Rapid Lysis Technique for Mycobacterial Species

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The lysis of mycobacterial cells typically has been difficult and time-consuming. We report a method for the physical rupture of Mycobacterium paratuberculosis and several other members of the family Mycobacteriaceae using a Mini-Beadbeater cell disruptor (Biospec Products; Bartlesville, Okla.) and zirconium beads, a process which yields DNA and RNA of high molecular weight and in greater quantity than that obtained by rupture in a Ribi pressure cell.

Technology has made possible the development of DNA probes useful for the identification of Mycobacterium leprae (3) and Mycobacterium bovis (11) and the evaluation of genetic relatedness of many members of the family Mycobacteriaceae through DNA homology studies (1, 2, 5, 6, 8, 9). However, the study of genetics and taxonomy of the Mycobacteriaceae has been hindered by the laborious lysis procedures available. The thick lipid-rich cell wall and slow replication of most mycobacterial species make chemical disruption of the cells relatively tedious. For these lysis procedures, the cell wall peptidoglycan is weakened by growth for several generations in the presence of 100 to 200 mM glycine, and lysis is achieved by lysozyme, proteinase K, and sodium dodecyl sulfate treatment (3, 10-12). Such procedures can take days, depending on the Mycobacterium species being grown. Mechanical lysis procedures, such as the French press, the high pressure, and the Ribi pressure cell, take less time than chemical methods (minutes compared with hours or days). Growing the cell cultures and achieving the appropriate concentration and volume can be time-consuming. In addition, impurities, such as those that might be present in feces or sputum, can damage the mechanisms.

We used the Mini-Beadbeater produced by Biospec Products, Bartlesville, Okla., to rupture Mycobacterium paratuberculosis; Mycobacterium phlei; several serovars and biovars of Mycobacterium avium, including the wood pigeon bacillus; Mycobacterium fortuitum; and Mycobacterium scrofulaceum. Mycobacterial strains and sources were as follows: M. paratuberculosis ATCC 19698 (Trudeau Mycobacterial Culture Collection [TMC], National Jewish Hospital, Denver, Colo.), and NADC 19698 and strain 18 (National Animal Disease Center [NADC], Ames, Iowa); M. avium TMC 715 serotype 2 (bovine) (TMC) and TMC 702 serotype 6 (avian), TMC 801 serotype 2 (avian), mycobactin dep 5, mycobactin dep 7, and mycobactin dep 8 (NADC); wood pigeon bacillus (M. F. Thorel) (NADC); M. intracellulare TMC 1479 serotype 9 (bovine) (TMC) and TMC 1472 serotype 6 (porcine) (NADC); M. scrofulaceum TMC 1320 serotype 43 (human) (TMC) and Anderson serotype 43, EW0407 serotype 42, CDC 1198 serotype 42, Cardiff serotype 41, Bridge serotype 41 (human), and M150 serotype 43 (National Jewish Hospital); M. bovis TMC 410 (bovine) (TMC); M. fortuitum TMC 1529 fortuitum 2 (human) (NADC); and M. phlei TMC 1548 (hay) (TMC) and NADC not typed (NADC).

Cultures grown in Middlebrook 7H9 broth were pelleted, and the cell pellet was suspended in sufficient 1 M Tris EDTA (TE) buffer (pH 8.0) to make a 50% cell suspension. The suspension was pipetted in 500- to 600-μl aliquots into 1.5-ml screwcap polypropylene tubes (Walter Sarstedt, Inc., Princeton, N.J.). A volume of TE-saturated phenol equal to the volume of the bacterial cell suspension was added to each tube. Then the tube was filled with 0.1-mm zirconium beads supplied by the manufacturer of the Mini-Beadbeater. The tubes were inserted into the arms of the Mini-Beadbeater. The tubes containing the disrupted cells, phenol, and zirconium beads were spun for 15 min at 8,000 x g, after which the aqueous layer containing the nucleic acids was removed. A modification of the method of Darby et al. was used to purify the mycobacterial DNA (4). The aqueous phase of the first phenol extraction, approximately 500 μl, was shaken with an equal volume of chloroform-isooamyl alcohol-phenol (24:1:25) for 25 min. The aqueous phase was again recovered by centrifugation, ether extracted four to six times, and precipitated in 3 volumes of chilled ethanol. DNA thus obtained was suspended and treated with RNase (Sigma Chemical Co., St. Louis, Mo.) at 50 mg/ml.

Aliquots of DNA which were subsequently used for homology studies were further processed to remove polysaccharides bound to mycobacterial DNA. A 5% solution of cetyltrimethylammonium bromide was added in a ratio of 200 μl/500 μl of DNA solution, and the mixture was allowed to incubate for 15 min at room temperature (7). A few additional drops of cetyltrimethylammonium bromide were added to ensure that all the DNA was precipitated. The tubes were spun at 8,000 x g for 15 min. The DNA pellet was washed with 0.4 M NaCl, suspended in 1 M NaCl, and extracted with 1 volume of chloroform-isooamyl alcohol (24:1). Chloroform-isooamyl alcohol extraction was repeated until there was no intermediate layer between the chloroform-isooamyl alcohol and the DNA-containing aqueous layer. The DNA was precipitated in 3 volumes of ethanol.
FIG. 1. Comparison of DNA and RNA prepared from cells ruptured in the Mini-Beadbeater with that from cells lysed in a Ribi pressure cell. Equal numbers of cells were processed by both methods. Lanes: 1, nucleic acids obtained by rupture of \textit{M. paratuberculosis} in a Mini-Beadbeater; 2, nucleic acids obtained by use of a Mini-Beadbeater and incubated with RNase for 1 h at 37°C; 3, nucleic acids obtained by rupture in a Ribi press; 4, nucleic acids from Ribi press-lysed cells incubated with RNase for 1 h at 37°C; 5, reference \textit{Hind}III-digested \lambda DNA. Fragment sizes shown on the right are in kilobase pairs. The 1% agarose electrophoresis gel was stained with ethidium bromide and photographed.

This procedure, which combines the phenol extraction with the physical rupture of the mycobacterial cells, produces DNA and RNA of high purity, with the DNA having an average molecular size of approximately 10 kilobases (Fig. 1). DNA obtained by the procedure described above was used to construct gene libraries and evaluate DNA homology among different mycobacterial isolates. In addition, \textit{M. paratuberculosis} cells added to bovine fecal material were ruptured by using this technique to expose the \textit{M. paratuberculosis} DNA, along with the DNA of other bacterial cells present in fecal material. The resultant DNA preparations were collected onto nitrocellulose by using the Minifold II Slot Blotter (Schleicher & Schuell, Inc., Keene, N.H.). These filters were tested with DNA hybridization probes that identify \textit{M. paratuberculosis} (Fig. 2). The description of the isolation of the \textit{M. paratuberculosis}-specific probes will appear elsewhere (S. S. Hurley, G. A. Splinter, and R. A. Welch, manuscript in preparation).

The \textsuperscript{32}P-labeled probes used produce positive signals when hybridized against as few as \(10^9\) organisms in bovine fecal material. To test the sensitivity and diagnostic specificity of the probes, 1 g of a \textit{M. paratuberculosis} cell paste containing approximately \(10^{11}\) cells was mixed thoroughly with 4 g of bovine fecal material (\(10^{11}\) \textit{M. paratuberculosis} cells per g of fecal material). The fecal material was vortexed in 20 ml of TE buffer (pH 8.0) and allowed to settle for 1 h. A 100-\mu l sample of the supernatant was added to a conical microcentrifuge tube. Of the 100 \mu l of supernatant, 10 \mu l was mixed in a second tube with 90 \mu l of fecal supernatant to which no mycobacterial cells had been added, and 10 \mu l of the material from the second tube was added to a third tube containing 90 \mu l of unadulterated fecal supernatant. This procedure was repeated to yield a series of 10-fold dilutions of \textit{M. paratuberculosis} cells in bovine fecal supernatants. After being mulled in the Mini-Beadbeater for 3 min with phenol and 0.1-mm zirconium beads and further DNA purification as described above, the DNA pellets were suspended in 100 \mu l of TE buffer, 25 \mu l of which was used in the hybridization reaction. Fecal samples containing equal numbers of \textit{M. phlei} cells were processed in an identical manner and collected onto the same blot.

Probes developed from \textit{M. paratuberculosis} DNA can detect this organism when it is present in bovine fecal material which has been processed as described in the Mini-Beadbeater to lyse the cells present. The probes do not hybridize to equal numbers of \textit{M. phlei} cells added to bovine fecal material and processed in the same manner. Lysis of mycobacterial cells using the Mini-Beadbeater yields DNA of sufficient quantity and size to participate in hybridization reactions with specific probes under stringent conditions (30% formamide, 65°C). We used DNA prepared in this manner for cloning and DNA-DNA homology studies. Mechanical shearing of the DNA does occur with this method; the range of fragment sizes is 23 to approximately 3 kilobases (Fig. 1). When equal numbers of cells were used in both the Ribi pressure cell and the Mini-Beadbeater, the Mini-Beadbeater preparations yielded three to four times more DNA, although the range of fragment sizes was greater (Fig. 1). Recent advances in mycobacterial genetics and the increased interest in the \textit{Mycobacteriaceae} stimulated by studies of Johne’s disease in cattle and opportunistic pathogens in patients with acquired immunodeficiency syndrome have pointed out the need for a rapid lysis procedure. This procedure for the physical rupture of mycobacterial cells will simplify the process of purifying DNA for use in many procedures for which time-consuming lysis procedures have been used in the past.

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LITERATURE CITED


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Volume 25, no. 11, p. 2227, column 2, lines 5 and 6: ‘‘1 M Tris EDTA’’ should read ‘‘10 mM Tris–1 mM EDTA.’’

Fusarium proliferatum as an Agent of Disseminated Infection in an Immunosuppressed Patient
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Volume 26, no. 1, p. 84, Table 1, column 1, line 3: ‘‘2.5’’ should read ‘‘12.5.’’