,000 × g for 3 min and analyzed by electrophoresis in 0.7% agarose gels (6) stained with ethidium bromide. When radiolabeled DNA was used as the substrate, radioactivity precipitated from the reaction mixture by 10% trichloroacetic acid was determined by standard methods (6).

Nuclease activity associated with intact *M. pulmonis* is shown by the disappearance of plasmid DNA from the reaction mixtures (Fig. 1, lanes 2 and 3). When [3H]DNA was used as the substrate, 90 to 95% of the radioactivity remained in the supernatant after the mycoplasmas were removed by centrifugation, indicating that the loss of DNA did not result from binding to the mycoplasmas. Both intact

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cells and purified membranes contained the endonuclease activity, but supernatants from membrane suspensions did not. Degradation of the supercoiled form of the plasmid (Fig. 1, lanes 4 through 7) indicated that DNase activities included endonuclease. In parallel experiments in which E. coli were substituted for the mycoplasmas, no degradation was observed, even at the lowest DNA concentration.

The membrane-associated nuclease activity was inhibited by either 5 mM EDTA or 10 mM zinc chloride (data not shown). A previous report of soluble Mollicutes DNase activities (10) showed that EDTA and zinc sensitivities were common features of these enzymes. Nuclease activity could also be destroyed by heating the organisms at 56°C for 15 min. This result allowed us to eliminate the possibility that the activity was due to nuclease adsorbed from the serum by the mycoplasmas, since serum was heated to 56°C for 1 h prior to use even though we could detect no nuclease activity.

Trypsin treatment of intact mycoplasmas resulted in loss of nuclease activity. The activity could be regenerated (Fig. 2) by incubation of the treated organisms in a medium which supported protein synthesis but not replication (8). These results provide further evidence that the nuclease activity is exposed at the cell surface and is synthesized by the mycoplasma. CFU were assayed after trypsin treatment and during the 5-h incubation period. No decrease in viability was observed in the mycoplasma preparations, indicating that significant lysis resulting in release of internal nucleases did not occur.

To determine the fraction of total M. pulmonis nuclease activity exposed at the cell surface, degradation of 32P-labeled DNA by intact cells and lysed cells was compared (Fig. 3). The cell lysates were prepared by sonicating mycoplasma suspensions (0°C) with a cup horn for 15 20-s bursts with 1-min rest periods between bursts. No viable mycoplasmas remained after treatment. Based on estimates of initial reaction rates obtained from these data by linear regression, approximately 20% of total DNase activity effective against the plasmid fragments resulting from nick trans-

![FIG. 1. Digestion of pACYC184 DNA. An M. pulmonis whole cell suspension containing 50 µg of protein was incubated with the indicated amounts of plasmid DNA for 30 min at 37°C. The DNA was precipitated, and equivalent fractions from each assay were analyzed in a 0.7% agarose gel. The amount of plasmid DNA added to the reaction mixtures were (lanes): 1, 0.6 µg and no mycoplasmas; 2, 0.6 µg; 3, 1.2 µg; 4, 1.8 µg; 5, 2.4 µg; 6, 3 µg; and 7, 3.6 µg. The bands represent the supercoiled (lower), open circular (upper), and linear (middle) forms of plasmid DNA. Small fragments of random sizes resulting from degradation of the linear form are not visible in the photograph. This figure demonstrates the endonuclease activity by conversion of the supercoiled plasmid DNA to its open circular and linear forms. Increased amounts of DNA show progressively less degradation.]

![FIG. 2. Regeneration of the membrane endonuclease. Membrane-associated endonuclease activity was destroyed by trypsin and regenerated as described in the text. The control (lane 1) contained no mycoplasmas. The times of incubation were (lanes): 2, 5 h; 3, 4 h; 4, 3 h; 5, 2 h; and 6, 1 h. At 0 h, the plasmid pattern was identical to the control and 1-h patterns (lanes 1 and 6, respectively).]

![FIG. 3. Comparison of total and membrane-associated nuclease activities. Reaction mixtures contained 5 µg of unlabeled plasmid DNA in addition to 100,000 cpm of 32P-labeled DNA. The mean and the standard deviation from triplicate experiments are shown. Open bars represent cell lysates containing both membrane fragments and the soluble fraction, and hatched bars represent whole cell suspensions. Controls consisted of identical reaction mixtures held at 0°C throughout the experiment. There was no difference between control acid-insoluble counts at 0 and 30 min (data not shown). Data were normalized with respect to protein concentration and expressed as counts per minute per mg of protein.]
loration can degrade these fragments even when mycoplasmas are intact.

The data presented above show that M. pulmonis contains substantial cell surface DNase activity, at least part of which is attributable to an endonuclease. This activity could play an important role in the acquisition of nucleic acid precursors by this organism. It should be possible to test this hypothesis by isolating nuclease-deficient mutants and devising a medium which contains only oligonucleotides as a source of these precursors. The ability of such mutants to cause disease will be of particular interest.

We discovered this activity while attempting to develop a useful system for genetic transformation of M. pulmonis. Degradation of DNA during the transformation process is clearly one barrier that must be overcome in devising such a system.

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