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NOTES

Rapid Method To Extract DNA from *Cryptococcus neoformans*

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A rapid and easy method for the extraction of total cellular DNA from *Cryptococcus neoformans* is described. This procedure modifies and considerably simplifies previously reported methods. Numerous steps were either eliminated or replaced, including preincubations with cell wall permeability agents such as β-mercaptoethanol and dithiothreitol. The commercially available enzyme preparation Novozyme 234 was found to contain a potent concentration of DNases which actively degrade DNA. Degradation and loss of DNA was prevented by maintaining a high concentration of EDTA in the lysing solution. This procedure resulted in high yields (150 to 200 μg of DNA from 100 ml of culture) of good-quality (undegraded), high-molecular-weight DNA which was readily digested by restriction endonucleases, making it suitable for use in various molecular applications.

Cryptococcus neoformans, an encapsulated basidiomycetous yeast, is one of the major fungal pathogens infecting immunocompromised patients. The recent development of a transformation system (4) would inevitably increase the demand for a rapid, high-yield method to extract good-quality DNA from *C. neoformans*. Protoplast formation is a prerequisite to DNA extraction and has previously been achieved by hydrolysis of the cell wall using an enzyme preparation, Novozyme 234 (5, 7, 9). Novozyme 234 is a multienzyme preparation containing an α-1,3-glucan glucanohydrolase produced by *Trichoderma harzianum*. The enzyme preparation, originally called Mutanase or Mutanase Novo (8), is marketed as Novozyme 234 (Novo Laboratories) or Mureinase (United States Biochemical Corp., Cleveland, Ohio). It also contains low quantities of cellulase, xylanase, β-1,3-glucan glucanohydrolase, and protease. Previous reports have observed protoplasting times ranging from 20 min to several hours and subsequent DNA extractions involving numerous steps. Since various fungi, including *C. neoformans*, are known to produce DNases (3, 6) which may also be present in the enzyme preparation, steps were incorporated into the procedure to reduce DNA loss. In this report we describe a modified, easy, and rapid protocol for the extraction of high-quality cellular DNA from *C. neoformans*. This procedure is derived and modified from our previous method by discarding and/or incorporating several steps.

Four cultures consisting of one isolate from each serotype of the two varieties of *C. neoformans*, NIH-68 (serotype A), B-3939 (serotype B), NIH-191 (serotype C), and B-3501 (serotype D), were used for the extraction of total cellular DNA. The cultures were maintained on YEPD (1% yeast extract, 2% Bacto-Peptone, and 2% glucose) agar slants, and 1- to 2-day-old cultures were used to inoculate YEPD broth. Wash solution (SCS) contained 20 mM sodium citrate (pH 5.8) in 1 M sorbitol. The protoplasting solution contained 10 mg of Mureinase (United States Biochemical Corp.) per ml in SCS buffer. The lysing solution consisted of 0.45 M EDTA (pH 8), 10 mM Tris hydrochloride (pH 8), 1% Sarkosyl (ICN, Costa Mesa, Calif.), and 2 mg of proteinase K (Boehringer, Mannheim, Federal Republic of Germany) per ml. Other solutions included 5 M ammonium acetate, 2-propanol, 2% sodium dodecyl sulfate, 10 mg of RNase A (Sigma Chemical Co. St. Louis, Mo.) per ml, phenol (Bethesda Research Laboratories, Gaithersburg, Md.) equilibrated with 0.5 M Tris (pH 8), chloroform (J. T. Baker, Phillipsburg, N.J.), 0.3 M sodium acetate, and TE (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA). The following steps are used in the protocol. (i) Inoculate a 100-ml YEPD broth with cells of the desired isolate (optical density at 600 nm, ca. 0.05) in a 250-ml Erlenmeyer flask. Agitate vigorously (200 rpm) overnight at 30°C. (ii) Harvest cells (ca. 10^7 to 10^8 cells per ml) by centrifugation at 4,000 × *g* for 5 min. (iii) Resuspend cells in 5 ml of SCS buffer, and pellet them by centrifugation at 4,000 × *g* for 5 min. (iv) Resuspend cells in 5 ml of protoplasting solution, and incubate the solution in a 37°C water bath. (v) Check for the formation of protoplasts, by adding 5 μl of 2% sodium dodecyl sulfate to 5 μl of cell suspension on a slide, by phase-contrast light microscopy. (vi) When there are more than 70% protoplasts (ca. 15 min), pellet them at 4,000 × *g* for 5 min and resuspend the protoplasts in 5 ml of lysing solution. Incubate the solution at 37°C for 30 min. (vii) Transfer the tube to a 65°C water bath for 15 min and then cool to room temperature. Spin the tube at 20,000 × *g* for 15 min at 4°C to pellet the cellular debris. (viii) Transfer the supernatant to a fresh tube and add 5 ml of 2-propanol. Place the tube at −20°C for 10 min. (ix) Pellet, dry, and resuspend the DNA in 0.5 ml of 0.3 M sodium acetate. Transfer the DNA suspension to a 1.5-ml Eppendorf tube. (x) Add 10 μl of RNase (10 mg/ml) and incubate at 37°C for 30 min. (xi) Add 10 μl of proteinase K (20 mg/ml) and incubate at 37°C for 15 min. (xii) Extract twice with 0.5 volume of phenol and then once with 0.5 volume of chloroform. Precipitate DNA with 2 volumes of cold ethanol. Spin, dry, and resuspend the DNA in 100 μl of TE.

The overnight culture that was started from a loopful of yeast cells in 250 ml of YEPD broth yielded about 10^8 cells per ml. The presence of sodium citrate in both the wash as well as the protoplasting solutions eliminates the need for any preincubations with either β-mercaptoethanol or dithiothreitol, as reported earlier (5, 7–9). We separately used both

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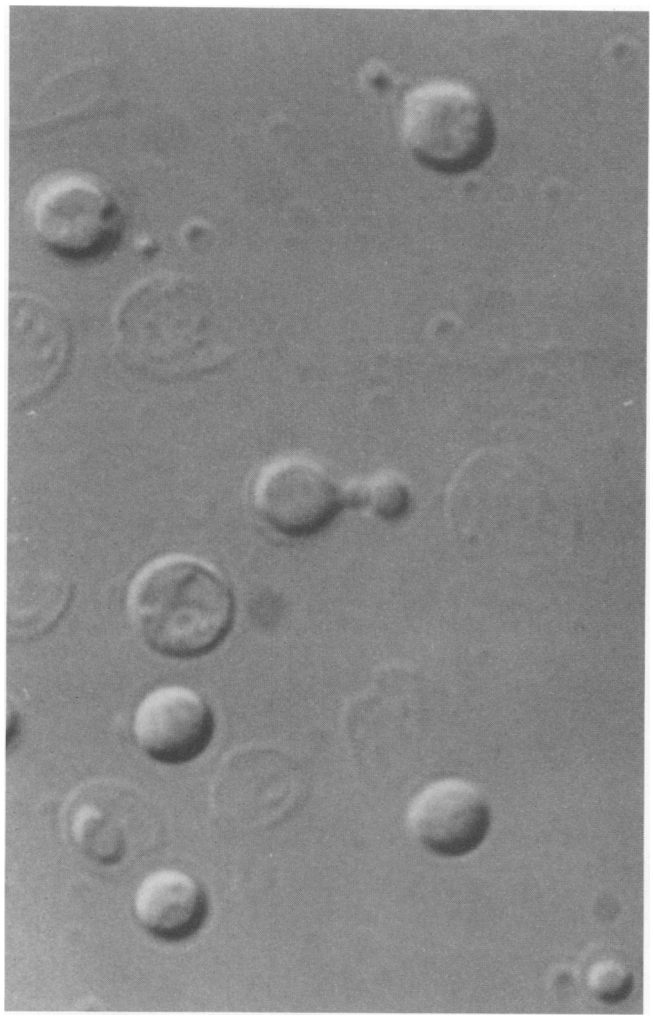

FIG. 1. Formation of *C. neoformans* protoplasts following incubation at 37°C in protoplasting solution containing Novozyme 234. Note the fragmented cell walls in the background. Magnification, ×1,600.

Novozyme 234 and Mureinase and did not record any detectable differences in either the protoplasting efficiencies or the final DNA yields. The reduced time of 10 to 15 min to generate more than 70% of the protoplasts was only slightly less than that reported by Rhodes and Kwon-Chung (7), but was significantly less than that observed by Restrepo and Barbour (5). Moreover, the production of a maximum number of good-quality protoplasts, as shown in Fig. 1, with a minimum amount of lysis during protoplasting contributed to high DNA yields. Generally, this was the case for most of the strains tested from both *C. neoformans* var. *neoformans* as well as *C. neoformans* var. *gattii*. However, there may be some strains which require a longer incubation period. While DNases are known to be produced by *C. neoformans* (3, 6), their presence in the commercially available enzyme preparation (Novozyme 234) has not been reported previously. A significantly potent concentration of DNases is apparently present in the preparation. At a concentration of greater than 3 mg/ml, most of the DNA appears to be degraded (Fig. 2B). This is important, since the concentration in the procedure is 10 mg/ml. The presence of high concentrations (0.45 M) of

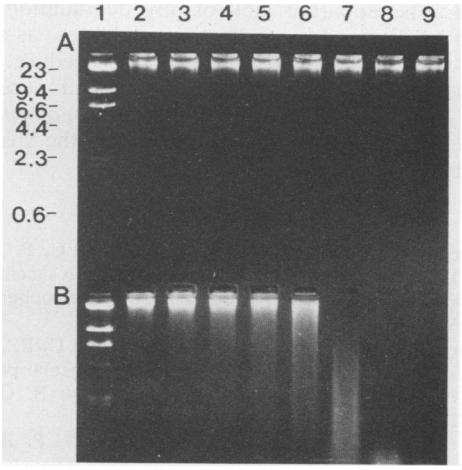

FIG. 2. Ethidium bromide-stained gel of 1 μg of total DNA from *C. neoformans* after incubation at 37°C in different concentrations of Mureinase with (A) and without (B) 0.45 M EDTA. Lane 1, Kilobase-pair size markers, using *Hin*dIII fragments of lambda DNA. Mureinase concentrations (in milligrams per milliliter) were as follows: lane 2, 0; lane 3, 0.4; lane 4, 0.8; lane 5, 1; lane 6, 2; lane 7, 3; lane 8, 4; lane 9, 5.

EDTA during lysis of the protoplasts is apparently sufficient to inhibit the activity of the DNases in the enzyme preparation (Fig. 2A).

Our procedure not only required many fewer steps after protoplast formation compared with those described in other reports (4, 5, 7, 9) but it also results in higher yields of undegraded high-molecular-weight DNA. A yield of about 150 μg of total DNA from approximately 10^9 yeast cells was

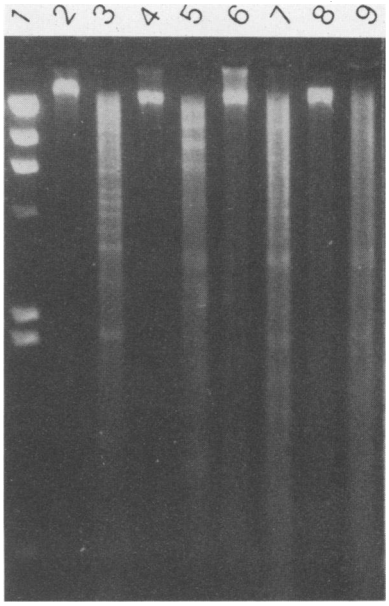

FIG. 3. Electrophoresis of total cellular DNA from an isolate of each serotype (A, B, C, and D) on 0.8% agarose either uncut (lanes 2, 4, 6, and 8) for strains of each serotype, respectively) or following digestion with *Eco*RI (lanes 3, 5, 7, and 9 for strains of each serotype, respectively). Lane 1, Kilobase-pair size markers, using *Hin*dIII fragments of lambda DNA.

observed. The apparent lack of any degradation and the ability to be digested by restriction nucleases, as shown in Fig. 3, attests to the superiority of this procedure. Furthermore, no special modification was deemed necessary in order to extract DNA from isolates of *C. neoformans* var. *gattii*, despite the increased complexity in the structure of their capsular polysaccharides (1, 2).

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