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-isoamyl 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 P. Lavalle and A. González Ochoa (Centro Dermatológico Pascua and Instituto de Salubridad y Enfermedades Tropicales, respectively, Mexico City, Mexico); 1583, MB 90-3, and MDL 7009-88 from the New Mexico Department of Health, Albuquerque; 1707 from the Waksman Institute (Piscataway, N.J.); 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 3 to 5 days. Then p-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

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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 isopropanol alcohol at room temperature. The solution was centrifuged at 27,000 × g at 4°C (J2-21 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 ρNase 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 95% cold ethanol, washed with 70% ethanol, and dissolved in a small volume of TE. A Beckman DU 20 spectrophotometer was used to determine the A260/A280 ratio. The total DNA content was estimated from the A260 reading. The A260/A280 ratio was interpreted as an index of DNA purity as described by Sambrook et al. (14). This procedure was repeated a minimum of two times for each strain used. In addition, samples from each DNA preparation were electrophoresed in 0.8% agarose gels containing ethidium bromide, visualized under a UV transilluminator, and photographed with Polaroid 667 type film and a Polaroid DS-34 camera (GIBCO-BRL, Gaithersburg, Md.).

Ten micromgrams 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 (TIPI 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 QC buffer (1.0 M NaCl, 50 mM MOPS, 15% ethanol [pH 7.0]), and the DNA was eluted with 5 ml of QF buffer (1.25 M NaCl, 50 mM Tris, 15% ethanol [pH 8.5]). The DNA was 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 KCl, 9 mM MgCl2, PCR primers [final concentration, 40 μM each], and deoxynucleoside 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 CAG TGT ATC CTG CCT CAG 3'; positions 8 to 28 of the Escherichia coli 16S rRNA sequence plus EcoRI, SmaI, and BamHI sites at the 5' end) and pH (5' GGA ATT CCC GGG ATC CAA GGA GAT GATCCA GCC GCA 3'; positions 1542 to 1522 of the E. coli 16S rRNA sequence plus EcoRI, SmaI, and BamHI sites at the 5' end). The overall average of the DNA yield was determined from the DNA preparations generated with undegraded DNA (data not shown) (14).

Each of the DNA preparations generated was run in 0.8% agarose gels, and all were found to be in an undegraded condition (Fig. 1). Further purification of the DNA was accomplished by different means. The phenol-chloroform-isooamyl alcohol extractions were repeated after the ρNase treatment, and then a final extraction was done with water-saturated chloroform. This eliminated remnants of bacterial cell wall lipids and phenol traces, both of which are known to interfere with other procedures, such as digestion with restriction enzymes (14). Six of the further-purified DNAs from different Nocardia strains were subjected to endonuclease digestion with PvuII. 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 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 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 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 N. asteroides N-58 and N. brasiliensis N-146-78 and MB90-3. Variation in cell wall composition among the species of 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 A260/A280 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 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 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 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 Nocardia clinical isolates.

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REFERENCES


