proteomics that resolve thousands of proteins on 2-D gels that need to be identified. Western blotting remains an important method in proteomics to identify specific proteins in complex protein mixtures (9,11). The staining/destaining procedure described here will increase the reliability of assigning immuno-stained protein spots to their silver-stained counterparts in the reference 2-D gel pattern.

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DNA Isolation from Chloroform/Methanol-Treated Mycobacterial Cells without Lysozyme and Proteinase K

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The recent resurgence of pathogenic mycobacteria including *Mycobacterium tuberculosis* (9) and *M. ulcerans* (5) increases the relevance of molecular genetic research on the elucidation of pathogenicity and the development of new prophylactic and therapeutic tools against mycobacterial diseases (3). However, the isolation of genomic DNA remains a laborious task despite the availability of several protocols (10) that are generally divided into two groups according to the procedure of cell wall lysis. The group of mechanical cell wall disruption includes short protocols that are often associated with DNA shearing, whereas the group of enzymatic cell wall lysis includes methods that require several hours or days to prepare genomic DNA. In addition, a few methods, based on organic solvent pretreatment (OSP) of cells before the DNA isolation procedure, have been reported for preparation of high molecular weight DNA from mycobacteria (4,6). Although these OSP methods apparently support the preservation of DNA integrity, they are time consuming and labor intensive, as they include refluxing and washing steps to remove traces of organic solvents before enzymatic lysozyme/proteinase K lysis steps. We have developed from existing methods (4,6) a new OSP method that is based on the guanidine thiocyanate disruption (1) of mycobacterial cells pretreated with chloroform/methanol (2:1, v/v). Modifications involve not only the omission of the enzymatic cell lysis including the washing/refluxing steps preceding lysing steps (4,6) but also the use of vortex mixing instead of a Teflon® homogenizer (6) and platform rocker (4). Our method is faster and easier to apply, requiring less than 1 h to purify high molecular weight DNA from mycobacteria, compared with the 6 and 20 h required by

Benchmarks

Table 1. Protocol for Genomic DNA Isolation from *Mycobacteria*

1. Harvest cells by centrifugation at 3500× *g* for 5 min in 30-mL Teflon screw cap tubes (25.5 × 92 mm; Nalgene, Rochester, NY, USA), and suspend 0.3–3 g wet pellets by vortex mixing for 2 min in 5 mL chloroform/methanol (2:1, v/v).
2. Centrifugate at 3500× *g* for 10 min and collect lipid lightened cells after pouring off solvents.
3. Add (1 mL/0.5–1 g wet cell pellets) the disruption solution [4 M guanidine thiocyanate, 0.025 M sodium citrate, pH 7.0, 0.5% (w/v) Sarkosyl, and 0.1 M 2-mercaptoethanol] before vortex mixing for 1 min.
4. Centrifugate at 4000× *g* for 15 min and transfer supernatants into new tubes before extracting with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v).
5. Add 0.1 volume of 3 M sodium acetate and 1 volume of isopropanol to aqueous phases. For thick solutions, DNA could be pulled, whereas for dilute solutions, DNA needed to be precipitated at 12 000× *g* for 15 min at 4°C.
6. Wash DNA with 70% ethanol, dry, and dissolve in sterile water before storage at 4°C.

Reagents: chloroform, methanol, guanidine thiocyanate, and phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) were purchased from Sigma (St. Louis, MO, USA).

methods from Imai et al. (6) and Belisle et al. (4), respectively.

To apply this newly developed method (Table 1), nine *Mycobacterium* spp. were statically cultured until late-log phase in 50 mL modified-Reid medium as previously described (7). The yield of purified DNA determined by measuring absorbance at 260 nm in a GeneQuant spectrophotometer (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) was an average of 20 µg/100 mg wet cell pellets (approximately 5% of theoretical yield of genomic DNA), which is comparable to the 17.2 µg previously achieved by the protocol of Imai et al. for the same wet weight of cells (6).

To determine the size distribution of extracted DNA, 3 µg were electrophoresed through a 0.3% (w/v) agarose gel running in Tris-borate-EDTA buffer at 2 V/cm. Each sample showed abundance in DNA molecules that are higher than the 48.5 kb (Figure 1A), thus corroborating the evidence, as previously described (5,8), that the application of chloroform/methanol lipid extraction before the DNA isolation procedure supports the yield of high molecular weight DNA.

To check the presence of traces of proteins or phenol in DNA preparations, the ratio of absorbance at 260 and 280 nm (A₂₆₀/A₂₈₀) was determined for

each DNA preparation and provided values varying from 1.6 to 2, indicating an acceptable level of purity, compared with the known A₂₆₀/A₂₈₀ ratio (1.8) corresponding to a pure DNA sample (8). In addition, we investigated whether the purity level of the extracted DNA displayed above interferes with the activities of DNA manipulation enzymes. One aliquot (50 ng) from each DNA preparation was used as template in a mycobacterial 16S RNA gene-specific PCR of 50 µL containing 20 pmol each of oligonucleotide primers (forward primer, 5'-GTGCTTAACACATGCAAGTCG-3' and reverse primer, 5'-GTGAGATTTCACGAACAACGC-3'), 200 µM dNTPs, 10 mM Tris-HCl (pH 8.6), 50 mM KCl, 1.65 mM MgCl₂, 1% (w/v) Triton® X-100 and 1 U *Taq* DNA polymerase (Promega, Madison, WI, USA). Amplification conditions were performed with the aid of a Thermojet thermal cycler (Eurogentec, Seraing, Belgium) as follows: 3 min at 94°C; 35 cycles of 1 min at 94°C, 45 s at 56°C, 1 min at 72°C; followed by a 7-min hold at 72°C. Electrophoretic analysis of PCR products was performed through a 1.5% (w/v) agarose gel and showed a product of expected size (557 bp) (Figure 1B), demonstrating the absence of inhibition of *Taq* DNA polymerase activity. We also tested the use of the restriction endonuclease *Sma*I (Roche

Benchmarks

Figure 1. Size distribution (A) and susceptibility to the *Taq* DNA polymerase (B) and the restriction endonuclease *Sma*I (C) of DNA isolated from nine *Mycobacterium* species. Lines line 1, *M. tuberculosis* H37Ra; line 2, *M. bovis* BCG (Pasteur); line 3, *M. ulcerans* ATCC 19423; line 4, *M. marinum* ATCC 927; line 5, *M. kansasii* ATCC 12478; line 6, *M. vaccae* ATCC 15483; line 7, *M. smegmatis* ATCC 1446; line 8, *M. fortuitum* ATCC 12790; and line 9, *M. avium* ATCC 15769. M1, M2, and M3 correspond respectively to the following molecular weight markers: λ DNA (48.5 kb; MBI Fermentas, Hanover, MD, USA), λ DNA/*Hind*III (Stratagene, Heidelberg, Germany), and Superladder-med2 200-bp ladder (GenSura, San Diego, CA, USA). Molecular size, in kb, of selected bands are indicated on the left of each figure.

Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions and observed through a 0.8% (w/v) agarose gel electrophoresis an effective cutting of extracted DNA

from each tested strain (Figure 1C). In addition to *Sma*I digestion, analogous results (not shown) were obtained with several other restriction enzymes including *Eco*RI, *Eco*RV, *Pst*I, *Bam*HI, and *Sau*3A, indicating the absence of lipoglycans and polysaccharides that are generally known as inhibitors of DNA manipulation enzymes (2).

The method described here, compared with existing protocols, is an excellent alternative to purify high-quality genomic DNA from mycobacteria because it is less time consuming and involves essentially two steps: (i) the chloroform/methanol (2:1, v/v) lipid extraction and (ii) the guanidine thiocyanate cell disruption. In addition, it is applicable without any modifications to a wide variety of pathogenic and non-pathogenic *Mycobacterium* species that are representative of all colony morphology types known at the genus level: rough, smooth, and intermediate type. It could be useful in applications such as Southern hybridization, generation of cosmid libraries, and others.

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