Enzyme-based DNA extraction from zoospores of ruminal fungi

K.-P. Tsai and R.E. Calza - Genetics and Cell Biology, Washington State University, Pullman, WA 99164-6332

We report here a rapid, efficient and simple method for the extraction of high molecular weight DNA from zoospores of *Neocallimastix frontalis* EB188. This anaerobic fungus, isolated from bovine digesta, effectively degrades plant fiber in vitro (Barichievich et al. 1990. Appl. Env. Micro. 56:43-48). Our interest in ruminal fungi stems from their ability to degrade wood materials (Joblin et al. 1989. FEMS Micro. Lett. 56:119-122) and their potential use in biomass saccharification. Zoospore DNA synthesis is of particular interest to our laboratory. It is these motile zoospores which colonize and degrade plant materials (Mountfort 1987. FEMS Micro. Rev. 46:501-508). To detail fully this metabolic event, it will be necessary to extract nucleic acids from zoospores. Such procedures have not been reported in the literature. Using acetone drying and enzymatic removal of cell walls, we have isolated high molecular weight DNA from very small amounts of culture. The procedure takes less than one hour and DNA yields are high. The DNA is readily cut with restriction endonucleases and religated efficiently but is otherwise stable. Electrophoretic analysis of the DNA confirmed the presence of repetitive sequences. This procedure will aid the study of DNA replication, DNA repair and DNA RFLP analysis of various strains using small (<1 ml) cultures.

All chemicals, unless otherwise stated, were purchased from U.S. Biochemicals (Cleveland, OH, USA) and were the best grade available. Zymolase (#100,000) was purchased from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan) and was a gift from A. Schroeder. The Lysing Enzyme (#L-2265) preparation was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA).

The ruminal fungus, *Neocallimastix frontalis* EB188 was isolated from bovine digesta and grown as described (Barichievich et al. 1990. Appl. Env. Micro. 56:43-48). The strain used (#76100) was obtained from the American Type Culture Collection (Rockville, MD, USA). Zoospores were separated from cultured rhizoidal mass using filtration through 8 layers of cheese cloth and collected using centrifugation at 10,000 x g for 10 min. Zoospores were free from rhizoidal or sporangial debris and counts determined using a Fuch/Rosenthal hemacytometer and microscope. Cultures of *N. frontalis* EB188 released few zoospores (less than 200/ml were present) until 60-63 h after starting cultures. Zoospore numbers increased rapidly afterwards and by 88 h achieved a maximum of 158,000 +/- 11,000/ml. Less than 1200 +/- 400/ml zoospores were found in cultures after 110-120 h. Large (and adequate) numbers of culture zoospores were therefore attainable at 60-110 h.

Zoospores (typically 1 to 2 million) rinsed in water in Eppendorf tubes were resuspended into 100 ul of 0.1 M Na Citrate, pH 7.5, containing 0.5 M sorbitol and 0.06 M Na2-EDTA. When appropriate, zoospores were rinsed in acetone (100%) before enzyme treatments. Acetone

treatment (Heath et al. 1986. Appl. Env. Micro. 51:1138-1140) included resuspending the rinsed zoospores into acetone (100%) (0-1 C) and holding on ice for 1-2 min. Zoospores were then collected by centrifugation at 5000 x g (at 4 C) for 2 min. Pelleted material was dried under N2 gas and finally resuspended in enzyme buffer. beta-mercaptoethanol (from a 10% v/v stock) was added to 0.05% (v/v), and Zymolase (or Lysing Enzyme) was added to 80 æg/ml. The sample was mixed and placed at 30 C for 5-10 min. Enzyme protein was determined using the dye binding assay (Bradford 1976. Anal. Biochem. 72:248-252) with BSA as standard. After incubation, Na-sarkosyl (from a 10% w/v stock) was added to 0.5% (S/v) and proteinase K (from a 10 mg/ml stock solution which had been autodigested several hours at 37 C) was added to 25 ug/ml. The sample was then placed at 62 C for several min. Phenyl methyl sulfonal fluoride (PMSF) (from a 10 mM stock in ethanol) was then added to 0.1 mM and the sample held at 20 C for 1-2 min. Diethyl pyrocarbonate (DEPC) (from a 10% v/v stock in ethanol) was then added to 0.1% v/v and incubation at 20 C was continued for 1-2 min. Tris-HCl, pH 8.0 (from a 1 M stock) was then added to 10 mM. An equal volume (100 ml) of chloroform/isoamyl (25:1 ratio) was then added and the tube gently mixed for 1-2 min to achieve a fine emulsion. Centrifugation at 10,000 x g (at room temperature) for 2 min was used to break the emulsion. The aqueous layer was carefully collected using a trimmed (200 ul) pipet tip. The sample volume was then increased to 500 ul using water. Sodium acetate, pH 6.0 (from a 3 M stock) was added to 0.25 M and isopropyl alcohol was added to 35% (v/v) final. The sample was gently mixed and chilled to 0-1 C (water/ice bath) for 2-3 min or held at room temperature for 10 min. Centrifugation at 10,000 x g (at 4 C or room temperature for 5 min was used to collect the precipitate. The pellet was rinsed with 100 ul of absolute ethanol (4 C) and respun at 10,000 x g (at 4 C) for 1 min. The pellet was finally dried under N2 gas and dissolved into a small volume (50 ul) of 10 mM Tris, pH 7.5, containing 1 mM Na2-EDTA. Where necessary, and before gel analysis, ribonuclease A (RNase A) was added (from a stock which was boiled for 10 min.) to 10 U/ml and samples placed at 37 C for 5 min. Quantification of total DNA in zoospores or extracted DNA was performed using the diphenylamine assay (Richards 1974. Anal. Biochem. 57:369-376). Calf thymus DNA was used as standard. Values reported represent the means of triplicate samples. Restriction digestion and ligation of DNA was as suggested by suppliers, and commercial buffers were used. Agarose gel electrophoresis was performed as described (Maniatis et al. 1982. Cold Spring Harbor Lab. Press, p. 545).

Zoospores each possess 2.2 +/- 0.6 pg of DNA. Extraction of high molecular weight DNA from enzymatically weakened zoospores (collected from cultures less than 110 h old) was possible. The median molecular weight of the extracted DNA was greater than the 49 kb marker. Based on ultraviolet absorbance determinations (A_{230}/A_{260}=2.5-3.1), DNA preparations contained large amounts of carbohydrate. Less protein (A_{230}/A_{260}=1.8-1.9) contaminated the DNA preparations. The yield of DNA extracted from zoospores was 61% +/- 7%. RNA contaminated all DNA preparations but was effectively removed using RNase A. The extracted DNA was stable for long incubations (several hours at 37 C or several days at 4 C) while in a variety of enzyme buffers. Deoxyribonuclease I (DNase I) completely degraded the extracted DNA and *Hin*dIII readily digested the DNA. A variety of other endonucleases including *Pst*I and *Eco*RI also readily digested the DNA. Using sequential incubations (within the same tube), *Hin*dIII digested zoospore DNA was religated efficiently with T4 DNA ligase. *Hin*dIII digested lambda phage DNA could also be effectively religated in the presence of *Hin*dIII digested zoospore DNA.

Fragments representing lambda phage DNA could be regenerated (digested) readily from such samples.

Notes:

A. Without Zymolase (or Lysing Enzyme) treatment of zoospores, high molecular weight DNA was not successfully extracted. The use of Zymolase was preferred since it was less expensive. Much lower amounts (less than 10%) of DNA was extracted from zoospores collected from cultures older than 120 h. Such DNA was also degraded (<20 kb) and was unstable (autodegraded) at 37 C. Moderately high molecular weight DNA (about 30-40 kb) in moderate yields (about 30%) could be recovered from overmature zoospores if acetone pretreatments were used before cell wall removal. This extracted DNA was digestible with *Eco*RI and *Hin*dIII. Significantly less RNA was isolated from overmature zoospores, and RNase A treatment was seldom necessary. Evidently, overmature zoospores (which microscopically appeared shrunken) presented cell walls which would not readily lyse (or only partially) without acetone drying (or disruption) of membranes.

B. The proteinase K treatment was important for DNA extraction. Yields of DNA were decreased if this step was excluded. Subsequent PMSF treatment of samples was necessary to inactivate proteinase K since DNA, from several extractions where PMSF was excluded, could only be incompletely digested with *Hin*dIII or *Eco*RI. The use of DEPC during the DNA extraction was necessary since in several preparations (of the more than 50 performed) the DNA was unstable at 37 C. This suggested that nuclease remained in these samples. SDS could be substituted for the Na-Sarkosyl during the lysis step, but additional isopropyl alcohol precipitations (at room temperature) were necessary before the DNA would digest completely with *Hin*dIII, *Eco*RI or DNase I.

C. We have used this procedure to extract DNA successfully from as few as 25,000 zoospores (160 ul of culture fluid from 88-h cultures). Such samples contained less than 0.02 mg dry weight (based on measurements of much larger samples) and provided DNA sufficient for several lane loadings. We have also successfully extracted DNA from 36 mission zoospores (the total of one 250-ml culture), and the extraction was done in two 1.5 ml Eppendorf centrifuge tubes. The DNA digested readily with *Hin*dIII or *Eco*RI and apparently possessed repetitive DNA sequences. This confirmed an earlier report of ruminal fungus DNA extracted from rhizoidal tissue (Brownlee 1989. Nucl. Acids Res. 17:1327-1335). The DNA extraction procedure described here has been used successfully on polyflagellated zoospores from several different (yet untyped) ruminal fungi. This procedure, with or without acetone, could not be successfully used with rhizoidal tissues from either early (65-80 h) or hold (120-160 h) cultures.

Acknowledgements. Support was provided by the ARC at Washington State University and the Washington State Dairy Commission. The authors gratefully acknowledge the assistance of M. Storms, D. Larson and Dr. P. Lurquin.