Phylogenetic relationships of *Nocardiopsis* species based on partial *gyrB* and 16S rRNA gene sequences

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Partial nucleotide sequences of the *gyrB* gene were determined for members of the genus *Nocardiopsis* and subjected to phylogenetic analysis. Interspecies DNA similarity values of partial *gyrB* gene sequences ranged from 79.9 to 99.7% among the *Nocardiopsis* species. The average similarity of the *gyrB* gene (87.7%) was significantly less than that of the 16S rRNA gene (96.65%), indicating a high discriminatory power of the *gyrB* gene. The topology of neighbor-joining, maximum likelihood and maximum parsimony phylogenetic trees based on nucleic acid sequences and protein sequences of *gyrB* gene were reconstructed. Compared with the 16S rRNA gene dendrogram, the *gyrB* gene provided more information for understanding the genetic relationships among *Nocardiopsis* species. Using the information derived from 16S rRNA and *gyrB* genes, a more comprehensive understanding of the evolutionary history of *Nocardiopsis* species was obtained.

INTRODUCTION

On the basis of the characteristic development of spores (including the specific zigzag formation of aerial hypha before spore dispersal and the lack of madurose), Meyer proposed a new genus, *Nocardiopsis* (Meyer, 1987). This genus harbors 24 species with validly published names (Li *et al.*, 2006). Although *Nocardiopsis* species are distributed ubiquitously in the environment, most of them have been isolated from areas with a high salt concentration (Tang *et al.*, 2003). The taxonomic positions and designations of some species currently belonging to the genus *Nocardiopsis* have been subject to change, reflecting steps in the development of actinomycete classification (Kroppenstedt & Evtushenko, 2002). Using polyphasic approaches, it is not difficult to determine the phylogenetic and taxonomic positions of some new microorganisms. Use of a 16S rRNA gene catalogue could supply useful information rapidly and effectively, though this information might be unavailable in some circumstances. For example in the genus of *Nocardiopsis*, the taxonomic positions of some species were confirmed by DNA-DNA reassociation, because of the high identity of their 16S rRNA gene sequences (97% (Stackebrandt & Ebers, 2006)). The low interspecies polymorphism exhibited in the 16S rRNA gene sequence in a certain taxon (Clarridge, 2004; Schloss & Handelsman, 2004), and its multiple ribosomal RNA (*rrn*) operon copies appearing in a single bacterial genome (Acinas *et al.*, 2004), might result in false phylogenetic relationships between species. Protein-coding genes with a higher sequence substitution rate than that of the 16S rRNA gene and a single copy in the genome, were used to obtain

a better insight into interspecific phylogenies (Cohan, 2001; Evtushenko *et al.*, 2000). Phylogenetic dendrogram topology comparisons have been published by different authors (Li *et al.*, 2006; Evtushenko *et al.*, 2000; Sabry *et al.*, 2004; Hozzein *et al.*, 2004; Schippers *et al.*, 2002; Li *et al.*, 2003), revealing that the relationships of some *Nocardiopsis* species were not identical, especially when a low bootstrap value was obtained.

To reveal more precise genetic relationships between them, the single copy *gyrB* gene (B-protein subunit of DNA gyrase) was utilized. The main goal of this study was to determine the phylogenetic relationships of *Nocardiopsis* species from an alternative perspective.

MATERIALS AND METHODS

Bacterial stains and culture conditions

Bacterial strains used in this study are listed in Table 1. All strains were grown at 28 °C on GYM agar (0.4% glucose, 0.4% yeast extract, 1.0% malt extract and 0.2% CaCO₃), or on alkaline GYM agar (adjusting pH value to 9.0 with saturated NaOH solution) for alkaliphilic strains, or on saline GYM agar with 10% (w/v) NaCl for halophilic and halotolerant strains.

DNA extraction, PCR amplification and sequencing

Extraction of genomic DNA was performed as described by Li *et al.* (2007) PCR reactions with degenerate primers UP1/UP2r for *gyrB* (Yamamoto & Harayama, 1995) were performed in a final volume of 50 µl containing 0.2 mM each of the four dNTPs (TaKaRa), 0.1 µM of each primer, 1 µl extracted DNA and 0.5 U *Taq* polymerase (TaKaRa)

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Table 1. Strains of *Nocardiopsis* analyzed and accession numbers for the *gyrB* gene determined in this study.

Type strains	Accession numbers	
	16S rRNA	<i>gyrB</i>
<i>Nocardiopsis prasina</i> ATCC 35940 ^T	X97884	EF565425
<i>Nocardiopsis listeri</i> DSM 40297 ^T	X97887	EF565422
<i>Nocardiopsis exhalans</i> DSM 44407 ^T	AY036000	EF565417
<i>Nocardiopsis xinjiangensis</i> YIM 90004 ^T	AF251709	EF565433
<i>Nocardiopsis chromatogenes</i> YIM 90109 ^T	AY619715	EF565414
<i>Nocardiopsis rhodophaeos</i> YIM 90096 ^T	AY619714	EF565426
<i>Nocardiopsis kunsanensis</i> DSM 44524 ^T	AF195412	EF565421
<i>Nocardiopsis gilva</i> YIM 90087 ^T	AY619712	EF565418
<i>Nocardiopsis halophila</i> DSM 44494 ^T	AJ421018	EF565419
<i>Nocardiopsis metallicus</i> DSM 44598 ^T	AJ420769	EF565424
<i>Nocardiopsis aegyptia</i> DSM 44442 ^T	AJ539401	EF565411
<i>Nocardiopsis alba</i> DSM 43377 ^T	X97883	EF565412
<i>Nocardiopsis lucentensis</i> DSM 44048 ^T	X97888	EF565423
<i>Nocardiopsis trehalosi</i> DSM 44380 ^T	AF105972	EF565430
<i>Nocardiopsis synnemataformans</i> DSM 44143 ^T	Y13593	EF565429
<i>Nocardiopsis tropica</i> DSM 44381 ^T	AF105971	EF565431
<i>Nocardiopsis baichengensis</i> YIM 90130 ^T	AY619716	EF565413
<i>Nocardiopsis composta</i> DSM 44551 ^T	AF360734	EF565415
<i>Nocardiopsis umidischolae</i> DSM 44362 ^T	AY036001	EF565432
<i>Nocardiopsis halotolerans</i> DSM 44410 ^T	AJ290448	EF565420
<i>Nocardiopsis rosea</i> YIM 90094 ^T	AY619713	EF565427
<i>Nocardiopsis salina</i> YIM 90010 ^T	AY373031	EF565428
<i>Nocardiopsis dassonvillei</i> subsp. <i>dassonvillei</i> KCTC 9190 ^T	X97886	EF565416
<i>Nocardiopsis alkaliphila</i> YIM 80379 ^T	AY230848	EF565434

with its reaction buffer. The thermal profile involved initial denaturation for 4 min at 94 °C, 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 53 °C and extension for 2 min at 72 °C followed by a final extension at 72 °C for 10 min. Negative controls without template DNA were included for each PCR experiment. Amplification products were analyzed by electrophoresis (5 V cm⁻¹) in 1.5% (w/v) agarose gels stained with ethidium bromide and purified using a DNA fragment purification kit (TaKaRa). Cloning of PCR products was carried out in the pMD18-T (TaKaRa) cloning vector. The partial nucleotide sequence of *gyrB* was obtained using an automated DNA sequencer (model 377; Applied Biosystems) and software provided by the manufacturer.

Phylogenetic analysis

16S rRNA gene sequences of all *Nocardiopsis* species were downloaded from GenBank and used in the phylogeny reconstruction of the genus *Nocardiopsis*. Genetic distances were calculated using the Kimura two-parameter-model with complete deletion of all gaps. 16S rRNA gene sequences were aligned using CLUSTALX (Thompson *et al.*, 1997) and aligned regions with gaps removed before phylogenetic analysis. The ExPASy translate tool (http://sosnick.uchicago.edu/translate_dna.html) allowed us to translate the *gyrB* gene sequence to the corresponding

amino acid sequences. Hierarchical likelihood-ratio tests were conducted with a batch file supplied with Modeltest 3.7 (Posada & Hasegawa, 1998) to provide the evolutionary models used in neighbor-joining (NJ) and maximum-likelihood (ML) methods. Phylogenetic trees were obtained by using the NJ method, maximum-parsimony (MP) method (with heuristic search option) and the ML method with heuristic search option in PAUP* version 4.0b10 (Swofford, 2002). Genetic distances are Kimura two-parameter-model (Kimura, 1980) distances with a 2:1 of transition/transversion ratio calculated in MEGA 4 (Tamura *et al.*, 2007). Bootstrap analysis (Felsenstein, 1985) was made with 1000 replicates except in ML, where only 100 replicates were generated. The incongruence length difference (ILD) test (Farris *et al.*, 1995; 1000 randomizations of datasets) was performed using PAUP* version 4.0b10 program. The 16S rRNA (Accession number: CP000088 Region: 2390559-2392029) and *gyrB* genes (Accession number: CP000088 Region: 6950-8935) of *Thermobifida fusca* YX were used as an outgroup.

RESULTS AND DISCUSSION

Phylogenetic analysis of the 16S rRNA gene

Pairwise interspecies DNA similarity values of 16S rRNA gene sequences ranged from 93.6 to 99.86%. 41.3%

of them were more than or equal to 97%, which is the criterion for confirming the species affiliation in prokaryotes. The highest interspecies similarity, 99.9%, was found between *N. halophila* and *N. baichengensis*. However, the DNA-DNA relatedness was 55.9% (Li *et al.*, 2006). The similarity between *N. metallicus* and *N. exhalans* was 99.6% with 18.2% of DNA-DNA relatedness, and the DNA-DNA relatedness between *N. metallicus* and *N. prasina* was 44.1% (Schippers *et al.*, 2002), but the similarity of 16S rRNA gene sequences was 99.5%. The interspecies similarity between *N. synnemataformans* and *N. dassonvillei* subsp. *dassonvillei* was 99.4% with 45.5% of DNA-DNA relatedness (Yassin *et al.*, 1997).

Phylogenetic trees based on 16S rRNA gene were calculated using the NJ (Fig. 1A), MP and ML methods (not shown). In the NJ and ML analyses, the Tamura-Nei model (Tamura & Nei, 1993) with gamma (Γ) variation and a proportion of invariable sites (I) was used. The comparison of the topology of phylogenetic trees based on different methods showed some differences among them. The distinctions appeared at the branches which is supported by the low bootstrap values. The genetic position of *N. composta* changed remarkably. All genetic species can now be assigned to three main subclades, including 4 species which had not yet been assigned to exact phylogenetic positions (*N. halotolerans*, *N. aegyptia*, *N. alba* and *N. lucentensis*). Main subclade I contains *N. trehalosi*, which is the deepest branching species in *Nocardiopsis*. Main subclade II contains *N. composta*, *N. chromatogenes*, *N. baichengensis*, *N. halophila*, *N. gilva*, *N. rhodophaea* and *N. rosea*. All these species can grow on agar plates at NaCl concentrations of up to 18% (w/v, except *N. composta* which only can grow at NaCl concentrations of up to 15% and *N. halophila* which has a prerequisite of 3% NaCl concentration (Al-Tai & Ruan, 1994)), with well growth at 5–8%. The other *Nocardiopsis* species are included in main subclade III. *N. salina*, *N. kunsanensis* and *N. xinjiangensis* are halophilic species and formed a branch with high supporting evidence in main subclade III. The phylogenetic relationships among *Nocardiopsis* species in main subclade III were indistinct, although some species formed reliable neighbor relationships such as *N. tropica* and *N. umidischolae*; *N. listeri* and *N. alkaliphila*; *N. prasina*, *N. exhalans* and *N. metallicus*. Although the genetic positions of some species changed, in the 16S rRNA gene phylogeny, many of the main groups (with reliable neighbor relationships) receive bootstrap support above 70% in the NJ analysis, and most of them are reproduced in the MP and ML tree. Generally, groups that have short basal branches are less well supported than those groups with longer branches.

Phylogenetic analysis of the *gyrB* gene

Partial *gyrB* gene sequences (about 1160 bp) obtained in this study and their accession numbers are listed in Table 1. The *gyrB* gene sequences were aligned and imported into

MEGA to calculate genetic distances. Pairwise interspecies DNA similarity values of *gyrB* gene sequences ranged from 79.9 to 99.7%. In these similarity values, 96.4% of them were less than or equal to 95%. The variability of *gyrB* gene is greater than that of 16S rRNA gene although there are several *Nocardiopsis* species exhibiting higher similarity of *gyrB* gene with their phylogenetic neighbors. For example, the *gyrB* gene sequence similarities between *N. halophila* and *N. baichengensis*, *N. metallicus* and *N. exhalans*, *N. synnemataformans* and *N. dassonvillei* subsp. *dassonvillei* were 98.9%, 98.08% and 97.54% respectively. Besides the DNA-DNA relatedness, the differences between *N. halophila* and *N. baichengensis* also appeared in the major menaquinones and NaCl concentration range for growth. The remarkable characteristic of *N. metallicus*, differentiating it from other *Nocardiopsis* species, is the ability to dissolve metals from siliceous, alkaline dump material (Willscher & Bosecker, 2001). *N. synnemataformans* differs from *N. dassonvillei* subsp. *dassonvillei* by its ability to utilize rhamnose and maltose and its inability to utilize trehalose and sucrose as carbon sources (Yassin *et al.*, 1997). The highest interspecies similarity, 99.7%, was found between *N. umidischolae* and *N. tropica* (16S rRNA gene similarity of 99.2%). Although the difference can not be found at a genetic level between *N. umidischolae* and *N. tropica*, the main evidence for description of *N. umidischolae* as a novel species was phenotypic differentiation (Peltola *et al.*, 2001).

Phylogenetic trees based on *gyrB* gene sequences were reconstructed by using the NJ (Fig. 1B), MP and ML methods (not shown). Some branches were supported by higher bootstrap values than the 16S rRNA gene. The F81 model (Felsenstein, 1981) with gamma (Γ) variation and a proportion of invariable sites (I) was used in NJ and ML analysis. Hierarchical likelihood-ratio tests were performed in the selection of the evolutionary model and in the optimization of parameters such as base frequencies, substitution model and among-site rate variation. The MP and ML trees shared an identical topological structure. The comparison of the topology of phylogenetic trees reconstructed by NJ and MP methods (or ML methods) showed that there are some differences among them. Compared with the 16S rRNA gene, phylogenetic relationships between the outgroup (*Thermobifida fusca* YX) and main subclade I (*N. trehalosi*) was so close that the function of the outgroup could not be exhibited in *gyrB* gene trees. In the main subclade II, the *gyrB* gene seems to give more factual resolution for *N. composta*. The spore chains, colony pigmentation on ISP 5, and DNA G+C content (mol %) of *N. composta* differentiate it from *N. gilva*, *N. rhodophaea* and *N. rosea* (Li *et al.*, 2006). These characteristics made *N. composta* exhibit a closer relationship with *N. chromatogenes*, *N. baichengensis* and *N. halophila*.

Nucleotide substitution which results in amino acid substitution is less frequent than synonymous substitution.

Fig. 1. Phylogenetic trees showing the genetic relationships among *Nocardiopsis* species. Strain numbers and sequence accession numbers are provided in Table 1. Bootstrap values (>50%) from 1000 analyses are shown at the nodes of the tree. Bar, 5 nucleotide substitutions per 100 nucleotides of 16S rRNA gene and *gyrB* gene sequences. A: 16S rRNA gene tree; B: *gyrB* gene tree; C: tree based on GyrB sequence. D: tree based on concatenated sequences of *gyrB* and 16S rRNA gene. All trees shown here were reconstructed using the NJ method.

The amino acid substitution may be influenced by the function of the protein directly, and each protein can be expected to have a rate of amino acid substitution differentiated from that of nucleotide substitution. The branching order of the GyrB-based tree (Fig. 1C) was different from that based on the *gyrB* nucleotide sequences especially in subclade III. However, the GyrB-based tree shared many

similarities with the tree based on concatenated sequence described below.

Concatenated sequence analyses

The *P* value generated from the ILD analysis was 0.02, which indicated that combination of the data would improve the phylogenetic accuracy. As a result, the

concatenated tree (Fig. 2D, made by MEGA 4) would give us a more comprehensive understanding of the phylogenetic relationships between *Nocardiopsis* species. Most of branches are supported by high bootstrap values in this concatenated tree. The phylogenetic position of *N. trehalosi*, which is the most ancient species of the genus *Nocardiopsis*, cannot be doubted. Moreover, the relationship between *N. trehalosi* and *T. fusca* is subtle and remarkable. Main subclade II includes *N. rhodophaea*, *N. rosea*, *N. gilva*, *N. composta*, *N. chromatogenes*, *N. baichengensis* and *N. halophila*. *N. composta*'s membership of this cluster was supported by other information, such as growth on agar plates at NaCl concentration of 15% (w/v). The phylogenetic positions of most of the species assigned to main subclade III can be determined in the concatenated tree. However, bootstrap values present on some nodes were still less than 50%. Subsequently, the genetic statuses of species located at the end of these branches have yet to be confirmed. Genetic information derived from another conserved housekeeping gene would be needed to resolve this problem.

The genus-specific rate of evolution of the 16S rRNA gene prevents its use in the determination of the precise branching order and in the evaluation of the evolutionary rates at genomic level. In the case of phylogenetic analysis of the genus *Nocardiopsis*, the *gyrB* gene proved useful due to its higher evolutionary rate. Use of the 16S rRNA gene cannot be abandoned since its low divergence facilitates the finding of universal PCR primers. Operational flexibility is one of the main reasons for the wide use of the 16S rRNA gene. The higher divergence of protein-coding genes makes finding suitable primers more difficult, further work will be necessary to identify an ideal clade.

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