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Characterization of *Nocardia asteroides* Isolates from Different Ecological Habitats on the Basis of Repetitive Extragenic Palindromic-PCR Fingerprinting

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Thirteen isolates of *Nocardia asteroides* from both soils and aquatic samples (lake and moat sediments, as well as scum from activated sludge), together with a type strain and two known clinical isolates of this species, were characterized by repetitive extragenic palindromic-PCR fingerprinting with the BOX-A1R primer. The resulting DNA fingerprint patterns proved to be strain specific, and cluster analysis distinguished the soil isolates, the aquatic isolates, and the known strains as being in separate groups.

Nocardia asteroides is a gram-positive, partially acid-fast, aerobic mesophile that characteristically produces primary a mycelium that often fragments into bacillary and coccoid elements (7). An aerial mycelium with short chains of arthrospores is usually formed. This actinomycete has been implicated as an opportunistic pathogen in a range of pulmonary diseases, including nocardiosis (6). Therefore, subtyping, or differentiation of isolates belonging to this taxon, is of epidemiological importance for investigating outbreaks of infection, determining the source of the infection, and recognizing especially virulent strains (12). DNA-based typing methods, including pulsed-field gel electrophoresis and randomly amplified polymorphic-DNA analyses, have been used to characterize clinically related *N. asteroides* strains (10, 11) and have shown that clinical isolates of *N. asteroides* are genetically heterogeneous.

N. asteroides is also involved in the saprophytic digestion and recycling of plant material in natural environments (5–7). The primary reservoir of *N. asteroides* is thought to be the soil, but this organism can also be found in lake and marine sediments. However, there is little published information on the genetic diversity of *N. asteroides* strains associated with these natural habitats (6). The purpose of the present study was to characterize and differentiate *N. asteroides* strains isolated from both terrestrial and aquatic environments by using the repetitive extragenic palindromic (REP)-PCR fingerprinting technique, in which PCR amplification of the DNA between adjacent repetitive extragenic elements is used to obtain DNA fingerprints (19). REP-PCR has recently proven to be highly discriminatory for subtyping or strain classification of several bacterial species (12).

***N. asteroides* isolates.** Sixteen *N. asteroides* strains were included in this study (Table 1). Of these, three strains used as reference strains for REP-PCR analysis were obtained from the Japan Collection of Microorganisms, RIKEN, Saitama Prefecture (JCM; $n = 1$) and the Institute for Fermentation, Osaka Prefecture (IFO; $n = 2$), Japan. All other strains were isolated from soil, lake, or moat sediments or from scum of activated sludge. Soil samples were collected from various locations in Yamanashi Prefecture, Japan. Sediment samples were taken from Lake Suwa, Nagano Prefecture, and from the moat surrounding Takeda Shrine, Yamanashi Prefecture, Japan, by use of an Ekman-Birge sediment surface sampler (Watanabe Keiki Co., Ltd., Tokyo, Japan). Lake Suwa is a eutrophic lake with a maximum depth of 7.2 m and a surface area of 13 km^2. The Takeda Shrine moat is about 18 m wide and has a total length of 1,000 m and a maximum depth of 1 m. A scum sample was obtained from activated sludge at a sewage treatment plant in Saitama Prefecture, Japan. The *Nocardia* isolation method is described in detail in a previous paper (21).

The identities of *N. asteroides* isolates were confirmed by morphological and chemotaxonomic characterization, restriction fragment length polymorphism (RFLP) analysis of 16S rRNA genes (rDNAs), and sequencing of 16S rDNA signature sequences (2) between positions 591 and 648 (*Escherichia coli* numbering) (1). Details of the taxonomic tests employed have been previously described (9, 13, 21). All 13 test isolates consistently developed a fragmenting substrate mycelium and a relatively short aerial mycelium with chains of smooth-surfaced arthrospores when inoculated onto oatmeal agar (16). All 13 test isolates were found to contain *meso*-diaminopimelic acid as a cell wall diamino acid and to have galactose and arabinose as characteristic whole-cell sugars. The predominant menaquinone component of these strains is MK-8 (H_4, ω cyclized), and they also contain phosphatidylethanolamine and nocardomycolic acid. These morphological and chemotaxonomic properties of the test strains are consistent with those of the genus *Nocardia* (6, 7). The 13 test isolates, together with three reference strains, were evaluated by amplification and restriction endonuclease analysis of a portion (1 kb; positions 30 to 1049) of the 16S rDNA (3, 21). PCR products were subjected to digestion with HhaI, NdeI, BstEII and HindIII, all of which have been recommended for use in identification of *Nocardia* species on the basis of RFLP analysis of 16S rDNA (3, 21). All 13 test isolates had RFLP patterns identical to those of *N. asteroides* (strains JCM 3384^T, IFO 3423, and IFO 3424) and could be differentiated from all other known *Nocardia* species,

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TABLE 1. *N. asteroides* strains used in this study

N. asteroides strain	Source of isolate or strain description[a]
Environmental isolates	
SO 008-1, SO 008-2	Vegetable field soil
SO 020-4	Paddy field soil
SO 026-1	Vegetable field soil
SO 027-1	Cornfield soil
SO 031-3	Flatland forest soil
LS 001-3, LS 001-4	Lake Suwa surface sediment (center area, 5 m depth)
LS 002-1, LS 002-2, LS 002-5	Lake Suwa surface sediment (near the east shore, 1 m depth)
MS 002-1	Takeda Shrine moat surface sediment (0.5 m depth)
AS 004-5	Scum of activated sludge
N. asteroides JCM 3384^T	ATCC 19247^T; proposed working type[b]
N. asteroides IFO 3423	FMJ 101; clinical isolate
N. asteroides IFO 3424	FMJ 102; clinical isolate

[a] ATCC, American Type Culture Collection, Rockville, Md.; FMJ, Faculty of Medicine, Juntendo University, Tokyo, Japan.
[b] See reference 17.

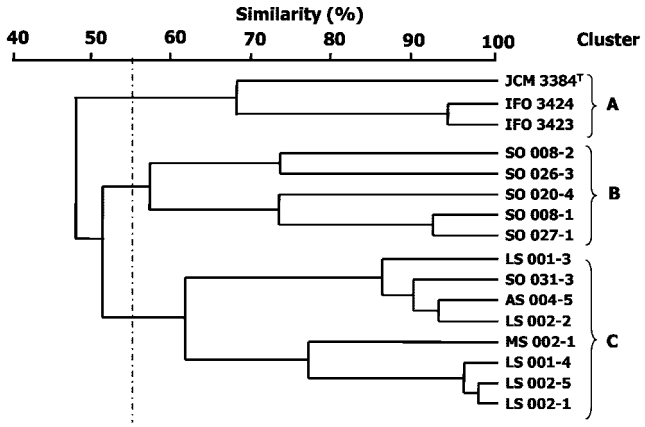

FIG. 2. Dendrogram of REP-PCR genomic fingerprints of *N. asteroides* strains. Percent similarity between patterns was calculated using Pearson coefficients. The clustering pattern was generated using the UPGMA method.

including the closely related species *N. abscessus*, *N. cyriacigeorgica*, *N. farcinica*, and *N. nova* (3, 10, 14, 21). For sequence analysis, a portion of 16S rDNA (0.8 kb; positions 9 to 802) was amplified by PCR and sequenced by a procedure described previously (21). It was confirmed that 16S rDNA nucleotide signatures typical of *N. asteroides* (2) were present between positions 591 and 648 in all test strains.

REP-PCR. Total genomic DNA was prepared from the 16 test *N. asteroides* strains by the method of Torres et al. (18) and used as a template in REP-PCR performed with the BOX-A1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′) (20). The reaction mixture (50 μl) contained 1× PCR buffer (Takara Shuzo Co., Ltd., Kyoto, Japan), a 2.5 mM concentration of each deoxynucleoside triphosphate (Takara), a 50 pM concentration of the BOX-A1R primer, 100 ng of genomic DNA, and 1 U of *Taq* polymerase. Amplification was performed in a Takara PCR Thermal Cycler PERSONAL as follows: after an initial denaturation step (95°C, 8 min), 30 cycles of denaturation (95°C, 1 min), annealing (53°C, 1 min) and extension (65°C, 4 min) were performed, followed by a single final extension (65°C, 8 min). PCR products were separated by horizontal gel electrophoresis on a 1.5% Seakem GTG agarose gel (FMC Bioproducts, Rockland, Maine) containing 0.5 μg of ethidium bromide/ml. The amplimers were visualized and photographed under UV light.

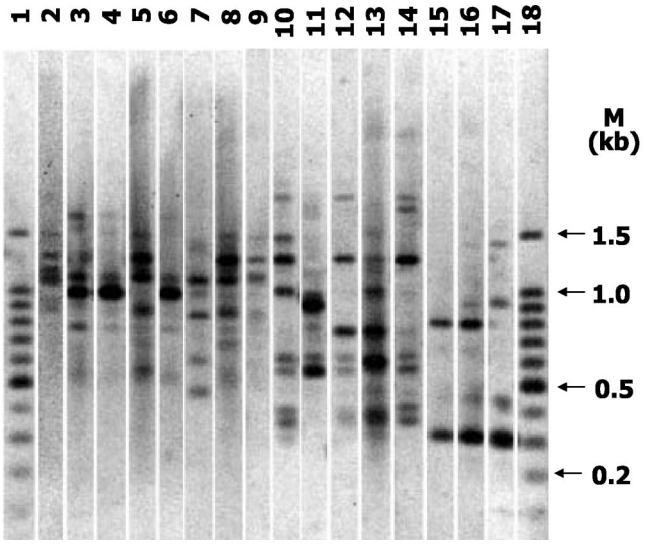

FIG. 1. Fingerprint patterns of *Nocardia asteroides* strains, obtained by REP-PCR with the BOX-A1R primer. Lanes: 1, 100-bp DNA ladder; 2, LS 001-3; 3, LS 001-4; 4, LS 002-1; 5, LS 002-2; 6, LS 002-5; 7, MS 002-1; 8, AS 004-5; 9, SO 031-3; 10, SO 008-1; 11, SO 008-2; 12, SO 020-4; 13, SO 026-3; 14, SO 027-1; 15, *N. asteroides* IFO 3384; 16, *N. asteroides* IFO 3424; 17, *N. asteroides* JCM 3384^T; 18, 100-bp DNA ladder.

Photographs of REP fingerprinting patterns were scanned, converted to digitized images, and analyzed by the fragment analysis software Diversity Database (*pdi*, Inc., New York, N.Y.). Figure 1 shows typical fingerprints for *N. asteroides* test isolates and reference strains, generated by REP-PCR with primer BOX-A1R. The profiles of the 16 strains revealed multiple DNA bands corresponding to sizes ranging from approximately 0.2 to 2.5 kb. The banding patterns were diverse and strain specific. However, it was evident that some of the strains are related to each other. Isolates from aquatic habitats (lake and moat sediments, as well as scum of activated sludge) showed a conserved major band at a position corresponding to a molecular size of about 1,085 bp. Most of the soil isolates showed major conserved bands at positions corresponding to molecular sizes of about 535 and 590 bp. Similarly, reference strains (a type strain and known clinical isolates) showed major conserved bands at about 230 and 485 bp. To determine the stability and reproducibility of DNA fingerprint patterns, DNA was extracted from three single-colony isolates of *N. asteroides* JCM 3384^T and analyzed by REP-PCR with the BOX-A1R primer. When the resulting patterns of DNA fragments were compared with that of the original *N. asteroides* JCM 3384^T culture, no significant difference in the DNA fingerprints was observed (data not shown). Similar results were obtained for other test *N. asteroides* strains, including SO 008-2 and SO 031-3.

Similarity between strains was determined by the Pearson product moment correlation coefficient with clustering by the unweighted pair group method with arithmetic mean

(UPGMA) algorithm. A dendrogram (Fig. 2), constructed by performing a cluster analysis of the BOX-A1R primer DNA fingerprint patterns with the Pearson similarity coefficient and UPGMA algorithm, produced three clusters (A, B, and C) defined at the 57% similarity level. Cluster A includes the type strain and two clinically related, known strains of *N. asteroides*. Cluster B is composed of five strains originating from soil. Cluster C includes all aquatic isolates and one soil isolate.

Data generated by the REP-PCR DNA fingerprint technique with the BOX-A1R primer in the present study show that genetic diversity exists among *N. asteroides* isolated from natural habitats and that *N. asteroides* isolates cluster by environment; most of the soil isolates and aquatic isolates of this taxon were assigned to separate groups (Fig. 2). Therefore, REP-PCR performed with the BOX-A1R primer could be useful for determining and characterizing *N. asteroides* strains endemic to specific natural habitats. This technique could also be useful for investigating an outbreak of *N. asteroides* infection. However, additional comparative studies involving larger numbers of judiciously selected environmental and virulent clinical *N. asteroides* strains are needed to devise methods for acute strain differentiation in epidemiological studies.

In conclusion, REP-PCR DNA fingerprint analysis can be used for differentiating *N. asteroides* isolates obtained from different ecological habitats. REP-PCR has already been successfully used to classify and differentiate strains of *E. coli* (4), *Bacillus sporothermodurans* (8), *Streptomyces* spp. (15), and several other bacteria (12), but the method has not been previously applied to *Nocardia* strain classification. REP-PCR DNA fingerprinting is simple and easy to perform and, thus, may prove to be a useful tool for rapidly determining *N. asteroides* strain identity and tracking specific strains for epidemiological and ecological studies.

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