Comparative sequence analyses of the ribonuclease P (RNase P) RNA genes from LL-2,6-diaminopimelic acid-containing actinomycetes

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Ribonuclease P (RNase P) RNA genes of LL-2,6-diaminopimelic acid (LL-A₂pm)-containing actinomycetes, except *Streptomyces* species, were sequenced after PCR-amplification and cloning. By using the sequence data, the relationships between species within genera and the relationships between taxa above genus level were investigated and the usefulness of the RNase P RNA gene as another phylogenetic marker was evaluated. RNase P RNA gene sequences of all strains used in this study contained relatively conserved regions along with highly variable regions. The mean RNase P RNA gene similarity value was approximately 82±18% and the mean RNase P RNA gene similarity value when gaps were included was approximately 76±24%. The nucleotide similarities between the RNase P RNA genes of different strains were mostly fewer than the 16S rDNA similarities. The RNase P RNA gene was more useful than 16S rDNA for clearly differentiating the relationships between species belonging to a genus and the relationships between some genera. However, nucleotide sequences of RNase P RNA genes were not necessarily appropriate for comparisons at all taxonomic levels (such as those between species, between genera and between families).

Keywords: RNase P RNA gene, LL-2,6-diaminopimelic acid, actinomycetes, phylogeny

INTRODUCTION

The best means of investigating the genetic relationship between organisms is to compare their total genomic sequences. However, this approach is not likely to be possible without innovation in the existing molecular biological and analytical techniques. In the meantime, DNA–DNA hybridization and some useful gene fragments are used for elucidating genetic relationships or in phylogenetic studies. Such gene fragments include genes for rRNAs (Woese, 1987), the β -subunit of ATP-synthase (Amann *et al.*, 1988), tRNA (Hofle, 1990), glutamine synthetase (Brown *et al.*, 1994), elongation factor Tu (Kamla *et al.*, 1996), a 65 kDa heat-shock protein (*hsp65*) (Swanson *et al.*, 1997) and a histone-like protein (*hbb*) (Valsangiacomo *et al.*, 1997). Among

these, 16S rRNA or 16S rDNA have been extensively studied and have become important as target genes in the phylogeny and classification of prokaryotic organisms (Woese & Fox, 1977; Woese, 1987; Stackebrandt *et al.*, 1997). Nevertheless, there is a general consensus that 16S rRNA gene sequences may be inappropriate for elucidating the relationships between closely related organisms and may be insufficient to guarantee species identity (Fox *et al.*, 1992). DNA–DNA relatedness is generally recognized as being very important for defining species in current bacterial systematics (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). However, despite the fact that it provides a decisive criterion in species definition, DNA–DNA reassociation also has some experimental problems, being affected by various factors such as DNA concentration, DNA purity, size of DNA fragments, incubation temperature, differences in the presence of RNA and so forth (Johnson, 1991); in addition, different methods are used to determine the levels of DNA–DNA relatedness (Vandamme *et al.*, 1996). Therefore, quantitatively

Abbreviations: LL-A₂pm, LL-2,6-diaminopimelic acid; ITS, internally transcribed spacer.

The GenBank accession numbers for the sequences of the RNase P RNA genes determined in this study are listed in Table 1.

identical results are not always obtained. This also makes it difficult to construct databases using DNA–DNA relatedness results.

Although 16S rDNA sequencing and DNA–DNA relatedness tests are indispensable tools in current bacterial systematics, additional genetic markers also may be necessary to support the results of 16S rDNA sequencing and DNA–DNA relatedness, in line with trends in polyphasic taxonomy. Recently, the sequences of the 16S–23S rDNA (16S–23S) internally transcribed spacer (ITS) have been shown to be useful for elucidating the relationships between closely related strains or species (Leblond-Bourget *et al.*, 1996; Yoon *et al.*, 1997a, 1998b). However, the 16S–23S ITS also has some disadvantages as a phylogenetic marker. The large variation in sequence and length of 16S–23S ITSs make it difficult to infer relationships between distantly related taxa (e.g. between taxa above genus level). Also, heterogeneities in sequence and length of 16S–23S ITSs within one organism have been found (Hain *et al.*, 1997; Yoon *et al.*, 1998b). Such heterogeneities, caused by the existence of multiple 16S–23S ITS alleles, may reduce the value of the 16S–23S ITS as a phylogenetic marker. Direct sequencing of heterogeneous PCR products of the 16S–23S ITS yields unreadable mixed sequence data above a certain position (Yoon *et al.*, 1998b). If the extent of heterogeneity in sequence and length is to be determined, the PCR products should be cloned before sequencing is begun. However, because the numbers of rRNA gene clusters vary according to the organisms (Baylis & Bibb, 1988; Pernodet *et al.*, 1989; Yoon *et al.*, 1996), it may not be easy to investigate the extent of the heterogeneity. Such heterogeneity within strains makes phylogenetic analysis of the 16S–23S ITS sequences rather complex.

Recently, the ribonuclease P (RNase P) RNA gene was evaluated for its potential as a new phylogenetic marker (Cho *et al.*, 1998); its application to the study of the genus *Saccharomonospora* showed that it could possibly be used effectively in systematic studies. The mean nucleotide similarity of RNase P RNA gene sequences between *Saccharomonospora* species was intermediate between those values obtained from the sequences of 16S rDNA and the 16S–23S ITS (Cho *et al.*, 1998). The members of the genus *Saccharomonospora* formed a single cluster distinct from some Gram-positive bacteria in a dendrogram based on comparative analysis of RNase P RNA gene sequences. Unlike the 16S–23S ITS, RNase P RNA is known to be encoded by a single-copy gene (*rnpB*) in Gram-positive bacteria (Haas *et al.*, 1996) and its size is relatively conserved (350–400 bp in bacteria; Pace & Brown, 1995). Despite the potential of the RNase P RNA gene as an additional phylogenetic marker, few sequence data have been determined, especially for actinomycetes, and few systematic studies based on RNase P RNA gene sequences have been performed. Therefore, RNase P RNA gene sequences from diverse taxa including actinomycetes are necessary to evaluate

its value as a marker for identification, classification and phylogeny. Such sequences will be used to extend RNase P RNA gene-based systematic studies to other genera. It is also necessary to investigate whether the RNase P RNA gene can be used to elucidate the relationships between distantly related taxa (such as those between genera or families).

In this study, LL-2,6-diaminopimelic acid (LL- A_2 pm)-containing actinomycete taxa, except *Streptomyces* species, were chosen for extending the phylogenetic study based on RNase P RNA gene sequences. LL- A_2 pm-containing actinomycetes are a taxonomically and biotechnologically interesting group. Representatives of LL- A_2 pm-containing coryneform and nocardioform actinomycetes, as well as the genus *Streptomyces*, have the ability to accumulate phosphate (Nakamura *et al.*, 1995), to produce the antibiotic erythromycin (Miller *et al.*, 1991) and to degrade some harmful aromatic compounds (Yoon *et al.*, 1997c, 1999). Currently, LL- A_2 pm-containing actinomycetes fall into five phylogenetic lineages, as shown by 16S rRNA gene sequence analyses (Stackebrandt *et al.*, 1997). The five lineages represent the following families (Stackebrandt *et al.*, 1997): the family *Nocardioidaceae* comprises the genera *Nocardioides* (Prauser, 1976) and *Aeromicrobium* (Miller *et al.*, 1991); the family *Intrasporangiaceae* comprises the genera *Terrabacter* (Collins *et al.*, 1989), *Terracoccus* (Prauser *et al.*, 1997) and *Intrasporangium* (Kalakoutskii *et al.*, 1967); the family *Propionibacteriaceae* comprises the genera *Propioniferax* (Yokota *et al.*, 1994), *Luteococcus* (Tamura *et al.*, 1994), *Microbium* (Nakamura *et al.*, 1995) and *Friedmanniella* (Schumann *et al.*, 1997); the family *Streptomycetaceae* comprises a single genus, namely *Streptomyces*; and the family *Sporichthyaceae* comprises a single genus, namely *Sporichthya* (Lechevalier *et al.*, 1968). In addition to genera such as *Streptomyces*, *Intrasporangium* and *Sporichthya*, which were described earlier, recently the numbers of LL- A_2 pm-containing coryneform and nocardioform taxa have increased considerably. Nevertheless, until now, only approximately 22 validly described species of the 11 genera (except for the genus *Streptomyces*) have been validly described as LL- A_2 pm-containing actinomycete taxa. There is evidence that LL- A_2 pm-containing coryneform bacteria may be relatively common in nature and that the majority of such organisms have not been described (Collins *et al.*, 1994; Keddie *et al.*, 1966). LL- A_2 pm-containing coryneform and nocardioform actinomycetes are important taxa that have received attention from the point of view of taxonomy, biodiversity and biotechnology. Therefore, additional useful taxonomic markers (whether genetic or chemotaxonomic) may be necessary to construct a polyphasic taxonomic approach to differentiating between existing LL- A_2 pm-containing taxa and to describing putative novel LL- A_2 pm-containing taxa.

The aim of this study was to investigate the relationships between taxa above genus level, as well as to

study the interspecific relationships of LL-A₂pm-containing actinomycetes, using RNase P RNA gene sequences. LL-A₂pm-containing taxa were also used to determine if the RNase P RNA gene is appropriate for clarifying the phylogenetic relationships between distantly related organisms (e.g. between genera or families).

METHODS

Bacterial strains and culture conditions. Table 1 summarizes the strains used in this study. Most strains were grown in shake flasks containing trypticase soy broth (BBL) supplemented with glucose (0.75%, w/v) at suitable temperatures. *Propioniferax innocua* was cultivated in shake flasks containing trypticase soy broth supplemented with yeast extract (0.3%, w/v) at 37 °C. *Friedmanniella antarctica* was cultivated in shake flasks containing R-medium at 22 °C. *Microlunatus phosphovorius* was cultivated in shake flasks containing microlunatus medium (Nakamura *et al.*, 1995) at 25 °C. The broth cultures were checked for purity before being harvested by centrifugation.

Isolation of DNA. Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996).

Amplification and cloning of the RNase P RNA gene. The RNase P RNA gene was amplified using two primers described previously (Cho *et al.*, 1998). Amplification was performed under the same conditions as those described previously (Yoon *et al.*, 1997b). The PCR products were purified using the GeneClean II kit (Bio 101) and cloned into T-vector prepared with pBluescript (Stratagene) (Marchuk *et al.*, 1991). General techniques for cloning were followed, as described previously (Kim *et al.*, 1995).

Sequencing of the RNase P RNA gene. Nucleotide sequences were determined using the T7 Sequenase version 2.0 DNA sequencing kit (Amersham) with primers T3 (5'-ATTAA-CCCTCACTAAAG-3') and T7 (5'-TAATACGACTCAC-TATAGGGCGA-3'). In some cases, reactions with dITP and the SequiTherm EXCEL II DNA sequencing kit (Epicentre Technologies) were performed to relieve compression artifacts.

Data analysis. The sequences of the RNase P RNA genes determined in this study were aligned by using CLUSTAL W software (Thompson *et al.*, 1994). Phylogenetic trees were inferred by using three tree-making algorithms, namely the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods, all of which were performed using the PHYLIP package (Felsenstein, 1993). Evolutionary distance matrices for the neighbour-joining method were

Table 1. Strains used in this study and accession numbers of RNase P RNA gene sequences

Species	Source*	Accession no.
<i>Nocardioides albus</i>	KCTC 9186 ^T	AF110036
<i>Nocardioides luteus</i>	KCTC 9575 ^T	AF110037
<i>Nocardioides jensenii</i>	KCTC 9134 ^T	AF110038
<i>Nocardioides plantarum</i>	NCIMB 12834 ^T	AF110039
<i>Nocardioides simplex</i>	KCTC 9106 ^T	AF110040
<i>Nocardioides pyridinolyticus</i>	KCTC 0074BP ^T	AF110041
<i>Nocardioides nitrophenolicus</i>	KCTC 0457BP ^T	AF110042
' <i>Nocardioides flavus</i> '	IFO 14396 ^T	AF110043
' <i>Nocardioides fulvus</i> '	JCM 3335 ^T	AF110044
<i>Kribbella flavida</i>	IFO 14399 ^T (KCTC 9580 ^T)	AF110045
<i>Kribbella sandramycini</i>	ATCC 39419 ^T (KCTC 9609 ^T)	AF110046
<i>Aeromicrobium erythreum</i>	NRRL B-3381 ^T	AF110047
<i>Aeromicrobium fastidiosum</i>	KCTC 9576 ^T	AF110048
<i>Terrabacter tumescens</i>	KCTC 9133 ^T	AF110049
<i>Terracoccus luteus</i>	DSM 44267 ^T	AF110050
<i>Intrasporangium calvum</i>	IFO 12989 ^T (KCTC 9796 ^T)	AF110051
<i>Friedmanniella antarctica</i>	DSM 11053 ^T	AF110052
<i>Microlunatus phosphovorius</i>	JCM 9379 ^T	AF110053
<i>Microlunatus phosphovorius</i>	JCM 9380	AF110054
<i>Propioniferax innocua</i>	DSM 8251 ^T	AF110055
<i>Luteococcus japonicus</i>	IFO 12422 ^T (KCTC 9794 ^T)	AF110056
<i>Luteococcus japonicus</i>	IFO 15385 (KCTC 9795)	AF110057
<i>Sporichthya polymorpha</i>	IFO 12702 ^T (KCTC 9797 ^T)	AF110058

* The following abbreviations are used: KCTC, Korean Collection for Type Cultures, Taejeon, 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, Manassas, VA, USA; IFO, Institute for Fermentation, Osaka, Japan; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA.

generated with the algorithm of Jukes & Cantor (1969) using the DNADIST program. The stability of relationships was assessed by a bootstrap analysis of 100 data sets by using the PHYLIP package.

RESULTS AND DISCUSSION

The genomic DNAs of strains used in this study were checked for contamination. The genomic DNAs of the *Nocardioide* species, the *Aeromicrobium* species and *Terrabacter tumescens* used in this work were those shown to be pure, by previous 16S rDNA analysis (Yoon *et al.*, 1998a). The DNAs extracted from other strains were identified as being uncontaminated, by direct determination of partial sequences of 16S rDNAs. The gene encoding the RNase P RNA (*rnpB*) was amplified by the PCR, using primers described previously (Cho *et al.*, 1998), and was cloned before being sequenced. At least two to three clones containing RNase P RNA gene from each strain were sequenced to obtain exact sequence data and to

confirm that the genuine sequence from each strain had been obtained.

The lengths of the RNase P RNA gene sequences determined ranged from 266 bp (in *Nocardioide luteus*, '*Nocardioide flavus*' IFO 14396^T and '*Nocardioide fulvus*' JCM 3335^T) to 326 bp (in *Nocardioide jensenii* and *Luteococcus japonicus*). The nucleotide sequences corresponding to annealing regions of PCR primers were excluded from subsequent analyses, since their sequences may be different from the original sequences of the genes. The sequences determined correspond to the region between positions 78 and 346 (approx. 71%), by comparison with the RNase P RNA gene (377 bp long) of *Escherichia coli* (Reed *et al.*, 1982; Lawrence *et al.*, 1987), and the region between positions 68 and 376 (approx. 75%), by comparison with the RNase P RNA gene (413 bp) of *Streptomyces bikiniensis* (Morse & Schmidt, 1992). The 5'-region of the RNase P RNA gene before the annealing region of the forward primer is not con-

Table 2. Percentage nucleotide similarity, with or without gaps included, for the RNase P RNA genes of the strains used in this study and *Streptomyces bikiniensis*

The values on the lower left are levels of nucleotide similarity and the values on the upper right are those with gaps included. Strains: 1, *Nocardioide albus*; 2, *Nocardioide luteus*; 3, *Nocardioide jensenii*; 4, *Nocardioide plantarum*; 5, *Nocardioide simplex*; 6, *Nocardioide pyridinolyticus*; 7, *Nocardioide nitrophenolicus*; 8, '*Nocardioide fulvus*' JCM 3335^T; 9, *Kribbella flavida*; 10, *Kribbella sandramycini*; 11, *Aeromicrobium erythreum*; 12, *Aeromicrobium fastidiosum*; 13, *Terrabacter tumescens*; 14, *Terracoccus luteus*; 15, *Intrasporangium calvum*; 16, *Friedmanniella antarctica*; 17, *Microtholatus phosphovor* JCM 9379^T; 18, *Microtholatus phosphovor* JCM 9380; 19, *Propioniferax innocua*; 20, *Luteococcus japonicus*; 21, *Sporichthya polymorpha*; 22, *Streptomyces bikiniensis*.

Strain	1	2*	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20†	21	22
1	—	89.0	66.9	70.8	80.1	71.3	76.8	89.3	68.6	71.6	67.0	71.8	72.3	71.4	70.9	67.8	67.2	67.0	72.5	67.2	56.5	70.6
2*	94.0	—	64.1	69.1	78.0	69.0	78.4	99.6	69.3	69.4	65.9	67.3	70.0	67.7	71.5	66.0	66.6	66.3	75.7	64.4	58.8	69.0
3	77.6	78.6	—	75.1	68.4	74.3	73.3	64.7	70.4	69.1	67.2	69.0	69.6	70.5	67.9	72.6	74.0	74.3	62.8	99.7	56.5	74.2
4	80.1	79.7	81.2	—	77.5	72.7	78.5	69.1	67.1	66.8	72.4	66.5	70.1	71.4	71.2	73.8	75.4	76.0	70.5	75.4	60.0	73.6
5	82.4	82.4	81.7	87.2	—	76.9	85.8	78.3	72.7	71.0	67.2	71.9	73.9	74.4	73.0	71.8	71.1	70.9	74.4	68.7	58.7	70.9
6	78.4	78.5	83.5	82.9	83.4	—	73.8	68.7	73.3	73.4	66.7	68.1	71.6	72.1	69.8	72.4	74.7	75.4	67.2	74.3	58.3	73.4
7	84.6	82.0	86.0	86.7	94.7	83.2	—	78.8	67.1	67.4	69.0	67.0	74.4	72.3	77.9	73.7	74.3	73.2	72.3	73.6	61.1	72.6
8	94.4	99.6	79.3	79.7	82.8	78.2	82.3	—	68.9	69.0	66.2	67.6	70.0	67.7	71.5	66.3	66.9	66.7	76.1	64.7	58.5	68.7
9	75.7	80.8	80.4	76.7	82.1	82.6	81.8	80.4	—	91.0	68.1	72.3	65.2	64.0	64.3	71.4	69.8	70.2	69.9	70.1	56.7	69.2
10	79.0	80.8	79.4	75.8	80.6	83.3	81.6	80.5	92.2	—	66.1	74.6	66.8	63.9	65.0	71.8	70.3	70.7	68.1	69.4	55.6	68.5
11	76.4	77.4	74.8	75.5	76.8	76.2	77.0	77.7	76.0	73.7	—	78.6	69.6	67.9	67.9	68.3	68.7	69.6	65.8	67.2	59.6	72.2
12	79.0	78.2	77.6	75.7	82.0	77.6	80.8	78.6	74.9	77.3	88.0	—	67.6	64.9	63.1	69.9	68.2	68.6	67.1	69.3	57.9	67.2
13	80.6	82.5	75.3	76.8	83.2	76.8	83.3	82.5	77.0	78.8	78.0	79.5	—	89.7	87.7	70.4	70.4	69.7	65.0	69.9	59.2	74.4
14	80.1	79.8	76.3	78.8	84.2	78.4	81.5	79.8	77.1	77.4	76.6	77.9	91.4	—	84.8	66.9	68.2	67.9	65.4	70.8	58.5	73.6
15	82.2	78.8	77.1	78.8	83.6	80.4	81.6	78.8	81.1	81.9	76.9	79.2	93.8	91.3	—	67.5	69.7	68.8	68.3	68.2	58.8	73.9
16	76.3	77.4	76.8	81.6	82.1	77.4	82.7	77.8	80.8	81.2	76.2	77.3	75.8	73.0	76.9	—	80.9	80.6	66.4	72.9	60.5	73.0
17	74.5	76.3	79.6	81.0	79.5	79.6	82.6	76.7	78.8	79.9	74.0	76.2	74.2	73.8	78.2	83.8	—	98.7	68.7	74.3	58.4	73.6
18	74.5	76.3	79.7	81.4	79.5	80.6	80.6	76.7	79.5	80.6	74.7	76.9	73.2	73.2	76.9	83.2	99.0	—	67.8	74.6	58.6	73.2
19	82.9	82.2	76.3	80.1	84.0	80.9	80.1	82.6	81.3	79.2	75.0	76.8	80.3	81.4	80.2	81.0	81.4	80.7	—	62.8	52.4	66.4
20†	77.9	78.9	99.7	81.6	82.1	83.5	86.3	79.3	80.1	79.7	74.8	77.9	75.7	76.6	77.4	77.1	79.9	80.0	76.3	—	56.5	73.9
21	63.9	68.4	64.8	66.2	66.1	65.1	68.0	68.1	69.7	68.4	66.8	70.9	64.9	63.7	65.1	67.4	65.7	64.5	64.3	64.8	—	59.3
22	78.2	80.8	78.3	80.6	80.2	79.2	81.2	80.4	81.6	79.7	80.8	78.0	76.3	76.5	79.5	77.2	77.0	76.4	81.9	78.0	65.9	—

* Identical results were obtained for *Nocardioide luteus* and '*Nocardioide flavus*' IFO 14396^T.

† Identical results were obtained for *Luteococcus japonicus* strains IFO 12422^T and IFO 15385.

served in sequence and length (Brown, 1998), unlike the 16S rRNA gene. Therefore, it was difficult to design a primer for the region close to the 5'-end of the RNase P RNA gene. In some recent studies, most primers used for PCR amplification of the RNase P RNA gene also have been designed around the annealing regions of the PCR primers used in this study (Haas *et al.*, 1996; Brown *et al.*, 1996).

The alignment of the RNase P RNA gene sequences of all strains exhibited relatively conserved regions along with highly variable regions and nucleotide gap-containing regions (data not shown). The regions corresponding to nt 116–138, 159–189 and 214–235, by comparison with the RNase P RNA gene sequence of *Streptomyces bikiniensis*, were relatively conserved, whereas the regions at nt 139–154, 239–275 and 350–376 were highly variable. The RNase P RNA gene sequence of *Sporichthya polymorpha* KCTC 9797^T exhibited many mismatched nucleotides when compared with those of other strains, as is also demonstrated by nucleotide similarities (Table 2). When the nucleotide sequence of *Sporichthya polymorpha* KCTC 9797^T was excluded from the alignment, the numbers of matched nucleotides were somewhat increased.

The mean RNase P RNA gene similarity value of LL-A₂pm-containing strains used in this study was approximately 82 ± 18% and the mean RNase P RNA gene-similarity value when gaps were included was approximately 76 ± 24% (Table 2). The between-strain nucleotide similarities of RNase P RNA genes were mostly fewer than the corresponding 16S rDNA similarities. The nucleotide sequences of RNase P RNA genes are unlikely to be appropriate for comparisons at all taxonomic levels (e.g. between species, between genera and between families). In particular, the nucleotide similarities between most species belonging to the genus *Nocardioide*s did not show conspicuous differences when compared with those between them and members of other genera; moreover, some species produced examples showing rather higher nucleotide similarities to members of other genera than to *Nocardioide*s species (Table 2). It appears that the RNase P RNA gene does not necessarily show much higher sequence differences between higher-ranked taxa than between lower-ranked taxa in the LL-A₂pm-containing strains used in this study. A phylogenetic tree based on the RNase P RNA gene sequences of all strains differed from that obtained using their 16S rDNA sequences. Therefore, phylogenetic trees based on RNase P RNA gene sequences of LL-A₂pm-containing strains used in this study were omitted because of the recognized limitations of RNase P RNA gene as a molecular clock.

The RNase P RNA gene sequences of validly described *Nocardioide*s species were relatively divergent when compared with levels of 16S rDNA similarity. The mean RNase P RNA gene similarity of the type strains of all validly described *Nocardioide*s species was 85.8 ± 8.2% (Table 2). The mean RNase P RNA gene

Fig. 1. Neighbour-joining tree showing the relationships among *Nocardioide*s species based on RNase P RNA gene sequences. Bootstrap values are shown at the branch points. Bar, 5 nucleotide substitutions per 100 nucleotides.

similarity including gaps of the type strains of all valid *Nocardioide*s species was approximately 76.6 ± 12.5% (Table 2). The relationships between *Nocardioide*s species, based on the RNase P RNA gene sequences, were examined with the trees inferred using the three different algorithms (Fig. 1). The topologies of the resultant trees were compared with the topology of a previously constructed tree based on 16S rDNA sequences (Yoon *et al.*, 1998a). The close relationships between some species in the tree based on the RNase P RNA gene sequences (Fig. 1) are mostly consistent with the result obtained from 16S rDNA sequences (Yoon *et al.*, 1998a). The type strains of *Nocardioide*s albus and *N. luteus* were phylogenetic neighbours with a nucleotide similarity of 94%. The clustering of *Nocardioide*s simplex KCTC 9106^T and *Nocardioide*s nitrophenolicus KCTC 0457BP^T was supported with low bootstrap resampling values only when the neighbour-joining and maximum-likelihood methods were used. *N. jensenii*, *Nocardioide*s plantarum and *Nocardioide*s pyridinolyticus formed phylogenetic lineages distinct from each species. However, in a phylogenetic analysis including other taxa used in this study, *Nocardioide*s species did not form a monophyletic cluster encompassed by the genus *Nocardioide*s. *N. pyridinolyticus* and *N. jensenii* exhibited phylogenetic lineages independent of the cluster containing the type species of the genus *Nocardioide*s, *N. albus* and most *Nocardioide*s species. In particular, it was interesting that *N. jensenii* showed only a 1 bp sequence difference with respect to *L. japonicus*. It was confirmed that the RNase P RNA

gene sequences of *N. jensenii* and *L. japonicus* were correctly determined, by using repeated tests. The type strains of two invalidly described *Nocardioide*s species, '*N. flavus*' IFO 14396^T and '*N. fulvus*' JCM 3335^T, were used to infer the relationships with other *Nocardioide*s species. '*N. flavus*' IFO 14396^T had an RNase P RNA gene sequence identical to that of *N. luteus* and '*N. fulvus*' JCM 3335^T showed only a 1 bp difference with respect to *N. luteus*. '*N. flavus*' IFO 14396^T has already been described as a strain of *N. luteus* (Prauser, 1989) and this has been confirmed recently by 16S rDNA and 16S–23S ITS sequence analyses (Yoon *et al.*, 1998a, b). '*N. fulvus*' JCM 3335^T was not clearly defined taxonomically until the 16S rDNA and 16S–23S ITS sequences were determined. Therefore, RNase P RNA gene sequences also support previous data showing that '*N. flavus*' IFO 14396^T and '*N. fulvus*' JCM 3335^T are suitable for description as members of *N. luteus*. Another strain (IFO 14399) of '*N. fulvus*' has been classified in a new genus (*Kribbella*) as *Kribbella flavida*, together with *Nocardioide*s sp. ATCC 39419, classified as *Kribbella sandramycini* (Park *et al.*, 1999). The two species of the new genus *Kribbella* formed a cluster independent of other strains used in this study and exhibited nucleotide similarity of 92.2%.

Two *Aeromicrobium* species formed a phylogenetic cluster that is clearly distinct from other taxa used in this study. The nucleotide similarity between the two species was 88% (78.6% when nucleotide gaps were included). The RNase P RNA genes of the two species are likely to be appropriate, as another genetic marker together with the 16S–23S ITS (Yoon *et al.*, 1998b), for clarifying the relationship between the two species. The two *Aeromicrobium* species were compared with the members of the genus *Nocardioide*s, a phylogenetic relative (Tamura & Yokota, 1994; Yoon *et al.*, 1998a). *Aeromicrobium* species formed a phylogenetic lineage distinguishable from the members of the genus *Nocardioide*s and exhibited less than 80% nucleotide similarity to *Nocardioide*s species, except in the case of the relationship between the type strains of *Aeromicrobium fastidiosum* and *N. simplex* (Table 2). Therefore, the RNase P RNA gene can be considered as a useful molecule that not only clearly differentiates *Aeromicrobium* species from *Nocardioide*s species but also shows the correct variation between the two *Aeromicrobium* species.

Three genera, *Intrasporangium*, *Terrabacter* and *Terracoccus*, which belong to the family *Intrasporangiaceae* and comprise only single species, formed a cluster distinct from other taxa, as shown in a phylogenetic analysis based on 16S rDNA sequences (Prauser *et al.*, 1997). *Intrasporangium calvum*, *Terrabacter tumescens* and *Terracoccus luteus* exhibited relatively high nucleotide similarity (91.3–93.8%) to each other, despite being of different genera, when compared with results between different genera or different species used in this study. Phylogenetically close relatedness among the three genera has already

been shown with 16S rDNA sequences (Prauser *et al.*, 1997; Park *et al.*, 1999). The RNase P RNA gene can be considered to be a more useful marker than 16S rRNA for differentiation of the three taxa at the genus level. On the other hand, it was shown in an original description that *Terracoccus luteus* has two types of colonies, i.e. the rough form (DSM 44275) and the smooth form (DSM 44274) (Prauser *et al.*, 1997). These forms were shown to be identical from a taxonomic point of view. We also isolated colonies of the rough form and the smooth form from the original culture (DSM 44267^T) and determined the RNase P RNA gene sequences from each colony type. The RNase P RNA gene sequences from the two types of colonies were found to be identical.

The genera *Friedmanniella*, *Luteococcus*, *Microbunatus* and *Propioniferax*, assigned to the family *Propionibacteriaceae*, also were compared. These genera also consist of single species. Among the four genera, *Luteococcus japonicus* formed a line of descent that is clearly different from the other three genera. As shown above, interestingly, *L. japonicus* had a nucleotide sequence nearly identical to that of *N. jensenii*. *F. antarctica*, *M. phosphovor* and *P. innocua* formed a line of descent that can be considered as one phylogenetic cluster. Although the three genera form a phylogenetic cluster, they exhibit levels of nucleotide similarity (81.0–83.8%) that makes possible their differentiation (Table 2).

Sporichthya polymorpha, one genus and one species that has been assigned to the family *Sporichthyaceae*, formed a line of descent that can be clearly distinguished from other LL-A₂pm-acid-containing taxa. *Sporichthya polymorpha* exhibited the lowest levels of nucleotide similarity to all strains when compared with those between other strains (Table 2).

From the above results it is clear that the RNase P RNA gene was useful for indicating phylogenetic relationships between species belonging to a genus and relationships between several genera, being target genetic material that exhibits lower nucleotide similarity than the 16S rRNA gene. Intraspecific variation of this gene was investigated for two species in this study. *L. japonicus* did not show intraspecific variation and two strains of *M. phosphovor* showed relatively minor variation of 4 bp differences. It was previously reported that the RNase P RNA genes of *Saccharomonospora* species also exhibited little or no intraspecific variation (Cho *et al.*, 1998). The RNase P RNA gene was found to have sequence variation intermediate between that of 16S rDNA and the 16S–23S ITS in data concerning the genera *Nocardioide*s, *Aeromicrobium* and *Kribbella* (Yoon *et al.*, 1998a, b), as was also shown for the genus *Saccharomonospora* (Cho *et al.*, 1998). Generally, 16S rDNA sequences have been known to be appropriate for comparisons between species that are not closely related and between taxa above genus level, and 16S–23S ITS sequences are thought to be appropriate

for indicating the relationships between closely related species and between strains belonging to a species. The RNase P RNA gene appears to be most suitable for indicating the relationships between species belonging to a genus and the relationships between several genera, because it is a genetic marker that shows sequence variation intermediate between that of 16S rDNA and the 16S–23S ITS. Most genera among the LL-A₂pm-containing actinomycetes have only a single species representative. Therefore, RNase P RNA gene sequences may be very useful for differentiation between existing species and putatively novel species of LL-A₂pm-containing genera, as it is one of several sources of valuable data in the polyphasic approach to taxonomy. Nevertheless, the RNase P RNA gene may be inappropriate for determining phylogenetic relationships between some genera of LL-A₂pm-containing taxa. It has already been shown that *Nocardioides* species did not form a monophyletic cluster and that some *Nocardioides* species have a higher level of nucleotide similarity to other genera. However, other genera, except *Nocardioides* and *Luteococcus*, showed lines of descent distinct from each other, although the topology of the tree is different from that of the tree based on 16S rDNA sequences and some genera did not form phylogenetic lineages within the radiation encompassed by the family. In a previous study using the genus *Saccharomonospora* and some representative genera in a phylogenetic analysis, RNase P RNA gene sequences were found to be useful for differentiating between genera as well as between species belonging to a genus. Each genus not only showed distinct interspecific sequence differences, but also formed distinct phylogenetic lineages (Cho *et al.*, 1998). Most genera in the phylogenetic tree obtained using RNase P RNA gene sequences from the RNase P database (Brown, 1998) are also shown to form distinct lines of descent.

It is still thought to be difficult to say whether it is worthwhile to use the RNase P RNA gene for inferring intergeneric relationships. To answer this question, it is necessary to extend the same approach to various genera. Even if subsequent tests prove that the RNase P RNA gene is less useful for comparisons of high-ranked taxa above genus level, it is clear that the RNase P RNA gene is useful for clearly differentiating species belonging to a genus. The species is the basic unit of bacterial systematics, but its definition has been a source of argument among microbiologists. For stable bacterial systematics, the application of a variety of target molecules or methods is very important because different information is obtained depending on the markers and methods used. The RNase P RNA gene showed greater sequence variation than 16S rDNA, in spite of its smaller size. The characteristics of the RNase P RNA gene, i.e. that it is encoded from a single-copy locus (Haas *et al.*, 1996) and is relatively conserved in size in bacteria (Pace & Brown, 1995), can be very useful in the determination and analysis of nucleotide sequences. Thus, the RNase P RNA gene is

a useful taxonomic marker that can be applied to a variety of taxa. Extension of the RNase P RNA gene database will contribute both to systematic study and the study of the function and secondary structure of the gene itself.

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