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Genetic analyses of the genus *Nocardioides* and related taxa based on 16S–23S rDNA internally transcribed spacer sequences

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The 16S–23S internally transcribed spacer (ITS) sequences were analysed to clarify inter- and intraspecific relationships among strains of the genus *Nocardioides* and the relationship between two *Aeromicrobium* species. The 16S–23S ITS regions from 33 *Nocardioides* strains, two *Aeromicrobium* species and *Terrabacter tumescens* were sequenced directly after polymerase chain reaction (PCR) amplification and λ exonuclease treatment. The genomes of some *Nocardioides* strains included two types of 16S–23S ITS sequences. The sizes of the 16S–23S ITS sequences of *Nocardioides* strains ranged from 328 to 539 bp. The 16S–23S ITS sequences of *Aeromicrobium erythreum* NSP37^{T}, *Aeromicrobium fastidiosum* NSP38^{T} and *T. tumescens* NSP39^{T} were 349, 355 and 386 bp long, respectively. Nucleotide similarity among 16S–23S ITS sequences of *Nocardioides albus* strains and of *Nocardioides simplex* strains was 84·1–100% and 97·7–100%, respectively. The 16S–23S ITS sequence of *Nocardioides luteus* was identical to that of '*Nocardioides fulvus*' NSP32^{T} and was only 1 bp different from that of '*Nocardioides flavus*' strains. However, the 16S–23S ITS sequences of '*N. fulvus*' NSP33 showed only a low degree of similarity to '*N. fulvus*' NSP32^{T} (54·8%). The degree of 16S–23S ITS similarity between *N. luteus* NSP20^{T} and *N. albus* strains ranged from 85 to 93%. The mean nucleotide similarity values between the type strains of validly described *Nocardioides* species were highly divergent at 68·1 16·8%. The two *Aeromicrobium* species showed a level of 16S–23S ITS similarity of 71·2%. In this study, 16S–23S ITS sequences of the members of the genera *Nocardioides* and *Aeromicrobium* were useful for inferring the relationships between closely related strains and species. However, they were not found to be appropriate for elucidating the phylogenetic relationships between distantly related organisms at the genus level.

Keywords: *Nocardioides*, rDNA, internally transcribed spacer

INTRODUCTION

The genus *Nocardioides* was proposed by Prauser (1976) for nocardioform actinomycetes containing LL-diaminopimelic acid, alanine, glutamic acid and glycine in the cell wall peptidoglycan (wall chemotype I; see Lechevalier & Lechevalier, 1970). The original type species of the genus is *Nocardioides albus* (Prauser, 1976). This organism is Gram-positive, non-acid-fast, aerobic and mesophilic and forms a mycelium that fragments into irregular rod- to coccus-like elements (Prauser, 1976). Several *Nocardioides* species, such as *Nocardioides jensenii*, *Nocardioides plantarum* and *Nocardioides simplex*, which have subsequently been reclassified and identified as members of the genus *Nocardioides*, do not form mycelia (O'Donnell *et al.*, 1982; Suzuki & Komagata, 1983; Collins *et al.*, 1989, 1994). The morphological heterogeneity found in the genus *Nocardioides* makes it necessary to use chemical markers to characterize the genus. The chemical markers that characterize the genus *Nocardioides* have

Abbreviation: ITS, internally transcribed spacer.

been described elsewhere (Prauser, 1976; O'Donnell *et al.*, 1982; Miller *et al.*, 1991; Collins *et al.*, 1994; Tamura & Yokota, 1994; Yoon *et al.*, 1997a).

There are currently five validly described *Nocardioides* species, namely *N. albus* (Prauser, 1976), *N. luteus* (Prauser, 1984), *N. jensenii* (Suzuki & Komagata, 1983; Collins *et al.*, 1989), *N. plantarum* (Collins *et al.*, 1994) and *N. simplex* (O'Donnell *et al.*, 1982; Suzuki & Komagata, 1983). In addition, there are three invalidly described *Nocardioides* species, namely '*Nocardioides flavus*' (Ruan & Zhang, 1979), '*Nocardioides fulvus*' (Ruan & Zhang, 1979) and '*Nocardioides thermolilacinus*' (Lu & Yan, 1983). The two strains of '*N. thermolilacinus*' display a *Streptomyces*-like life cycle, susceptibility to *Streptomyces* phages and lack of susceptibility to *Nocardioides* phage and are, hence, regarded as streptomycetes (Prauser, 1989). Our recent 16S rDNA sequence analysis also showed that the two strains are members of the genus *Streptomyces* (Yoon *et al.*, 1998). *Nocardioides fastidiosa* (Collins & Stackebrandt, 1989) has recently been transferred to the genus *Aeromicrobium* as *Aeromicrobium fastidiosum* based on chemotaxonomic characterization and the 16S rDNA sequence (Tamura & Yokota, 1994).

The genus *Aeromicrobium* was proposed by Miller *et al.* (1991) for *Arthrobacter* sp. NRRL B-3381 (French *et al.*, 1970). This organism differs in many ways from *Arthrobacter globiformis*, the type species of the genus *Arthrobacter*, and was therefore reclassified as a new taxon, the genus *Aeromicrobium* as *Aeromicrobium erythreum* sp. nov., based on additional taxonomic data (Miller *et al.*, 1991). The second *Aeromicrobium* species is *Aeromicrobium fastidiosum* (Tamura & Yokota, 1994). Thus, there are currently two *Aeromicrobium* species. The genus *Aeromicrobium* has a cell wall type based on LL-diaminopimelic acid (wall chemotype I; see Lechevalier & Lechevalier, 1970). The additional chemical markers that characterize the genus *Aeromicrobium* have been described elsewhere (Miller *et al.*, 1991; Tamura & Yokota, 1994). The genus *Aeromicrobium* has been included in the family *Nocardioidaceae* together with the genus *Nocardioides*, the type genus of the family (Stackebrandt *et al.*, 1997).

Recently, 16S rDNA sequence analysis has shown that *N. luteus* is closely related to the *N. albus* strains (Yoon *et al.*, 1998). *N. luteus* 16S rDNA exhibits 100% similarity to *N. albus* strain JCM 5854 and 99·0–99·9% similarity to the other 17 *N. albus* strains in the region corresponding to positions 28–1524 by comparison with 16S rRNA of *Escherichia coli* (Brosius *et al.*, 1991). According to 16S rRNA or rDNA sequences and DNA–DNA reassociation data (Stackebrandt & Goebel, 1994), strains belonging to the same species normally exhibit similarity levels of 16S rRNA or rDNA of more than 97%. Thus, taxonomic relationships between *N. albus* strains and *N. luteus* should be re-evaluated in detail. Moreover, '*N. flavus*' strains IFO 14396^T and IFO 14397 and '*N. fulvus*' JCM 3335^T show 100% 16S rDNA similarity to *N. luteus*. '*N. flavus*' IFO 14396^T has been assumed to be a synonym for *N. luteus* (Prauser, 1989), but the taxonomic position of '*N. fulvus*' strains has not been clearly defined. The mean intraspecific 16S rDNA similarities of *N. albus* and *N. simplex* were $99{\cdot}5 \pm 0{\cdot}5\%$ and 100%, respectively. *Nocardioides*-like isolate NSP41 has been shown to be related to *N. simplex* with a 16S rDNA similarity value of 98·6%. However, 16S rDNA similarity of 100% found between existing *N. simplex* strains makes the identification of isolate NSP41 problematic. Although DNA–DNA relatedness constitutes the most important criterion for determining the exact taxonomic relationships between closely related strains and species (Stackebrandt & Goebel, 1994), additional taxonomic markers also may be necessary for polyphasic taxonomic studies (Vandamme *et al.*, 1996).

16S rDNA and 16S rRNA sequencing is a powerful method for elucidating phylogenetic relationships among prokaryotic organisms (Woese, 1987; Stackebrandt *et al.*, 1997) and has been used to determine inter- and intraspecific relationships (Yoon *et al.*, 1998). Nevertheless, 16S rDNA/16S rRNA sequences may be insufficient to define phylogenetic relationships among closely related species and among strains belonging to a species because of the evolutionary conservation of the molecule (Woese, 1987). In this study, we determined the nucleotide sequences of 16S–23S rDNA (16S–23S) internally transcribed spacer (ITS) sequences as a more variable genetic marker for assessing the phylogenetic relationships of the genus *Nocardioides* and related taxa. Recently, sequencing (Leblond-Bourget *et al.*, 1996; Zavaleta *et al.*, 1996; Yoon *et al.*, 1997b), restriction analysis (Navarro *et al.*, 1992) and molecular typing (Dolzani *et al.*, 1994) of 16S–23S ITS regions have been shown to be useful in inferring the phylogenetic relationships between closely related organisms (Gürtler & Stanisich, 1996). The 16S–23S ITS region can be a useful target for polyphasic taxonomic analysis (Vandamme *et al.*, 1996). The sizes of 16S–23S ITS regions in many organisms are not conserved, unlike those of 16S rDNA/rRNA molecules (Gürtler & Stanisich, 1996; Leblond-Bourget *et al.*, 1996; Yoon *et al.*, 1997b). This property may also be useful for discriminating between genera, between species and between strains of a given species.

The aim of this study was to investigate intra- and interspecific relationships of the genus *Nocardioides* and between two *Aeromicrobium* species based on 16S–23S ITS sequences. In particular, the 16S–23S ITS sequence was thought to be helpful for studying the phylogenetic relationships between closely related strains and species of the genus *Nocardioides*. An additional objective was to investigate whether it is possible to clarify intergeneric relationships between the genera *Nocardioides* and *Aeromicrobium*, which are in fact phylogenetically related to each other despite differences in chemotaxonomic characteristics, by sequence difference and size differentiation of 16S–23S

ITS sequences. The ITS sequences determined in this study were also compared with those of representative members of other genera to investigate whether ITS sequences are appropriate for inferring phylogenetic relationships between distantly related organisms.

METHODS

Organisms and culture conditions. The strains used in this study are listed in Table 1. All strains used in this study were grown in shake flasks containing tryptone soy broth supplemented with glucose (0·75%, w/v) at temperatures appropriate for each strain. The broth cultures were checked for purity before they were harvested by centrifugation.

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

PCR amplification and sequencing of 16S–23S ITS regions. The primers for amplification of the DNA fragment containing the 16S–23S ITS region have been described previously (Yoon *et al.*, 1997b). The sequences of the oligonucleotide primers annealing to the 16S rDNA and 23S rDNA regions were 5′-CAGCMGCCGCGGTAATSC-3′ [primer 16SF, positions 519–536 (*Escherichia coli* 16S rRNA numbering)] and 5′-AGGCATCCACCGTGCGCCCT-3′ [primer 23SR, positions 34–14 (*E. coli* 23S rRNA numbering)], respectively. The 5′ end of primer 16SF was phosphorylated by using T4 polynucleotide kinase (New England Biolabs). PCR was performed as described previously (Yoon *et al.*, 1997b, c) using phosphorylated primer 16SF and primer 23SR. The PCR product was recovered by precipitation with 2-propanol, and the strand containing phosphorylated primer from the PCR product was selectively digested using λ exonuclease according to the instructions included with the Strandase ssDNA preparation kit (Novagen). Sequencing of the 16S–23S ITS region was performed with the ssDNA template produced as described previously (Kim *et al.*, 1995). The primers used to sequence the 16S–23S ITS region include several primers designed from 16S–23S ITS regions in addition to universal primers within 16S rDNA, namely 1392F [5′-GYACACACCGCCCGT-3′, positions 1392–1406 (*E. coli* 16S rRNA numbering)] (Lane *et al.*, 1985) and 1525F [5′-GGCTGGATCACCTCCTTTCT-3′, positions 1525–1542 (*E. coli* 16S rRNA numbering)] (Stackebrandt & Liesack, 1993), which have been described previously.

Cloning and sequencing of the 16S–23S ITS region from *Nocardioides* sp. NSP36. The PCR product of the 16S–23S ITS region for cloning was amplified by using a primer annealing to the 3′ end of 16S rDNA, primer 1525F and primer 23SR. Cloning and sequencing of the PCR product was performed using the methods described previously (Sambrook *et al.*, 1989; Kim *et al.*, 1995). The sequence of the 16S–23S ITS region was determined using three to four clones.

Data analysis. The sequences of the 16S–23S ITS sequences determined in this study were aligned by using CLUSTAL W software (version 1.7) (Thompson *et al.*, 1994). The 16S–23S ITS sequences of representatives strains or all strains of the genus *Nocardioides*, two *Aeromicrobium* species and *Terrabacter tumescens* were aligned with those of some other taxa whose 16S–23S ITS sequences have been determined. Reference sequences used in this study were obtained from the GenBank databases, where the nucleotide sequences are available under the following accession numbers: M27245 (*Streptomyces ambofaciens*), M76388 (*Streptomyces griseus* subsp. *griseus*), M20148 (‘*Streptomyces lividans*’), M55343 (*Frankia* sp.), U09517 (*Bifidobacterium bifidum*) and U09520 (*Bifidobacterium breve*). The nucleotide similarity values were calculated from the alignment, and a tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) from a distance matrix calculated with CLUSTAL W (Thompson *et al.*, 1994).

RESULTS AND DISCUSSION

The DNA fragments containing the 16S–23S ITS regions from 33 strains of the genus *Nocardioides*, two *Aeromicrobium* species and *Terrabacter tumescens* were amplified by PCR using two primers designed from the conserved regions of 16S and 23S rDNAs. All of the strains produced one PCR product for the 16S–23S ITS-containing DNA fragment as determined by agarose gel electrophoresis of PCR amplicons (data not shown). The presence of one PCR product for the 16S–23S ITS has been described for some *Bifidobacterium* species (Leblond-Bourget *et al.*, 1996) and *Saccharomonospora* species. However, the 16S–23S ITS sequences of some strains used in this study were found to differ in terms of size and sequence from *rrn* loci, as determined by subsequent sequence analysis. Variation in the length in 16S–23S ITS regions due to heterogeneity among rDNA alleles of a given strain has been found in some organisms (Gürtler & Stanisich, 1996; Hain *et al.*, 1997). In particular, the 16S–23S ITS sequences of *Staphylococcus aureus* and *Haemophilus influenzae*, for which the sequence of complete sets of rRNA operons has been determined, vary from 303 to 551 bp and from 478 to 723 bp, respectively (Gürtler & Stanisich, 1996). The heterogeneity of size and number of 16S–23S ITS sequences of rDNA alleles has been used to discriminate between strains of some *Streptomyces* species (Hain *et al.*, 1997). PCR products of heterogeneous size and sequence present in a single PCR amplicon can yield unreadable mixed sequence data when PCR products of the 16S–23S ITS region are sequenced directly.

The nucleotide sequences of the 16S–23S ITS regions have been determined by directly sequencing non-phosphorylated single strands of the PCR amplicon obtained using λ exonuclease, which selectively digests 5′-phosphorylated strands of double-stranded DNA fragments. The 5′ ends of the 16S–23S ITS regions of strains used in this study were determined from the 3′ ends of the 16S rDNAs of *Bacillus subtilis* (Green *et al.*, 1985), *Frankia* sp. (Normand *et al.*, 1992) and some *Streptomyces* species (Baylis & Bibb, 1988; Suzuki & Yamada, 1988; Suzuki *et al.*, 1988; Pernodet *et al.*, 1989; Van Wezel *et al.*, 1991; Kim *et al.*, 1993). The 3′ ends of the 16S–23S ITS regions were deduced by comparison with the similar sequence of the 5′ end regions of the 23S rDNAs of *Bacillus subtilis* (Green *et al.*, 1985), *Frankia* sp. (Normand *et al.*, 1992) and some *Streptomyces* species (Baylis & Bibb, 1988; Suzuki *et*

Table 1. Strains used in this study, and sizes and accession numbers for 16S–23S ITS sequences

Lab. no.	Species	Source*	Size (bp)	Accession no.
NSP01^T	*Nocardioides albus*	KCTC 9186^T	514	AF017470
NSP02	*Nocardioides albus*	JCM 5851	499	AF017471 (*rrn* 1)
			500	AF017472 (*rrn* 2)
NSP03	*Nocardioides albus*	JCM 5852	513	AF017473
NSP04	*Nocardioides albus*	JCM 5853	468	AF017474
NSP05	*Nocardioides albus*	JCM 5854	520	AF017475
NSP06	*Nocardioides albus*	JCM 5855	512	AF017476
NSP07	*Nocardioides albus*	JCM 5856	512	AF017477
NSP08	*Nocardioides albus*	JCM 5857	533	AF017478
NSP09	*Nocardioides albus*	JCM 5858	510	AF017479
NSP10	*Nocardioides albus*	DSM 46107	509	AF017480
NSP11	*Nocardioides albus*	JCM 5859	514	AF017481
NSP12	*Nocardioides albus*	JCM 5860	507	AF017482
NSP13	*Nocardioides albus*	JCM 5861	539	AF017483
NSP14	*Nocardioides albus*	JCM 5862	509	AF017484 (*rrn* 1)
				AF017485 (*rrn* 2)
NSP15	*Nocardioides albus*	JCM 5863	531	AF017486
NSP16	*Nocardioides albus*	JCM 5864	509	AF017487
NSP17	*Nocardioides albus*	DSM 43873	505	AF017488
NSP18	*Nocardioides albus*	DSM 43874	511	AF017489 (*rrn* 1)
			523	AF017490 (*rrn* 2)
NSP19^T	*Nocardioides jensenii*	KCTC 9134^T	344	AF017491
NSP20^T	*Nocardioides luteus*	KCTC 9575^T	473	AF017492
NSP21^T	*Nocardioides plantarum*	NCIMB 12834^T	438	AF017493
NSP22^T	*Nocardioides simplex*	KCTC 9106^T	386	AF017494
NSP23	*Nocardioides simplex*	JCM 1366	386	AF017495
NSP24	*Nocardioides simplex*	JCM 1367	386	AF017496
NSP25	*Nocardioides simplex*	NCIMB 12919	387	AF017497 (*rrn* 1)
			388	AF017498 (*rrn* 2)
NSP27	*Nocardioides simplex*	ATCC 15799	386	AF017499
NSP30^T	'*Nocardioides flavus*'	IFO 14396^T	473	AF017500
NSP31	'*Nocardioides flavus*'	IFO 14397	473	AF017501
NSP32^T	'*Nocardioides fulvus*'	JCM 3335^T	473	AF017502
NSP33	'*Nocardioides fulvus*'	IFO 14399	473	AF017503
NSP36	*Nocardioides* sp.	ATCC 39419	429	AF017504 (*rrn* 1)
			439	AF017505 (*rrn* 2)
NSP40	'*Nocardioides pyridinolyticus*'	OS4, S.-K. Rhee	392	AF017506
NSP41	*Nocardioides*-like isolate	Y. K. Cho	328	AF017507
NSP37^T	*Aeromicrobium erythreum*	NRRL B-3381^T	349	AF017508
NSP38^T	*Aeromicrobium fastidiosum*	KCTC 9576^T	355	AF017509
NSP39^T	*Terrabacter tumescens*	KCTC 9133^T	386	AF017510

* KCTC, Korean Collection for Type Cultures, Taejon, Korea; JCM, Japan Collection of Microorganisms, Institute of Physical and Chemical Research, Saitama, Japan; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; NCIMB, National Collection of Industrial and Marine Bacteria, NCIMB, Aberdeen, UK; ATCC, American Type Culture Collection, Rockville, MD, USA; IFO, Institute for Fermentation, Osaka, Japan; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA.

al., 1988; Pernodet *et al.*, 1989; Kim *et al.*, 1993). The lengths of the 16S–23S ITS sequences of the *Nocardioides* strains determined ranged from 328 bp in *Nocardioides*-like isolate NSP41 to 539 bp in *N. albus* NSP13 (Table 1). Large size differences between 16S–23S ITS sequences from different strains have also been found in some *Bifidobacterium* species (Leblond-Bourget *et al.*, 1996), with 16S–23S ITS sequences from some strains being only half the size of those found in other strains belonging to the same species.

The size variation of 16S–23S ITS sequences, together with sequence divergence, can be useful in discriminating between strains belonging to the same species and between species belonging to the same genus, especially in the case of closely related organisms. The lengths of the 16S–23S ITS sequences of *N. simplex* strains showed a small intraspecific variation of 386–388 bp when compared with *N. albus* strains (Table 1). The 16S–23S ITS regions of *N. jensenii* NSP19^T, *N. luteus* NSP20^T and *N. plantarum* NSP21^T were 344, 473 and 438 bp, respectively (Table 1). The 16S–23S ITS sequences of '*N. flavus*' strains NSP30^T and NSP31 and '*N. fulvus*' NSP032^T were the same size as those of *N. luteus* NSP20^T (Table 1). *N. albus* NSP05, *N. luteus* NSP20^T, '*N. flavus*' strains NSP30^T and NSP31 and '*N. fulvus*' NSP032^T were shown to have the same 16S rDNA sequence according to our recent 16S rDNA sequence analysis (Yoon *et al.*, 1998). However, the 16S–23S ITS of *N. albus* NSP05 was 47 bp longer. The 16S–23S ITS sequences of *A. erythreum* NSP37^T and *A. fastidiosum* NSP38^T were 349 and 355 bp long, respectively, which is smaller than those of *Nocardioides* strains except *N. jensenii* NSP19^T and *Nocardioides* sp. NSP41 (Table 1). The length of the 16S–23S ITS region of *T. tumescens* NSP39^T was 386 bp (Table 1).

Sequence analysis revealed that some *Nocardioides* strains used in this study displayed heterogeneity in the 16S–23S ITS sequences between rRNA gene clusters. From direct sequencing of the 16S–23S ITS region, unreadable mixed sequence data were produced above position 237 of the 16S–23S ITS sequence for *N. albus* NSP02, above position 124 for *N. albus* NSP18, between positions 169 and 173 and above position 183 for *N. simplex* NSP25 and above position 15 for *Nocardioides* sp. NSP36. However, *N. albus* NSP14 yielded unreadable mixed sequence data only between positions 373 and 393 of the 16S–23S ITS sequence, which indicates that the region exhibits sequence divergence but not size divergence between rDNA alleles. When we performed sequence analysis of several clones containing 16S–23S ITS sequences to investigate the extent of heterogeneity of 16S–23S ITS sequences from the five strains, all five strains were found to have two types of 16S–23S ITS sequences. In our previous ribotyping study, all of the *Nocardioides* strains and two *Aeromicrobium* species used in this study were shown to have two copies of rDNA alleles on their genomes (data not shown). Thus, *N. albus* strains NSP02, NSP14 and NSP18, *N. simplex* NSP25 and *Nocardioides* sp. NSP36 were thought to have two types of 16S–23S ITS sequences derived from the two rRNA gene clusters. However, in our study, the sequence and size divergence of two rRNA gene clusters of the five *Nocardioides* strains were not investigated in two *rrn* loci distinguished by the use of restriction endonucleases. The rRNA gene cluster containing the smaller 16S–23S ITS and the rRNA gene cluster containing the larger 16S–23S ITS from *N. albus* strains NSP02 and NSP18, *N. simplex* NSP25 and *Nocardioides* sp. NSP36 were designated *rrn* 1 and *rrn* 2, respectively. In *N. albus* NSP14, the rRNA gene cluster with a 16S–23S ITS containing the nucleotide sequence GGCTGTATGGGTTTACCTGTC and the rRNA gene cluster with a 16S–23S ITS containing the nucleotide sequence of AGCCGTGGGTTTAGGCC-TGGT between positions 373 and 393 were designated *rrn* 1 and *rrn* 2, respectively. *N. albus* NSP02 was found to have 16S–23S ITS sequences that were 499 bp long (*rrn* 1) and 500 bp long (*rrn* 2), and the 16S–23S ITS sequence of *rrn* 2 was identical to that of *rrn* 1 except that it contained an additional G nucleotide between positions 236 and 237 of the *rrn* 1 16S–23S ITS. *N. albus* NSP18 had two types of 16S–23S ITS sequences which differed in size by 12 bp (Table 1). The larger 16S–23S ITS of *N. albus* NSP18 (*rrn* 2) differed in size and sequence from the smaller one (*rrn* 1) between positions 124 and 129, resulting in a nucleotide similarity level of 99·6 % and a nucleotide similarity level, with gaps included, of 97·3 %. *N. simplex* NSP25 had two types of 16S–23S ITS, which show sequence divergence between positions 169 and 173, and the larger type (*rrn* 2) of 16S–23S ITS contains an additional A nucleotide between positions 183 and 184 of the smaller type (*rrn* 1). The nucleotide similarity and the nucleotide similarity level with gaps included between two types of 16S–23S ITS sequences of *N. simplex* NSP25 are 98·4 and 98·2 %, respectively. It was interesting that the two types of 16S–23S ITS sequences found in *Nocardioides* sp. NSP36 differed in size by 10 bp and showed high sequence divergence, with the nucleotide similarity level being 80·8 % (Table 2) and 78·4 % with gaps included.

All strains were analysed to investigate the existence of tRNA-like structures within their 16S–23S ITS regions. Comparisons of 16S–23S ITS sequences determined in this study with 21 tRNA sequences published previously (Green & Vold, 1983) showed no similarities. Even sequences within 16S–23S ITS sequences of certain strains showing few matches with tRNA sequences did not form the secondary structure characteristic of tRNA. Moreover, the sequences did not contain the trinucleotide sequence (-CCA) that has been found at the 3′ end of most tRNA sequences. Thus, it was concluded that no strain used in this study contains tRNA sequences in intergenic spacers between 16S and 23S rDNAs, as shown previously for some *Streptomyces* species (Baylis & Bibb, 1988; Suzuki *et al.*, 1988; Pernodet *et al.*, 1989; Kim *et al.*, 1993), *Frankia* strain (Normand *et al.*, 1992), some *Bifidobacterium* species (Leblond-Bourget *et al.*, 1992) and *Saccharomonospora* strains (Yoon *et al.*, 1997b). Our results are consistent with the previous finding that there are no tRNA genes in the 16S–23S ITS sequences of members of the order Actinomycetales.

The strains of *N. albus* showed a high degree of sequence divergence among their 16S–23S ITS sequences. *N. albus* strains NSP01^T and NSP11 and *N. albus* strains NSP06 and NSP07 were found to have identical 16S–23S ITS sequences. The mean intra-

Table 2. Percentage nucleotide similarity for 16S–23S ITS sequences of representatives of the genus *Nocardioides*, two *Aeromicrobium* species, *Terrabacter tumescens* and representatives of some other taxa

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1. *N. albus* NSP01^{T}																				
2. *N. jensenii* NSP19^{T}	65·8																			
3. *N. luteus* NSP20^{T}	84·8	63·3																		
4. *N. plantarum* NSP21^{T}	64·9	53·8	59·6																	
5. *N. simplex* NSP22^{T}	55·1	51·3	57·9	66·7																
6. ‘*N. flavus*’ NSP30^{T}	85·0	63·0	99·8	59·8	58·6															
7. ‘*N. fulvus*’ NSP32^{T}	84·8	63·3	100	59·6	58·1	99·8														
8. ‘*N. fulvus*’ NSP33	63·6	68·0	54·8	56·6	55·3	54·4	54·8													
9. *Nocardioides* sp. NSP36 (*rrn* 1)	57·6	70·2	55·3	58·1	56·1	55·6	55·3	75·4												
10. *Nocardioides* sp. NSP36 (*rrn* 2)	54·8	71·5	49·2	53·1	56·6	49·4	49·2	73·6	80·8											
11. *Nocardioides* sp. NSP40	63·9	64·2	66·8	65·3	54·4	66·2	66·8	58·4	58·4	57·7										
12. *Nocardioides* sp. NSP41	53·6	48·4	54·0	71·5	82·8	53·2	54·0	61·0	70·4	59·0	57·6									
13. *A. erythreum* NSP37^{T}	66·8	73·1	67·4	57·4	52·9	67·7	67·4	55·4	64·9	62·5	66·4	52·7								
14. *A. fastidiosum* NSP38^{T}	56·5	70·1	54·0	54·5	56·8	58·4	54·0	68·6	65·4	64·6	57·0	49·5	71·2							
15. *T. tumescens* NSP39^{T}	62·5	66·3	57·1	60·1	52·8	57·4	57·1	66·9	61·6	60·6	59·4	55·1	68·7	65·3						
16. *S. ambofaciens*	57·6	66·6	62·7	58·3	54·4	61·1	62·7	65·9	72·6	75·8	62·7	53·0	65·3	65·4	67·9					
17. *S. griseus* subsp. *griseus*	64·7	66·4	64·7	58·1	52·4	65·1	64·7	58·2	67·8	66·6	56·6	50·9	62·9	65·3	66·8	76·7				
18. ‘*S. lividans*’	67·4	69·9	67·8	56·5	67·2	68·5	67·8	61·8	66·2	73·5	67·5	46·9	70·0	67·2	70·0	81·0	77·9			
19. *Frankia* strain ORS 020606	58·0	67·2	54·1	62·4	52·5	54·3	54·1	65·6	62·8	61·4	48·7	53·9	68·8	65·6	66·4	71·3	68·4	72·5		
20. *B. bifidum*	55·6	49·7	59·7	57·6	61·5	59·4	59·7	50·4	53·4	51·4	51·5	65·6	51·7	53·7	53·9	56·8	54·6	58·4	54·3	
21. *B. breve*	54·6	56·0	51·2	54·1	58·8	51·7	51·2	50·6	50·6	50·1	58·0	57·1	52·3	56·3	58·2	55·8	57·0	55·1	56·6	70·6

specific nucleotide similarity value for 16S–23S ITS sequences of *N. albus* was 92·1 ± 7·9 %. The most distant relationship was that between *N. albus* NSP04 and *N. albus* strains NSP06 and NSP07, for which a level of similarity of 84·1 % was obtained. The 16S–23S ITS sequence has been found to have a high frequency of insertion and deletion, resulting in a high rate of evolution (Gürtler & Stanisich, 1996; Leblond-Bourget *et al.*, 1996; Yoon *et al.*, 1997b). The number of insertions and deletions in alignments of 16S–23S ITS sequences can constitute important differences between organisms. Thus, nucleotide similarities including gap sites for each strain were also calculated in our study. The mean level of nucleotide similarity including gap sites for *N. albus* strains was 87·3 ± 12·7 %. The most distant relationship, including gaps, was the relationship between *N. albus* NSP05 and *N. albus* NSP09, which exhibited a level of nucleotide similarity of 74·5 %. Including gap sites in the calculation of the nucleotide similarity values helps clarify the relationships among *N. albus* strains. The high degree of sequence divergence of 16S–23S ITS sequences among *N. albus* strains has been shown to be useful in discriminating *N. albus* strains. Also, despite their sequence divergences, *N. albus* strains form phylogenetic lineages that are distinct from other *Nocardioides* species, except *N. luteus* NSP20^{T}, ‘*N. flavus*’ strains NSP30^{T} and NSP31 and ‘*N. fulvus*’ NSP32^{T}, and from members of the other genera in the phylogenetic tree (data not shown). The 16S–23S ITS sequences of *N. luteus* NSP20^{T} are 85·0–93·0 % similar to those of *N. albus* strains, and 76·0–88·6 % similar to *N. albus* strains when gaps are included. When the 16S–23S ITS of *N. luteus* NSP20^{T} was compared with that of *N. albus* NSP05 (JCM 5854), whose 16S rDNA sequence has been shown to be identical to that of *N. luteus* NSP20^{T} (Yoon *et al.*, 1998), the level of nucleotide similarity and of nucleotide similarity including gaps was 90·0 and 77·0 %, respectively. The 16S–23S ITS of *N. luteus* NSP20^{T} was only 1 bp different from those of ‘*N. flavus*’ strains NSP30^{T} and NSP31, whose nucleotide sequences are identical, and had an identical sequence to that of ‘*N. fulvus*’ NSP32^{T}. ‘*N. flavus*’ strains NSP30^{T} and NSP31 and ‘*N. fulvus*’ NSP32^{T} are thought to be closely related to *N. luteus* NSP20^{T} from the results of the 16S–23S ITS sequence analysis and from that fact that the 16S rDNA similarity value is 100 %, as found previously. Organisms with an identical sequence or only a 1 bp sequence difference in the 16S–23S ITS sequences, although showing high sequence divergence from other *Nocardioides* species, should be regarded as members of same species. Thus, it is proposed that ‘*N. flavus*’ strains NSP30^{T} and NSP31 and ‘*N. fulvus*’ NSP32^{T} be described as members of *N. luteus*. ‘*N. flavus*’ NSP30^{T} has been assumed to be a synonym of *N. luteus* (Prauser, 1989). *N. luteus* NSP20^{T} clearly showed a lower 16S–23S ITS similarity to *N. albus* strains, when compared with the results of 16S rDNA analysis. However, *N. albus* strains and *N. luteus* NSP20^{T} form a combined phylogenetic group in a 16S–23S ITS sequence-based tree (data not shown). The degree of 16S–23S ITS similarity (85·0–93·0 %) between *N. luteus* NSP20^{T} and *N. albus* strains falls within in the range of 16S–23S ITS similarity found in *N. albus* strains (84·1–100 %). In addition, the 16S–23S ITS sequences of *N. luteus* NSP20^{T} are 473 bp, which falls within the range of lengths of 16S–23S ITS sequences found in *N. albus* strains (468–539 bp). However, the nucleotide similarity level between *N. luteus* NSP20^{T} and *N. jensenii* NSP19^{T}, *N. plantarum* NSP21^{T} and *N. simplex* NSP22^{T} was 63·3, 59·6 and 57·9 %, respectively. The sequence divergences of the 16S–23S ITS between *N. luteus* NSP20^{T} and *N. albus* strains show clearer results than those of 16S rDNAs, which have nucleotide similarity of 99·5 ± 0·5 %. Nevertheless, the taxonomic relationship between the two taxa has been vague until now. Re-examination of DNA–DNA relatedness is thought to be the ultimate solution to elucidating the relationships between *N. albus* strains, *N. luteus* NSP20^T, '*N. flavus*' strains NSP30^T and NSP31 and '*N. fulvus*' NSP32^T.

'*N. fulvus*' NSP33 showed a low 16S–23S ITS similarity to '*N. fulvus*' NSP32^T – 54·8 % (Table 2). In the previous 16S rDNA sequence analysis, '*N. fulvus*' NSP33 also showed low nucleotide similarity to *Nocardioides* strains, including '*N. fulvus*' NSP32^T, and *Aeromicrobium* species, which are phylogenetically related to the genus *Nocardioides*, and its highest nucleotide similarity, 99 %, was with *Nocardioides* sp. NSP36 (Yoon *et al.*, 1998). Thus, '*N. fulvus*' NSP33 and *Nocardioides* sp. NSP36 have been considered to be members of a new genus based on phylogenetic inference and subsequently determined chemosystematic characteristics of the two strains. In this study, the 16S–23S ITS of '*N. fulvus*' NSP33 was found to exhibit a higher degree of similarity to the 16S–23S ITS sequences of *Nocardioides* sp. NSP36 (75·4 %, *rrn* 1; 73·6 %, *rrn* 2) than to those of other *Nocardioides* strains and *Aeromicrobium* species (Table 2). The degree of similarity between 16S–23S ITS sequences from '*N. fulvus*' NSP33 and *Nocardioides* sp. NSP36 and those of other *Nocardioides* strains and *Aeromicrobium* species are similar those between *Nocardioides* species and between *Nocardioides* species and *Aeromicrobium* species.

The levels of nucleotide similarity of 16S–23S ITS sequences between *N. simplex* strains varied from 97·7 to 100 %. The sizes of the 16S–23S ITS sequences were identical (386 bp) in all *N. simplex* strains except *N. simplex* NSP25, which has two types of 16S–23S ITS sequences – 387 and 388 bp. *N. simplex* strains NSP22^T and NSP27 shared an identical 16S–23S ITS sequence, and *N. simplex* strains NSP23 and NSP24 also shared an identical 16S–23S ITS sequence. It was interesting that the nucleotide similarity value (98·7 %) between the 16S–23S ITS sequences of *N. simplex* strains NSP23 and NSP24 and the 16S–23S ITS of *rrn* 1 of *N. simplex* NSP25 was higher than that (98·4 %) between the two types of 16S–23S ITS sequences found in *N. simplex* NSP25. The sequence divergences of 16S–23S ITS sequences found between *N. simplex* strains were much smaller than those found between *N. albus* strains. Previous additional *N. simplex* strains, namely *N. simplex* strains ATCC 13260, ATCC 19565 and ATCC 19566, were reclassified as *Rhodococcus erythropolis* Gray and Thornton (Gray & Thornton, 1928) from phylogenetic inference based on 16S rDNA sequences and chemotaxonomic characteristics (Goodfellow, 1989; Yoon *et al.*, 1997d). Consequently, they were not used in this study. *Nocardioides*-like isolate NSP41, whose 16S rDNA is 98·6 % similar to that of *N. simplex* strains (Yoon *et al.*, 1998), was also analysed with respect to the 16S–23S ITS region to investigate the taxonomic relationships with *N. simplex* strains. Additionally, *N. simplex* strains NSP22^T, NSP23, NSP24, NSP25 and NSP27 were shown to have identical 16S rDNA sequences (Yoon *et al.*, 1998). Strain NSP41 has a 16S–23S ITS of 328 bp, which is much smaller than those of *N. simplex* strains, and was 81·8–84·6 % similar to that of *N. simplex* strains (68·4–70·1 % with gaps included). The divergence of the 16S–23S ITS of strain NSP41 from those of *N. simplex* strains show that this strain should be studied in detail by taxonomic comparison with *N. simplex* strains.

The 16S–23S ITS sequences in the type strains of validly described *Nocardioides* species were highly divergent, with mean nucleotide similarity values of 68·1 % ± 16·8 % and 59·0 % ± 7·7 %, except in relation to *N. albus* NSP01^T and *N. luteus* NSP20^T. These nucleotide similarity values were sufficiently divergent to justify evaluation of the phylogenetic relationships between *Nocardioides* species even if nucleotide similarity values including gap sites were not calculated separately (Table 2). Moreover, 16S–23S ITS similarity values, gaps included, were too low to allow meaningful comparison between species. The most distant relationship was the relationship between *N. jensenii* NSP19^T and *N. simplex* NSP22^T, for which a nucleotide similarity level of 51·3 % was obtained (Table 2). *N. jensenii* NSP19^T showed a higher 16S–23S ITS similarity to *Aeromicrobium* species, *T. tumescens*, '*N. fulvus*' NSP33 and *Nocardioides* sp. NSP36, which have been considered to be members of another new genus, than to other *Nocardioides* strains (Table 2). Strain NSP40, which had been identified previously as *Pimelobacter* sp. (Lee *et al.*, 1994) but has recently been described as a new species of the genus *Nocardioides*, '*Nocardioides pyridinolyticus*' (Yoon *et al.*, 1997a), showed a 16S–23S ITS similarity level of 54·4 % (*N. simplex* NSP22^T) to 66·8 % (*N. luteus* NSP20^T) to the type strains of validly described *Nocardioides* species. The 16S–23S ITS similarity value between *Aeromicrobium erythreum* NSP37^T and *Aeromicrobium fastidiosum* NSP38^T was 71·2 % (Table 2). The two *Aeromicrobium* species exhibited a mutually high level of 16S rRNA similarity of 98·2 %, despite low DNA–DNA relatedness values (12–14 %) between the two species (Tamura & Yokota, 1994). Thus, the relationship between the two *Aeromicrobium* species was clarified by the comparison of 16S–23S ITS sequences. It is interesting that *A. erythreum* has a higher 16S–23S ITS similarity level to *N. jensenii* NSP19^T at 73·1 % than to *A. fastidiosum*. *N. jensenii* NSP19^T was found to be more closely related to *Aeromicrobium* species, and even to *T. tumescens*, *Frankia* strain and *Streptomyces* species, than to *Nocardioides* strains from the sequence analyses of 16S–23S ITS sequences. *N. jensenii* NSP19^T was thought to be phylogenetically in the middle of the genera *Nocardioides* and *Aeromicrobium* from 16S rDNA sequence analysis, despite the fact that it has chemotaxonomic properties characteristic of the genus *Nocardioides*.

The 16S–23S ITS sequences of the representative strains of the genus *Nocardioides*, *Aeromicrobium* species and *T. tumescens* determined in this study, together with some representative species of the genera *Streptomyces* and *Bifidobacterium* and the *Frankia* strain, were analysed to investigate whether 16S–23S ITS sequences are also appropriate for inferring the relationships between distantly related organisms, such as intergeneric relationships. The levels of 16S–23S ITS similarity between *Nocardioides* species, except that between *N. albus* strains and *N. luteus* NSP20^T, are similar to the levels of 16S–23S ITS similarity between *Nocardioides* species and members of other genera used for sequence analysis in this study (Table 2). Moreover, in some instances, some *Nocardioides* species showed a lower 16S–23S ITS similarity level when compared with certain species of the genus *Nocardioides* than when compared with members of other genera (Table 2). For example, *N. jensenii* NSP19^T showed a lower similarity level to *Nocardioides* species (51·3–65·8 %) than to members of the genera *Aeromicrobium* (70·1–73·1 %) and *Streptomyces* (66·4–69·9 %), *T. tumescens* (66·3 %) and the *Frankia* strain (67·2 %) (Table 2). *N. luteus* NSP20^T also showed a 16S–23S ITS similarity level of 57·9 % to *N. simplex* NSP22^T but showed similarity of 62·7–67·8 % to three species of the genus *Streptomyces* used in this study (Table 2). Additional inconsistent cases can be found in Table 2. There are also instances of *Aeromicrobium* species showing higher similarity to *Streptomyces* species and the *Frankia* strain than to certain species of the genus *Nocardioides*, which are members of the same family (Stackebrandt *et al.*, 1997) (Table 2). For example, *A. erythreum* NSP37^T showed nucleotide similarity values of 57·4 and 52·9 % to *N. plantarum* NSP21^T and *N. simplex* NSP22^T, respectively, but showed nucleotide similarities of 68·7 % to *T. tumescens*, 62·9–70·0 % to *Streptomyces* species and 68·8 % to the *Frankia* strain (Table 2). *A. fastidiosum* NSP38^T displayed nucleotide similarity values of 54·0–56·8 % to the type strains of validly described *Nocardioides* species, except *N. jensenii* NSP19^T, but showed nucleotide similarities of 65·3 % to *T. tumescens*, 65·3–67·2 % to *Streptomyces* species and 65·6 % to the *Frankia* strain (Table 2). In the dendrogram based on 16S–23S ITS sequences, the genus *Nocardioides*, the genus *Aeromicrobium*, *T. tumescens* and '*N. fulvus*' NSP33 and *Nocardioides* sp. NSP36, which have been considered to be members of a new genus, did not form distinct phylogenetic lineages separate from each other or from other genera used in this study. Therefore, the dendrogram is not shown.

From the above results, 16S–23S ITS sequences were not thought to be appropriate for inferring phylogenetic relationships between distantly related organisms and between high-ranked taxa. These observations are caused by large sequence variations of the 16S–23S ITS, which does not allow us to determine the relationships among distantly related organisms, such as among the genus or among the family. The large variations in 16S–23S ITS sizes, resulting from a high frequency of insertions and deletions, and the lack of nucleotide similarity make accurate alignments of 16S–23S ITS sequences difficult and, in the long run, cause unfavourable phylogenetic inferences to be made among organisms, in particular among distantly related organisms. The existence of heterogeneous 16S–23S ITS sequences within a strain, which can occur as a result of lack of conservation, also makes it complicated to compare 16S–23S ITS sequences among organisms. These properties reduce the value of 16S–23S ITS sequences for database and BLAST search purposes. Nevertheless, 16S–23S ITS sequences have been shown to be reliable for inferring phylogenetic relationships among closely related organisms in this study and in previous studies (Zavaleta *et al.*, 1996; Yoon *et al.*, 1997b). In addition, sequence determination of the 16S–23S ITS region is relatively easy on account of its small length, irrespective of whether direct sequencing or sequencing following cloning is used, when compared with sequencing of 16S rDNA.

Concluding remarks

In conclusion, the relationships between *N. albus* strains, among *N. albus* strains, *N. luteus* NSP20^T, '*N. flavus*' strains and '*N. fulvus*' NSP32^T, between *N. simplex* strains, between *Nocardioides*-like NSP41 and *N. simplex* strains and between *Aeromicrobium* species, based on 16S–23S ITS sequences, are more distinct than the results obtained when 16S rDNA sequences were used. The properties of 16S–23S ITS sequences also make relationships between validly described *Nocardioides* species, except that between *N. albus* and *N. luteus*, clearer. Nevertheless, the large sequence variabilities and length polymorphisms of 16S–23S ITS sequences make it difficult to infer relationships between the genera *Nocardioides* and *Aeromicrobium* and *T. tumescens*, and among the three taxa and other genera used in this study. It is thought that 16S–23S ITS sequences must not be used to elucidate phylogenetic relationships between high-ranked taxa or distantly related taxa such as between genera or between families.

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