Application of multiplex PCR using species-specific primers within the 16S rRNA gene for rapid identification of *Nocardioides* strains

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For the rapid identification of *Nocardioides* strains, multiplex PCR, using 16S rDNA as target gene, was used and its value was evaluated. Forward primers specific for *Nocardioides albus*, *Nocardioides jensenii*, *Nocardioides plantarum* and *Nocardioides simplex*, among the five validly described *Nocardioides* species, were designed from the alignment of 16S rDNA sequences. *Nocardioides luteus* has been shown to be a member of the same species as *N. albus* by recent molecular systematic studies and preliminary DNA–DNA relatedness tests. Therefore, *N. albus* and *N. luteus* were considered as members of the same species in this study. Each primer was found to be species-specific by specificity testing. *N. albus* NSP01^T, *N. jensenii* NSP19^T, *N. plantarum* NSP21^T and *N. simplex* NSP22^T could be clearly differentiated by PCR products characteristic for each species in the multiplex PCR assay. *N. luteus* gave an identical result to *N. albus* NSP01^T. The additional 17 strains of *N. albus* and the additional four strains of *N. simplex* gave PCR products identical to those of *N. albus* NSP01^T and *N. simplex* NSP22^T, respectively. Multiplex PCR was found to be rapid, species-specific and reproducible. The technique evaluated in this study proved to be effective for rapidly identifying *Nocardioides* strains to species level.

Keywords: multiplex PCR, 16S rRNA gene, rapid identification, *Nocardioides*

INTRODUCTION

The genus *Nocardioides* was proposed by Prauser (1976) for nocardioform actinomycetes that form a mycelium which fragments into irregular rod- to coccus-like elements. The organisms are Gram-positive, non-acid-fast, aerobic and mesophilic and have a cell wall based on LL-diaminopimelic acid, the diagnostic diamino acid of wall chemotype I (Prauser, 1976). However, the inclusion of previously described *Pimelobacter* species within the genus *Nocardioides* and the description of *Nocardioides plantarum* have rendered the genus morphologically heterogeneous (Collins *et al.*, 1989, 1994). Therefore, additional chemical markers are important to characterize the taxon. The genus *Nocardioides* has complex cellular fatty acid profiles containing iso-, anteiso-, straight chain, unsaturated and 10-methyl-branched acids. The major fatty acid characterizing the genus is iso-C_{16:0} (Miller *et al.*, 1991; O'Donnell *et al.*, 1982; Tamura & Yokota, 1994). The genus lacks mycolic acids (Prauser, 1976) and has a tetrahydrogenated menaquinone with

eight isoprene units [MK-8(H₄)] as the predominant isoprenoid quinone (O'Donnell *et al.*, 1982; Tamura & Yokota, 1994).

There are currently five validly described *Nocardioides* species, namely *Nocardioides albus* (Prauser, 1976), *Nocardioides luteus* (Prauser, 1984), *Nocardioides jensenii* (Collins *et al.*, 1989; Suzuki & Komagata, 1983), *Nocardioides plantarum* (Collins *et al.*, 1994) and *Nocardioides simplex* (O'Donnell *et al.*, 1982). *Nocardioides fastidiosa* was proposed by Collins & Stackebrandt (1989) but was recently transferred to the genus *Aeromicrobium* as *A. fastidiosum* by Tamura & Yokota (1994).

Recent developments in molecular biology have resulted in new methods that can be used for the identification of micro-organisms. In particular, the introduction of PCR methodology has provided a vast range of techniques for rapid identification or differentiation at species and intraspecies levels (Vandamme *et al.*, 1996). PCR-based methods, such as randomly amplified polymorphic DNA (RAPD; Welsh &

Table 1. Strains used in this study

Laboratory no.	Species	Source*
NSP01 ^T	<i>Nocardioides albus</i>	KCTC 9186 ^T
NSP02	<i>Nocardioides albus</i>	JCM 5851
NSP03	<i>Nocardioides albus</i>	JCM 5852
NSP04	<i>Nocardioides albus</i>	JCM 5853
NSP05	<i>Nocardioides albus</i>	JCM 5854
NSP06	<i>Nocardioides albus</i>	JCM 5855
NSP07	<i>Nocardioides albus</i>	JCM 5856
NSP08	<i>Nocardioides albus</i>	JCM 5857
NSP09	<i>Nocardioides albus</i>	JCM 5858
NSP10	<i>Nocardioides albus</i>	DSM 46107
NSP11	<i>Nocardioides albus</i>	JCM 5859
NSP12	<i>Nocardioides albus</i>	JCM 5860
NSP13	<i>Nocardioides albus</i>	JCM 5861
NSP14	<i>Nocardioides albus</i>	JCM 5862
NSP15	<i>Nocardioides albus</i>	JCM 5863
NSP16	<i>Nocardioides albus</i>	JCM 5864
NSP17	<i>Nocardioides albus</i>	DSM 43873
NSP18	<i>Nocardioides albus</i>	DSM 43874
NSP19 ^T	<i>Nocardioides jensenii</i>	KCTC 9134 ^T
NSP20 ^T	<i>Nocardioides luteus</i>	KCTC 9575 ^T
NSP21 ^T	<i>Nocardioides plantarum</i>	NCIMB 12834 ^T
NSP22 ^T	<i>Nocardioides simplex</i>	KCTC 9106 ^T
NSP23	<i>Nocardioides simplex</i>	JCM 1366
NSP24	<i>Nocardioides simplex</i>	JCM 1367
NSP25	<i>Nocardioides simplex</i>	NCIMB 12919
NSP27	<i>Nocardioides simplex</i>	ATCC 15799

* ATCC, American Type Culture Collection, Rockville, MD, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; JCM, Japan Collection of Micro-organisms, Institute of Physical and Chemical Research, Saitama, Japan; KCTC, Korean Collection for Type Cultures, Taejeon, Korea; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK.

McClelland, 1990; Williams *et al.*, 1990), amplified rDNA restriction analysis (Laguerre *et al.*, 1994; Yoon *et al.*, 1997a) and amplified fragment length polymorphism (AFLP; Zabeau & Vos, 1993), have attracted much interest because of their universal applicability, simplicity and rapidity. The combination of PCR techniques and synthetic primers designed from particular target genes have also made possible applications in rapid diagnosis and detection of pathogenic micro-organisms from natural samples (Persing *et al.*, 1993). Multiplex PCR is another method based on PCR and specific primers and has been employed for the differentiation and identification of bacterial strains (Bourque *et al.*, 1993; Geha *et al.*, 1994; Yoon *et al.*, 1996b). For instance, multiplex PCR using the 16S rRNA gene as the target was recently introduced and successfully applied to the rapid identification of *Saccharomonospora* strains (Yoon *et al.*, 1996b). The method proved itself to be species-specific, rapid and simple (Yoon *et al.*, 1996b).

In this study, 26 strains of the genus *Nocardioides* were analysed on the basis of multiplex PCR targeting of the

16S rRNA gene. The usefulness of multiplex PCR analysis for rapid identification of *Nocardioides* strains was examined.

METHODS

Bacterial strains. Table 1 summarizes the bacterial strains used in this study. All of the test strains were grown in shake flasks containing trypticase soy broth (BBL) supplemented with glucose (0.75%) at suitable temperatures. The broth cultures were checked for purity before they were harvested by centrifugation.

Selection of primers. The forward primers were selected from 16S rDNA sequences of *Nocardioides* species determined in this laboratory. The single reverse primer was a universal primer corresponding to the 3' end of 16S rDNA. Table 2 summarizes the DNA sequences and annealing positions for PCR primers used in this study.

Isolation of DNA. Chromosomal DNAs were isolated by the method described previously (Yoon *et al.*, 1996a).

PCR. PCR was performed in a final reaction volume of 50 µl and the reaction mixture contained 0.5 µM each primer, 200 µM each dNTP, 50 mM KCl, 10 mM Tris/HCl (pH

Table 2. Primers used in this study

Primer	Sequence (5'–3')	Position*	Characteristic
1fAL	ACCGGATACGACAACCGATT	174–193	Specific to <i>N. albus</i> and <i>N. luteus</i>
2fJE	TGTAAACCTCTTTTCAGCGGG	427–446	Specific to <i>N. jensenii</i>
3fPL	AGGCGGTTCTCTGGCAATGTT	729–749	Specific to <i>N. plantarum</i>
4fSI	ATAGGGGTCTCTTTGATACT	1016–1038	Specific to <i>N. simplex</i>
5rE	AGAAAGGAGGTGATCCAGCC	1542–1525	Universal

* *E. coli* 16S rRNA numbering.

8.3), 1.5 mM MgCl₂, 0.01 % gelatin and 1.25 U *Taq* DNA polymerase (Perkin Elmer). The conditions used for thermal cycling were as follows: denaturation of DNA at 94 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min and extension at 72 °C for 2 min. The final cycle included an additional extension time of 10 min at 72 °C to ensure full extension of the products. On completion of the reaction, each reaction tube was frozen at –70 °C for 10 min and then the mineral oil was removed with a pipette tip. The PCR products were analysed by electrophoresis of a 5 µl aliquot of the reaction mixture through a 1.5 % (w/v) agarose gel in Tris/acetate buffer (0.04 M Tris/acetate, 0.001 M EDTA, pH 8.3) at 100 V followed by staining with ethidium bromide and visualization of DNA bands by UV transillumination.

RESULTS AND DISCUSSION

By using the alignment of 16S rDNA sequences of *Nocardioides* species determined recently (Yoon *et al.*, 1998a), species-specific primers were designed. Species-specific signature nucleotides or sequence regions were determined from the alignment and were positioned at the 3' end of primers for PCR. Our recent 16S rDNA sequence analysis showed that *N. luteus* exhibited a high level of 16S rDNA similarity to strains of *N. albus*: 99.0 % to the type strain of *N. albus*, 100 % to *N. albus* JCM 5854 and 99.0–99.9 % to 16 other *N. albus* strains in the region corresponding to positions 28–1524 (*Escherichia coli* numbering). 16S–23S internally transcribed spacer sequence analysis has also shown that *N. luteus* is related to strains of *N. albus* (Yoon *et al.*, 1998b). *N. luteus*, first published as *Nocardioides* sp. IMET 7830, was differentiated from *N. albus* by DNA–DNA reassociation analysis and was therefore published as a new species of the genus *Nocardioides* (Prauser, 1984). However, it has been thought that the taxonomic relationship between *N. albus* and *N. luteus* should be re-evaluated from results of 16S rDNA and 16S–23S internally transcribed spacer sequence analyses. Preliminary DNA–DNA reassociation studies showed that *N. albus* strains and *N. luteus* have enough DNA–DNA relatedness to be described as one species, according to the current phylogenetic definition of a species (strains with approx. 70 % or greater DNA–DNA relatedness; Wayne *et al.*, 1987). Therefore, *N. albus* and *N. luteus* were considered as the same species in this study and primer 1fAL annealed at the same position in the 16S rDNAs of both species.

Each primer was tested for primer specificity and size differentiation of the PCR fragment specific for each species. The nucleotide sequences of the four forward primers differ from the corresponding 16S rDNA sequences of non-target species by only a few nucleotides positioned at the 3' end of primers. Therefore, primer specificity tests, which investigate whether each forward primer anneals at the corresponding sequence regions of non-target species, are an important step. The resultant data showed that each primer set generated species-specific PCR products and non-target species did not produce PCR products identical to those of target species (Fig. 1). As shown in Fig. 1, primer set 1fAL and 5rE from 16S rDNA yielded a 1353 bp and a 1352 bp PCR product for *N. albus* NSP01^T and *N. luteus* NSP20^T, respectively. However, the two PCR products cannot be differentiated by agarose gel electrophoresis (Fig. 1). Primer set 2fJE and 5rE yielded a 1099 bp PCR product for the identification of *N. jensenii* NSP19^T, 3fPL and 5rE yielded a 827 bp PCR product for *N. plantarum* NSP21^T, and 4fSI and 5rE yielded a 529 bp PCR product for *N. simplex* NSP22^T (Fig. 1). In this study, several differently sized non-specific background PCR products were generated from certain species other than the target species at which each primer set annealed and their presence could not be completely avoided despite changing the annealing temperature (Fig. 1). These bands, arising through undesired primer annealing, appear weak and differ in size from the species-specific PCR products generated by each primer set (Fig. 1). However, they did not create confusion in discrimination of species-specific PCR products. Moreover, these bands were not found in subsequent multiplex PCR assays which was the ultimate objective of this study (Fig. 2). Since it was shown that each forward primer did not anneal to the corresponding 16S rDNA regions of other non-target species, each primer was found to be species-specific.

For rapid identification, the multiplex PCR assay in which the species-specific forward primers and reverse primer 5rE were mixed in a single reaction tube was performed for the discrimination of members of the genus *Nocardioides*. *N. albus* NSP01^T, *N. luteus* NSP20^T, *N. jensenii* NSP19^T, *N. plantarum* NSP21^T and *N. simplex* NSP22^T were first used as representative strains. The multiplex PCR product profiles of

Fig. 1. Agarose gel electrophoresis of amplification products obtained from primer sets 1fAL and 5rE (a), 2fJE and 5rE (b), 3fPL and 5rE (c), and 4fSI and 5rE (d). Lanes: M, 100 bp DNA ladder (BRL); 1, *N. albus* NSP01^T; 2, *N. luteus* NSP20^T; 3, *N. jensenii* NSP19^T; 4, *N. plantarum* NSP21^T; 5, *N. simplex* NSP22^T.

Fig. 2. Agarose gel electrophoresis of amplification products obtained by multiplex PCR from representative strains of the genus *Nocardioide*s. Lanes: M, 100 bp DNA ladder (BRL); 1, *N. albus* NSP01^T; 2, *N. luteus* NSP20^T; 3, *N. jensenii* NSP19^T; 4, *N. plantarum* NSP21^T; 5, *N. simplex* NSP22^T; N, species-specific PCR products obtained from four primer sets electrophoresed together.

representative strains of *Nocardioide*s species gave the same results as when individual primer sets were used separately (Fig. 2). The multiplex PCR products of *N.*

albus NSP01^T and *N. luteus* NSP20^T appeared at the same positions (Fig. 2). *N. jensenii* NSP19^T, *N. plantarum* NSP21^T and *N. simplex* NSP22^T gave PCR products of 1099, 827 and 529 bp, respectively (Fig. 2). The representative strains only showed PCR products amplified by primers specific to each *Nocardioide*s species and the PCR products were clearly distinguishable from each other by their different sizes in agarose gel electrophoresis (Fig. 2). The lower part of the agarose gel shows extra non-specific primers which were not annealed to templates (Fig. 2).

Seventeen additional strains previously identified as *N. albus* and four strains previously identified as *N. simplex* were subjected to the multiplex PCR assay. Their multiplex PCR products were compared with those of their corresponding representative strains. The resultant data showed that the additional 17 strains of *N. albus* gave PCR products identical to that of *N. albus* NSP01^T (Fig. 3). The sizes of multiplex PCR products of the additional *N. albus* strains may be slightly different from that of *N. albus* NSP01^T and may differ from each other because of intraspecific sequence divergence. However, probable size differences are not large enough to be differentiated by agarose gel electrophoresis. The additional four strains

Fig. 3. Agarose gel electrophoresis of amplification products obtained by multiplex PCR from *N. albus* strains. Lanes: M, 100 bp DNA ladder (BRL); 1, NSP01^T; 2–18, NSP02–NSP18, respectively; N, species-specific PCR products obtained from four primer sets electrophoresed together.

Fig. 4. Agarose gel electrophoresis of amplification products obtained by multiplex PCR from *N. simplex* strains. Lanes: M, 100 bp DNA ladder (BRL); 1, NSP22^T; 2, NSP23; 3, NSP24; 4, NSP25; 5, NSP27; N, species-specific PCR products obtained from four primer sets electrophoresed together.

of *N. simplex* gave PCR products identical to that of *N. simplex* NSP22^T (Fig. 4). The type strain and the additional four strains of *N. simplex* used in this study were found to share identical 16S rDNA sequences in a recent study (Yoon *et al.*, 1998a). Therefore, our multiplex PCR assay was confirmed as being a species-specific method for the identification of *N. albus* and *N. simplex* strains. Other *N. simplex* strains are listed in the American Type Culture Collection (ATCC 13260, ATCC 19565 and ATCC 19566) but these were reclassified as *Rhodococcus erythropolis* in a previous taxonomic study (Yoon *et al.*, 1997b) and were not used in this study. Since *N. jensenii* and *N. plantarum* are represented by only one strain each, species specificity tests for these species were not performed.

Our multiplex PCR assay was found to be successful in the identification of *Nocardioides* species. However, *N. albus* strains and *N. luteus* exhibited identical multiplex PCR results (Fig. 2), but these have been shown to be

members of the same species by molecular systematic studies (Yoon *et al.*, 1998a, b) and a preliminary DNA–DNA relatedness test. When compared with previous methods, including classical approaches, the multiplex PCR method is very effective in terms of saving time and experimental effort. Recently, it has been reported that the amplification of 16S rDNA and multiplex PCR is possible using crude cell lysates extracted from a single colony or from a small quantity of bacterial cells (Frothingham *et al.*, 1991; Hiraishi *et al.*, 1994; Yoon *et al.*, 1996b). This is one of many approaches that make rapid identification possible, although such a trial was not performed in this study.

Bacterial identification to species level is time-consuming and difficult when compared with identification at genus level which is generally possible by studying colonial traits and chemotaxonomic characterization such as isoprenoid quinone composition, fatty acid composition, cell wall composition, etc. (Vandamme *et al.*, 1996). 16S rDNA sequences have been mainly used for phylogenetic identification and classification of bacterial strains (Stackebrandt *et al.*, 1997; Woese, 1987) and such sequencing is performed routinely. Nevertheless, multiplex PCR is thought to have advantages in terms of speed and cost when compared to 16S rDNA sequencing. Our multiplex PCR assay is very effective for rapidly identifying large numbers of *Nocardioides* strains. As discussed previously (Yoon *et al.*, 1996b), the use of the 16S rRNA gene is thought to provide many advantages when compared with other genes. The existence of variable regions together with highly conserved regions (Woese, 1987) and a large amount of information available from databases make the 16S rRNA gene valuable as a target gene for multiplex PCR.

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