ethanol. By this method, we could routinely isolate 80–100 µg of RNA per 8 mL of culture. RNA isolated by this method or with TRIzol (manufacturer's recommended procedure) exhibited an $A_{260-280}$ ratio of 1.6–1.8. When needed, RNA samples were supplemented with RNA loading dye [0.72 mL formamide, 0.16 mL 10× MOPS buffer, 0.26 mL 37% formaldehyde, 0.18 mL distilled water (DEPC-treated), 0.10 mL 80% glycerol, and 8.0 mg bromophenol blue] and resolved on 1.2% formaldehyde-agarose gels. Alternatively, bromophenol blue (50 mg per 100 mL) could be added to the lysis buffer, and RNA, isolated as described above, could be directly applied to formaldehyde-agarose gels.

RNA isolated from *P. gingivalis* and *E. coli* using this new method was fractionated on formaldehyde-agarose gels along with preparations obtained by the TRIzol method. Figure 1 compares the quality of RNA isolated by this new protocol relative to the TRIzol method. No differences are seen in Figure 1, indicating no visible contamination in the new procedure. In the TRIzol method, chromosomal DNA contamination was detected for both organisms and was removed by DNase (RNase free) digestion prior to electrophoresis. RNA samples resolved on a formaldehyde-agarose gel were then transferred to a Hybond™-N+ nitrocellulose membrane (Amersham, Arlington Heights, IL, USA) by standard methods (3). This Northern blot was subjected to hybridization with a 0.4-kb *Pst*I-*Bam*HI DNA fragment internal to the *P. gingivalis hemR* gene (Karunakaran and Kuramitsu, unpublished results) as a probe utilizing nonradioactive ECL™ direct nucleic acid labeling and detection systems (Amersham). Figure 2 shows the results obtained following exposure of the blot to Hyperfilm™-ECL (Amersham). The results clearly indicate a transcript size of 3.1 kb for the *P. gingivalis hemR* gene with RNA isolated by both methods.

These results indicate that the newly described method represents a rapid and simple method for isolating high-quality RNA from gram-negative bacteria. Northern blot analysis readily detects specific mRNA transcripts in these preparations.

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Improved Isolation of Genomic DNA from Mycobacteria in Agarose Plugs by Rapid Lysis with a Combination of *N*-Acetylglucosaminidase and Lysozyme

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Pulsed-field gel electrophoresis (PFGE) allows the separation of large DNA fragments. The technique, originally developed by Schwartz et al. (6) for the separation of yeast chromosomes, has been applied to the analysis of bacterial genomes. After digestion by low-frequency cleavage restriction endonucleases, bacterial chromosomes provide DNA patterns composed of a few, usually well separated fragments.

Isolation of genomic DNA from mycobacteria is a time-consuming and tedious process (1,6). Most pathogenic mycobacteria grow slowly in culture media and their cells are difficult to lyse. The tough mycobacterial cell walls contain thick layers of lipopolysaccharide-protein complexes that make them resistant to the standard protocols (1,6). Due to the resurgence of tuberculosis, the molecular fingerprinting of mycobacterial isolates by restriction fragment length polymorphism (RFLP) analysis (7,8) is gaining importance in mycobacterial research and epidemiology. This involves handling of a large number of strains at a time. The time-consuming procedure in the isolation of DNA from mycobacteria is the disruption of the thick, lipopolysaccharide-rich cell wall without causing damage to the genomic DNA. We report a rapid and gentle method to extract sufficient quantity of unsheared genomic DNA from mycobacterial cells. Until now, several groups had isolated mycobacterial genomic DNAs from *Mycobacterium tuberculosis*, *M. bovis* BCG strains, *M. paratuberculosis* and *M. avium* using the usual method by utilizing lysozyme or zymolase for preparation of intact genomic DNAs in agarose plugs and analyzing by PFGE (2–4,7,8). However, these suffered from impurity of

Table 1. A Comparison of Our Method with That of the Primary Method of Bose et al. (1)

	Yield of DNA[a]	Purity[b]	Total time[c]	Preincubation requirements[d]	Mechanical/ physical method[e]	Utility in PFGE[f]
Method being reported	1.5–2.5	1.9–2.0	< 4.5 h	No	No	Yes
Bose et al. (1)	1.2–2.0	1.8–2.0	< 7 h	No	No	NA

[a] mg/g wet wt. of cells
[b] O.D. 260/280 nm
[c] Time in hours for DNA isolation from bacterial pellet
[d] Cell wall modifying agent necessary (Yes/No)
[e] Method of disruption employed, e.g, French pressure cell or bead beater (Yes/No).
[f] Utilization using agarose block for PFGE (Yes/No)
NA, Data not available.

the prepared genomic DNAs and the attendant processing problems. To solve these problems in preparation of mycobacterial plugs, we found that treating mycobacterial cells with a mixture of lysozyme and *N*-acetylglucosaminidase remarkably facilitated the successive digestion of these cells and further extraction.

Ordinary lysis procedures of mycobacteria, requiring inhibitors of cell wall synthesis and actively growing mycobacteria, could not be used due to its characteristic cell wall structures. We have devised a lysis method in which DNA is extracted from agarose-embedded bacteria in order to prevent mechanical, nonspecific chromosomal fragmentation and have developed a gentle lysis procedure that ensures suitable yields of entire DNA from immobilized, nondividing cells.

Agarose plugs were made with heat-treated cells prepared essentially by a slightly modified method of that of Park et al. (5). Briefly, mycobacterial cells cultured in liquid broth (Middlebrook 7H9) were harvested at the early exponential phase. Cells (100 mg wet weight) were washed twice with 10 mL TC lysis buffer (10 mM Tris-HCl and 1 M NaCl, pH 7.6) containing 1% sodium lauryl sarcosine followed by centrifugation (6000× *g* for 10 min). After resuspension of the cells in 2 mL of suspension buffer (0.01 M Tris-HCl, pH 8.0, 0.1 M Na-EDTA and 0.02 M NaCl), the suspension was warmed in an incubator at 30° to 40°C and then diluted with an equal volume of 1% low-melting-temperature agarose (FMC BioProducts, Rockland, ME, USA) made up in sterile water at 42°C. The resulting solution was then poured into a mold chamber (Bio-Rad, Hercules, CA, USA). Solidified blocks were incubated at 37°C for 1, 2 or 5 h in 1 mg/mL lysozyme, 1 mg/mL *N*-acetyl-β-D-glucosaminidase (Boehringer Mannheim, Seoul, Korea), 1 mg/mL *N*-acetyl-α-D-glucosaminidase (Boehringer Mannheim) and 50 µg/mL RNase A (Sigma Chemical, St. Louis, MO, USA) solution (in TC lysis buffer), and it was kept at 60°C for 30 min in a slow-speed shaker water bath to remove the bound polysaccharides. It was then treated for 2 h at 50°C with an equal volume of buffer containing proteinase K (1 mg/mL) (Boehringer Mannheim, Mannheim, Germany), 0.5% *N*-laurylsarcosine (Sigma Chemical) and 1 mM EDTA, pH 8.0. Proteinase activity was inhibited by washing the blocks twice for 1 h at room temperature in 40 µg/mL phenylmethylsulphonyl fluoride (PMSF). The

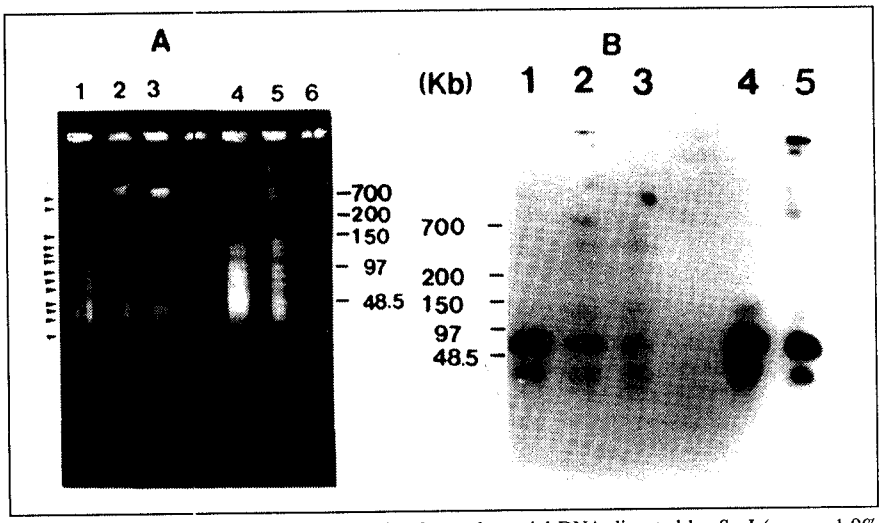

Figure 1. A) Pulsed-field gel electrophoresis of mycobacterial DNA digested by *Spe*I (run on 1.0% agarose gel in PFGE). B) Southern blot hybridization of *Spe*I-digested genomic DNA with *Leu*B gene of *M. bovis* BCG (Y. K. Choe et al., unpublished result), labeled with DIG-kit. Lane 1: *M. tuberculosis* R68 genomic DNA (10 µg) prepared by a newly developed procedure described above with incubation for 2 h; lane 2: *M. tuberculosis* R68 genomic DNA (10 µg) prepared by lysozyme only with incubation for 2 h; lane 3: *M. tuberculosis* R68 genomic DNA (10 µg) prepared by zymolase (Seikagaku Koukyo K. K., Tokyo, Japan) only with incubation for 2 h; lane 4: *M. tuberculosis* KIT10181 genomic DNA (20 µg) prepared by a newly developed procedure described above with incubation for 5 h; lane 5: *M. tuberculosis* KIT10181 genomic DNA (20 µg) prepared by lysozyme only with incubation for 5 h. The numbers in the middle show the positions for the DNA size standard markers of the sizes indicated. The ramped pulse times were 5–25 s for 24 h at 14°C and 200 V. The gel was 1.0% SeaPlaque agarose in 0.5× TBE.

Table 2. Mycobacterial Strains Used in This Study and Sensitivity to *N*-Acetylglucosaminidase

Strains	Sources	Sensitivity to Enzymes		
		Lys[a]	NAC[b]	Lys + NAC[c]
M. tuberculosis				
H37Rv	ATCC 27294	-	-	+
R68	clinical isolate	-	-	+
K30	clinical isolate	-	-	+
H23	clinical isolate	-	-	+
KIT10181	clinical isolate	-	-	+
KIT10202	clinical isolate	-	-	+
KIT10211	clinical isolate	-	-	+
KIT10215	clinical isolate	-	-	+
KIT10468	clinical isolate	-	-	+
KIT10110	clinical isolate	-	-	+
M. avium-intracellulae complex				
573	clinical isolate	-	-	+
569	clinical isolate	-	-	+
M. fortuitum				
551	clinical isolate	-	-	+
547	clinical isolate	-	-	+
M. gordonae				
560	clinical isolate	-	-	+
571	clinical isolate	-	-	+
M. terrae complex				
545	clinical isolate	-	-	+
479	clinical isolate	-	-	+

Results are obtained from 2-h exposure of bacteria only to lysozyme or *N*-acetylglucosaminidase and the combination prior to the remaining steps of lysis procedure described below. For measurement of effective lysis, mycobacterial DNAs were isolated in 3 separate conditions and DNA concentrations were measured. In brief, cells (5-mL culture) were killed by heating at 70°C in a water bath for 30 min and pelleted by centrifugation at 3000× *g* for 15 min. Mycobacteria were washed with 2 mL of TC lysis buffer containing 1% sodium dodecyl sulfate (SDS) and resuspended in 600 µL of suspension buffer. Cells were divided in 3 aliquots (200 µL) and separately incubated with 1 mg/mL lysozyme or 1 mg/mL *N*-acetylglucosaminidase and the combination at 37°C for 2 h. Lysates were extracted by phenol and phenol-chloroform for nucleic acids that were precipitated by adding 0.1 volumes of 3 M sodium acetate and 1 volume of 2-propanol. Washed and dried DNA pellets were suspended in 0.2 mL TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). RNA was digested by RNase A (Sigma Chemical). After adding proteinase K (1 mg/mL), samples were incubated for 30–60 min at 37°C. DNA was purified by extraction with phenol, phenol-chloroform and chloroform, and precipitated by adding 2.5 volumes of ethanol and the DNA concentration was measured. "+" indicates effective lysis of mycobacterial cell, showing 1.5–2.5 mg yield of DNA in mg/g (wet wt. of cells). "-" indicates 0.1 mg > yield of DNA.

[a]Lys: Lysozyme (results from exposure of bacteria only to lysozyme)

[b]NAC: *N*-Acetylglucosaminidase (results from exposure of bacteria only to *N*-acetylglucosaminidase)

[c]Lys + NAC: Lysozyme + *N*-acetylglucosaminidase (results from exposure of bacteria to the combination of lysozyme and *N*-acetylglucosaminidase)

blocks were then stored in 0.05 M Na-EDTA (pH 8.0) at 4°C. The yield was 1.5 to 2.5 mg/g wet weight of cells (Table 1). Table 1 shows a comparison of this method with the primary method of Bose et al. (1) for isolation of genomic DNA from mycobacteria. Furthermore, the procedure could be completed in 4.5 h and may be scaled up if required.

For digestion of DNA in agarose plug, 10 U restriction endonuclease *Spe*I (Boehringer Mannheim) were used. After digestion, blocks were mounted on the teeth of an electrophoresis comb. The gel was cast with 1.0% (wt/vol) SeaPlaque® agarose (FMC BioProducts) at 55°C in 0.5× TBE buffer (10 mM Tris-borate and 1 mM EDTA). The gel was electrophoresed at 14°C in a CHEF DR™ II apparatus (Bio-Rad). The gel was run for 24 h at 200 V with a ramped pulse time from 5 to 25 s. *Saccharomyces cerevisiae* chromosomes (Bio-Rad) and λ-DNA concatamer (Bio-Rad) were used as size markers for high-molecular mass DNA fragments. The DNA plug isolated by this method was pure enough for restriction analysis and PFGE analysis. The recovered chromosomal DNA was unsheared as ascertained by the restriction analysis (Figure 1A). To avoid the problem of quantitative hybridization of large DNA fragments due to inefficient transfer onto filters, the gel was treated with 0.5 M HCl and 0.5 M NaOH and hybridized under stringent conditions. Autoradiographs of Southern blot of different mycobacteria as representative strains are shown in Figure 1B. DNA plug was prepared as described in the text, restriction-digested with *Spe*I and hybridized with a *Leu*B probe (Y-K. Choe, unpublished result) labeled using a multipriming method (Boehringer Mannheim, Korea).

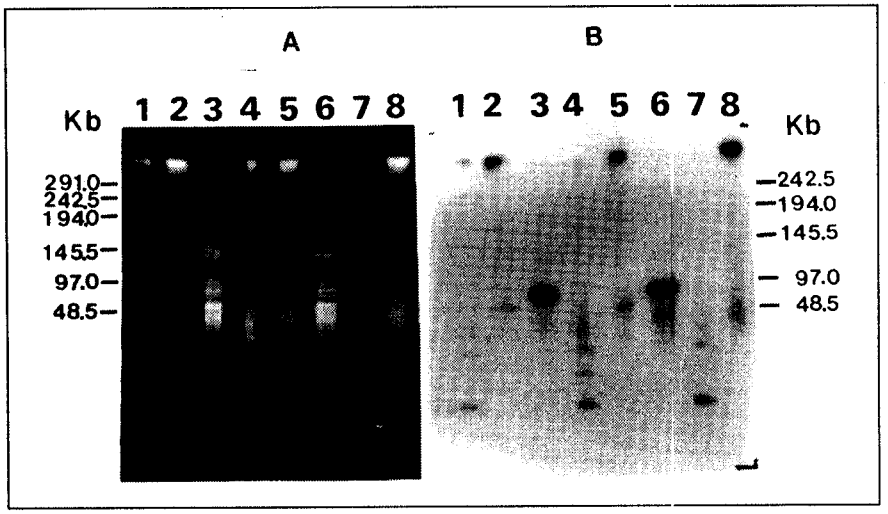

Figure 2. A) Pulsed-field gel electrophoresis of mycobacterial DNA digested by *Xba*I (run on 1.0% agarose gel in PFGE). B) Southern blot hybridization of *Xba*I-digested genomic DNA with *Leu*B gene of *M. bovis* BCG. Lanes 1 and 7: *M. tuberculosis* R68 genomic DNA (10 µg) prepared by zymolase (1-h incubation); lanes 2, 5 and 8: *M. tuberculosis* R68 genomic DNA (10 µg) prepared by lysozyme (1-h incubation); lanes 3 and 6: *M. tuberculosis* R68 genomic DNA (10 µg) prepared by a newly developed procedure (1-h incubation); lane 4: *M. tuberculosis* KIT10181 genomic DNA (10 µg) prepared by lysozyme (1-h incubation). The numbers on the left and right show the positions for the DNA size standard markers of the sizes indicated. Other conditions are the same as in Figure 1.

For further analysis of the superiority of this method, cells were treated for 1 h with the enzymes as described above and DNAs were analyzed by restriction enzyme *Xba*I (Boehringer Mannheim) and by Southern hybridization. Results clearly showed that this method produces more efficient lysis of mycobacteria or less fragmentation of the resultant DNA even in condition of a one-hour incubation with the enzyme system (Figure 2). Exactly the same results were obtained when mycobacterial strains of *M. tuberculosis, M. avium-intracellulae* complex, *M. fortuitum, M. gordonae* and *M. terrae* complex, listed in Table 1, were used for isolation of genomic DNA by means of agarose plugs (data not shown).

In conclusion, the method described here provides a simple, rapid and gentle procedure for lysis of mycobacterial cells, which is superior to previously used techniques. Because of short incubation times and ambient temperatures required for lytic activity, the inevitable nuclease activities present in crude lysates were negligible. This should be especially valuable for studies on genomic DNA. The concept of using *N*-acetylglucosaminidases of high specificity and activity for research and biotechnological applications is presently being extended to other bacteria that have some *N*-acetylglucosaminyl linkages in cell wall structures and will be reported in the near future.

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