A Rapid and Gentle Method for Isolation of Genomic DNA from Pathogenic *Nocardia* spp.

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The lack of simple and efficient methods for extraction of DNA from *Nocardia* spp. has hampered molecular manipulation of the DNA for diagnostic purposes. In the present study, a method for the rapid extraction of undegraded genomic *nocardial* DNA was established. Briefly, 14 pathogenic *Nocardia* strains were grown at 37°C for 3 to 5 days in Sauton broth containing 0.05% Tween 80. Subsequently, the cultures were treated for 48 h with 1.2 mg of cycloserine per ml (final concentration). Cells were then harvested by centrifugation and treated with a lysis solution containing 3 mg of lysozyme per ml. This was followed by the addition of proteinase K and sodium dodecyl sulfate to final concentrations of 0.2 mg/ml and 0.5%, respectively, and incubation for 1 h at 50°C. DNA was precipitated with isopropanol after phenol-chloroform-isomyl alcohol extractions and RNase treated before being quantitated and analyzed by agarose gel electrophoresis. The average undegraded DNA yields obtained were 101 μg for *Nocardia brasiliensis* and 121 μg for *N. asteroides*. This DNA was suitable for restriction endonuclease digestion and PCR amplification, which are methods being applied to the characterization and diagnosis of slowly growing organisms such as *Nocardia* spp.

*Nocardia* spp. are primary or opportunistic pathogens of the human host. Opportunistic infections by *Nocardia* spp., i.e., *nocardiosis*, may be pulmonary, systemic, or of the central nervous system. They affect mostly immunocompromised individuals and are associated with a high mortality, especially when an appropriate antibiotic treatment is delayed (2, 5, 11, 13). Thus, a timely diagnosis of these conditions is needed.

Because *Nocardia* spp. are slow growers, their isolation and identification by available microbiological methods require several weeks (6, 13). Moreover, when susceptibility tests are to be performed on a given isolate, another 2 to 3 weeks is needed. The above difficulties associated with the current cultural diagnosis of *Nocardia* infections may be circumvented by the use of molecular methods of amplification and/or detection of the pathogens’ specific nucleic acids. However, in order to develop these methods, a rapid and simple procedure for genomic DNA extraction is required (3). In 1989, Loeffelholz and Scholl established a cumbersome method for extraction of DNA from *Nocardia* spp. which is long, tedious, and unapplicable to a large number of strains (12).

In the present paper, we report an efficient and gentle method for the extraction of sufficient quantities of unsheared genomic DNA from *Nocardia* spp. The recovered DNA was suitable for molecular manipulations potentially applicable to the characterization and/or identification of *Nocardia* spp., such as restriction endonuclease analysis and PCR amplification.

The following *Nocardia brasiliensis* strains used in this study were obtained from the indicated sources: ATCC 19296 from M. Goodfellow (University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom); 267-78 and 146-78 from M.G. Goodfellow (University of Newcastle upon Tyne, United Kingdom); 267-78 and 146-78 from M. Goodfellow (University of Newcastle upon Tyne, United Kingdom); 267-78 and 146-78 from M. Goodfellow (University of Newcastle upon Tyne, United Kingdom); and GUH-1 from B. Beaman (University of California, Davis). *Nocardia asteroides* strains N-58, N-63, and N-71 were obtained from the Centers for Disease Control (Atlanta, Ga.); ATCC 19247 was obtained from M. Goodfellow (University of Newcastle upon Tyne); and MDL 874-90, MDL 1705-89, and MDL 2151-90 were from the California Department of Health, Berkeley. *N. asteroides* 555H was a bovine mastitis isolate from Puerto Rico. All strains were maintained on Sabouraud dextrose slants at room temperature until use.

For DNA extraction, 25 ml of Sabouraud dextrose broth was seeded with the growth recovered from a Sabouraud dextrose slant of each strain. These seed cultures were placed at 37°C in a controlled-environment incubator shaker (New Brunswick Scientific Co., Edison, N.J.) for 5 to 7 days. Five milliliters of each seed culture was transferred to 100 ml of Sauton-Dubos modified broth containing 0.05% Tween 80 (Sigma Chemical Co., St. Louis, Mo.). The cultures were incubated under the same conditions for 2 to 5 days. Then β-cycloserine (Sigma) was added to a final concentration of 1.2 mg/ml. The cultures were further incubated under similar conditions for 48 h (8).

Bacterial cells were pelleted by centrifugation at 757 × g for 20 min at 4°C (Centra-MP4R; IEC, Needham Heights, Mass.) in preweighed 50-ml conical centrifuge tubes, and their wet weights were determined. The cells were then resuspended in 4 ml of lysis buffer (15% sucrose, 0.05 M Tris [pH 8.0], 0.05 M EDTA) per g (wet weight) of bacteria. Lysozyme was added to a final concentration of 3 mg/ml of lysis buffer, and the suspension was reincubated at 37°C for 30 min. Sodium dodecyl sulfate was added to final concentration of 1%, and then 1 volume of sterile water and 0.4 mg of proteinase K per ml were added. The solution was gently mixed and further incubated for 1 h at 50°C.

The procedure for DNA purification was similar to that described by Sambrook et al. (14). Briefly, 2 volumes of Tris...
buffer (pH 8.0)-saturated phenol were added and the mixture was gently combined by inverting the tubes until an emulsion was formed. The solution was centrifuged at 757 × g for 20 min at 4°C. The aqueous phase was transferred to a clean tube, and the procedure was repeated with phenol-chloroform-isooamyl alcohol (25:24:1) until a clear interface was observed. A final extraction with chloroform-isooamyl alcohol (24:1) was performed. The aqueous phase was then transferred to a clean tube, and the DNA was precipitated with 0.25 volume of 10 M ammonium acetate and 0.6 volumes of isopropl alcohol at room temperature. The solution was centrifuged at 27,000 × g at 4°C (J2-1 centrifuge; Beckman Instruments, Irvine, Calif.) for 30 min. The pellets were washed with 70% ethanol, allowed to dry, and treated for 1 h at 37°C with RNase buffer, consisting of 10 μg of RNase per ml of TE (0.01 M Tris [pH 8], 0.001 M EDTA). DNA was extracted once with phenol-chloroform and again with chloroform-isooamyl alcohol. The nucleic acids were precipitated with 0.1 ml of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% cold ethanol, washed with 70% ethanol, and dissolved in a small volume of TE. A Beckman DU 20 spectrophotometer was used to determine the DNA content by the procedure recommended by the manufacturer (GIBCO-BRL, Gaithersburg, Md.).

Ten micrograms of purified Nocardia DNA was subjected to restriction endonuclease digestion with PvuII according to the procedure recommended by the manufacturer (GIBCO-BRL). Briefly, 5 U of the enzyme was added per μg of DNA in 60 μl of reaction buffer. The solution was incubated at 37°C for 1 h, and the reaction was stopped by addition of 0.1 volume of 0.1 M EDTA. Ten microliters was combined with 2 μl of 6× gel loading buffer (0.25% bromphenol blue, 0.25% xylene cyanole, 30% glycerol) and loaded into a 0.8% agarose gel containing ethidium bromide. The samples were electrophoresed at 60 V for 2.5 h in a horizontal mini-gel. Nocardia DNA preparations used for PCR amplification were further purified by the use of ion-exchange columns (Qiagen, Chatsworth, Calif.). Briefly, Nocardia DNA was ethanol precipitated and resuspended in 1/5 of the original volume in 10 mM EDTA (pH 8). This solution was added to a Qiagen column (TIP 100 or Midi) equilibrated with 3 ml of QBT buffer (750 mM NaCl, 50 mM MOPS [morpholinepropanesulfonic acid], 15% ethanol, 0.15% Triton X-100 [pH 7.0]). The column was washed with OC buffer (1.0 M NaCl, 50 mM MOPS, 15% ethanol [pH 7.0]), and the DNA was eluted with 5 ml of GF buffer (1.25 M NaCl, 50 mM Tris, 15% ethanol [pH 8.5]), precipitated with 0.7 volume of isopropanol, and resuspended in TE buffer. Dilutions of Nocardia DNA (1 μg/ml) were prepared in 50 μl of sterile water. A PCR template kit was diluted 1:10 in reaction buffer (10 mM Tris [pH 8.0], 1 mM EDTA, and 10 mM NaCl) and used as a control according to the instructions of the manufacturer (Perkin-Elmer Cetus, Norwalk, Conn.).

To the Nocardia DNA, 10 μl of 10× reaction buffer (100 mM Tris [pH 8.3], 500 mM KC1, 9 mM MgCl2, PCR primers [final concentration, 40 μM each], and dioxynucleoside triphosphates [final concentration, 200 μM] in a total volume of 100 μl) was added. Water was then added to bring the volume to 100 μl. The mixture was subjected to a hot start at 95°C for 5 min. Then 2.5 U of Taq polymerase (Perkin-Elmer Cetus) and sterile mineral oil (50 μl) were added to each tube, and all of the tubes were placed in a DNA thermal cycler (Perkin-Elmer Cetus). The temperature was cycled to 94°C for 1 min, 55°C for 1 min, and then 72°C for 2 min. The cycle was repeated approximately 36 times. The universal primers used were pA (5′ GGA ATT CCC GGG ATC CAA TTG ATC ATG GTT CCT CAG CAG 3′) and pB (5′ GGA GAT TCT CAC CTA CGT CAC 3′); positions 8 to 28 of the Escherichia coli 16S rRNA sequence plus EcoRI, SmaI, and BamHI sites at the 5′ end) and pC (5′ GGA ATT CCC GGG ATC CAA GTG GAT TCC ACC GCA GCC GCA 3′; positions 1542 to 1522 of the E. coli 16S rRNA sequence plus EcoRI, SmaI, and BamHI sites at the 5′ end) (7).

The average amounts of DNA recovered by the method described above for 14 Nocardia strains are presented in Table 1. As can be appreciated, there are marked differences in the DNA yield depending on the strain used. The average yields ranged from 14 to 368 μg/g (wet weight) of cells. These differences are due to the distinct rate of growth of Nocardia organisms in broth and to the refractivity to lysis exhibited by some strains (1, 10). The overall A260/A280 ratio for the DNA preparations ranged from close to 1.7 to 1.97, which falls within the range of pure DNA (data not shown) (14).

DNA further purified by using a resin was amplified with universal 16S rRNA primers and the PCR. Figure 2 shows the complex band pattern obtained, ranging from 125 bp to <23 kb. However, no DNA degradation or inhibition of the enzyme’s activity was observed.

Nocardia DNA further purified by using a resin was amplified with universal 16S rRNA primers and the PCR. Figure 3 shows the bands obtained, which corresponded to a product of 1,500 bp, as predicted from the 16S rRNA sequence of N. asteroides (4).
The above results show that our modification of the method used by Eisenach et al. (8) for extraction of DNA from mycobacteria allows simple and reproducible extraction of DNA from \textit{Nocardia} spp. The method is based on the gentle cell lysis of bacteria whose cell wall synthesis had been inhibited by D-cycloserine. In our study, the cultures of \textit{Nocardia} spp. subjected to cycloserine treatment were 3 to 5 days old. The variability in the length of incubation is related to the different rates of growth and to the fact that turbid cultures were required for D-cycloserine addition. Contact with this antibiotic was for a total of 48 h. An apparent change in the growth of \textit{Nocardia} organisms (i.e., from compact clumps to a loose or flaky appearance) or a drop in turbidity was correlated with effective cell lysis and good DNA yields. However, there were strains that proved to be very difficult to lyse, such as \textit{N. asteroides} N-58 and \textit{N. brasiliensis} 146-78 and MB90-3. Variation in cell wall composition among the species of \textit{Nocardia} as well as among strains of the same species has been reported previously (1, 9). Differences in the cell wall compositions of these three strains may account for their relative resistance to lysis.

The recovered DNAs were found to be relatively pure, according to their $A_{260}/A_{280}$ ratios, and to be suitable for molecular manipulations when further purified by chemical and/or chromatographic methods. This is very encouraging, as it has been envisioned that a rapid diagnostic method for \textit{Nocardia} infections may result from the development of DNA probes (3). The PCR amplification procedure which yielded the expected-molecular-size product is currently allowing the analysis of \textit{Nocardia} rRNA (4).

In summary, the procedure described here provides a relatively fast and efficient method for the extraction of sufficient quantities of genomic DNA from \textit{Nocardia} spp. The method is gentle enough to produce undegraded DNA, yet efficient enough to eliminate bacterial lipids, polysaccharides, proteins, and chemical contaminants which interfere with molecular methods such as digestion with restriction enzymes and/or PCR amplification. These could be applied to the characterization and identification of \textit{Nocardia} clinical isolates.

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REFERENCES


