Application of the ribonuclease P (RNase P) RNA gene sequence for phylogenetic analysis of the genus *Saccharomonospora*

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Sequences of the RNase P RNA gene were investigated for the phylogenetic analysis of the genus *Saccharomonospora*. Aligned nucleotide sequences, determined from the PCR-amplified RNase P RNA gene of representative strains of the genus *Saccharomonospora*, displayed $94.2 \pm 1.3\%$ interspecific variances. The intraspecific similarity value was $99.7\text{--}100\%$ in all species tested. *Saccharomonospora azurea* K161^T and '*Saccharomonospora caesia*' K76^T displayed identical RNase P RNA gene sequences in the region that was determined and *Saccharomonospora* sp. K180 showed sequences distinct from validly described species with a similarity value of $94.6 \pm 1.0\%$. The phylogenetic trees constructed by aligning the sequences either within the genus *Saccharomonospora* or with other Gram-positive bacteria were similar to the ones derived using sequences of the 16S rDNA gene. Advantageous features of this gene for application as a molecular phyletic marker are discussed.

Keywords: *Saccharomonospora*, RNase P RNA gene sequence, phylogeny

INTRODUCTION

Bacterial taxonomy for the classification and nomenclature of micro-organisms has progressed rapidly over the last 20 years (Pace, 1997). Recently, determination of taxa has required the integration of different kinds of data for the purposes of delineation or classification. Thus, 'polyphasic data' combining phenotypic and genotypic data have now been widely applied for the classification of prokaryotic organisms or for the determination of the evolutionary distances between micro-organisms (Vandamme *et al.*, 1996).

Of all the methods applied for bacterial phylogeny, the method based on sequence comparison of 16S rDNA or rRNA has contributed most to modern taxonomy (Gutell *et al.*, 1994; Woese, 1987). This has largely been made possible as a result of recent progress in molecular biology and database management techniques. Now, nearly full length sequences of 16S rDNA or rRNA from over 3600 different strains have been determined and are available through their own database (Maidak *et al.*, 1997). This database supplies

other relevant information such as secondary structure and taxonomic significance in addition to the sequences. The comparison of 16S rDNA or rRNA sequence is a generally accepted tool which gives the most reliable pattern for the classification of prokaryotic organisms. However, such data should not be used as the sole criteria for evaluating a particular species (Clayton *et al.*, 1995). Usually two strains having rRNA similarity greater than 97% are very likely to be identical (Stackebrandt & Goebel, 1994). However, the application of this rule for the classification of bacterial species sometimes does not give satisfactory results and many instances have been reported where two species, found to have distinct features by other standard methods, can still have over 97% nucleotide similarity in 16S rRNA sequences (Fox *et al.*, 1992; Kim *et al.*, 1995; Stackebrandt & Goebel, 1994).

Lately, the sequences of the 16S–23S internally transcribed spacer (ITS) region in the rRNA operon has received attention as a suitable tool for the identification and typing of bacteria and has been frequently applied (Gürtler & Stanisich, 1996; Leblond-Bourget *et al.*, 1996; Yoon *et al.*, 1997a). In many cases, it has been reported that the ITS region shows a higher variation rate than 16S rRNA and can be applied as an

Abbreviation: ITS, internally transcribed spacer.

The GenBank accession number for the RNase P RNA gene sequences reported in this paper are AF012792–AF012808 (see Table 1).

alternative marker for the identification of species and closely related strains in cases where 16S rDNA or rRNA sequences have a very high similarity value (over 97%) (Yoon *et al.*, 1997a). However, the presence of multiple rRNA alleles per single genome has often caused difficulties in sequence interpretation due to mixed sequence data. Researchers have sought alternative markers which may compensate for such problems, and the elongation factor Tu (Kamla *et al.*, 1996), tRNA (Hofle, 1990), histone-like proteins (Valsangiacomo *et al.*, 1997) and others (Vandamme *et al.*, 1996) have been applied for this purpose.

In this study, we used the gene for the ribonuclease P (RNase P) RNA to evaluate whether this marker can provide rapid identification with reliable divergence between species and between strains belonging to the same species. RNase P is a ribonuclease which cleaves the 5' leader sequences of tRNA precursors and is a ribonucleoprotein composed of RNA of about 400 nucleotides and a protein subunit of about 120 amino acids (Pace & Brown, 1995). In a diverse range of bacteria, the RNA component of the RNase P is reported to be sufficient to catalyse the reaction at high ionic strength *in vitro*, and its biochemical characters as a ribozyme have been widely studied.

The genes encoding the RNA component of the RNase P have been cloned from the archaea to man and now the sequences from about 200 organisms have been determined. Updated and compiled data containing sequences, sequence alignments, secondary structures and three dimensional models have become available through a database (Brown, 1997). Examination of the sequence variation between different organisms has revealed that the secondary structure, as well as its function, has been conserved throughout the evolutionary pathway. Comparative phylogenetic analysis based on secondary structures has demonstrated its evolutionary significance (Haas *et al.*, 1994). Thus, the higher-order structure required to preserve its *in vivo* function seems to be maintained through the covariation of the sequences in helical regions. The phylogenetic tree based on the secondary structure of a diverse group of bacteria displays a core structure embedded in all types of bacterial RNase P RNA structures. However, each group still has its own distinct structure with respect to the number or length of the helical structures (Pace & Brown, 1995). Sequence comparison has also revealed that there is a conserved core sequence, composed of approximately 225 nucleotides, which has a significant role as the minimum structure to maintain function over the course of evolution. Taken together with other studies, RNase P is believed to be an ancient molecule with a conserved function. It also contains characters suitable for its use as a phylogenetic marker, especially in prokaryotic taxa. However, its significance as a molecular phyletic marker has not been thoroughly tested in its application for interspecific or the intraspecific delineation of bacteria.

The genus *Saccharomonospora* was chosen to test the suitability of the RNase P RNA gene as a phylogenetic marker since the classification of this genus has been extensively studied in our laboratory and elsewhere. In particular, molecular phyletic aspects have been analysed using genomic DNA Southern blotting with rDNA probes (Yoon *et al.*, 1996a) 16S rRNA gene sequence comparison (Warwick *et al.*, 1994; Kim *et al.*, 1995), RFLP analysis (Yoon *et al.*, 1997b), multiplex PCR application (Yoon *et al.*, 1996b) and sequence comparison of ITS in 16S–23S and 23S–5S regions (Yoon *et al.*, 1997a). *Saccharomonospora viridis*, the type species of the genus *Saccharomonospora*, was isolated by Nonomura & Ohara from soil in 1971 as a monosporic actinomycete containing meso-diaminopimelic acid, and possessing arabinose and galactose in the cell wall peptidoglycan (Nonomura & Ohara, 1971). Features such as a non-fragmenting branched mycelium and aerial hyphae formation with single spores typify this genus. Other physical and chemical characters, including the composition of fatty acids and quinones, have also been determined. *Saccharomonospora azurea* (Runmao *et al.*, 1987), *Saccharomonospora cyanea* (Runmao *et al.*, 1988), *Saccharomonospora glauca* (Greiner-Mai *et al.*, 1988) and '*Saccharomonospora caesia*' (Greiner-Mai *et al.*, 1987) are continuously isolated, although '*Saccharomonospora caesia*', reported as the fifth taxon of this genus, has not been included in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) or on subsequent Approved Lists or Validation Lists. Recent sequence analyses revealed that *Saccharomonospora azurea* and '*Saccharomonospora caesia*' have identical sequences with respect to 16S rDNA and ITSs, which strongly suggests that these two species should be unified as a single species (Kim *et al.*, 1995; Yoon *et al.*, 1997a). Since some kinds of saccharomonosporae are known to cause hypersensitivity pneumonitis and *Saccharomonospora viridis* has been implicated as a causal agent of farmer's lung disease (Greene *et al.*, 1981), rapid identification methods for differentiation of these species are realized as being important.

In this study, we present the classification of the *Saccharomonospora* strains based on the sequence of the RNase P RNA gene and describe the significance and advantageous characters in the use of this gene as a phylogenetic marker.

METHODS

Organisms and culture condition. The growth conditions of the type strains and other related strains of the genus *Saccharomonospora* and the method for the isolation of chromosomal DNA are described elsewhere (Kim *et al.*, 1995; Yoon *et al.*, 1996a). The strains used in this study and their descriptions are shown in Table 1.

Amplification and cloning of RNase P RNA gene. PCR primers to amplify the gene for the RNase P RNA component were designed based on those of Brown *et al.* (1996) with some modifications. Modifications were introduced by considering the sequence of '*Streptomyces lividans*' (GenBank accession no. M64552) and *Streptomyces*

Table 1. Strains used in this study and GenBank accession numbers for RNase P RNA genes

Lab. no.	Species	Strain designation and source*	Accession No.
K161 ^T	<i>Saccharomonospora azurea</i>	H. Runmao, NA128 ^T	AF012792
K76 ^T	' <i>Saccharomonospora caesia</i> '	KCTC 9152 = INMI 19125 ^T	AF012793
K163	' <i>Saccharomonospora caesia</i> '	DSM 43068	AF012794
K182	' <i>Saccharomonospora caesia</i> '	E. Greiner-Mai, Ko18	AF012795
K200	' <i>Saccharomonospora caesia</i> '	J. Lacey, A1932	AF012796
SB-01	' <i>Saccharomonospora caesia</i> '	S.-B. Kim	AF012797
SB-22	' <i>Saccharomonospora caesia</i> '	S.-B. Kim	AF012798
K169 ^T	<i>Saccharomonospora glauca</i>	DSM 43769	AF012799
K202	<i>Saccharomonospora glauca</i>	J. Ruan, 350	AF012800
K194	<i>Saccharomonospora glauca</i>	J. Lacey, A66	AF012801
K195	<i>Saccharomonospora glauca</i>	J. Lacey, A1450	AF012802
K180	<i>Saccharomonospora</i> sp.	A. J. McCarthy	AF012803
K168 ^T	<i>Saccharomonospora cyanea</i>	H. Runmao, NA-134	AF012804
K73 ^T	<i>Saccharomonospora viridis</i>	NCIMB 9602	AF012805
K197	<i>Saccharomonospora viridis</i>	J. Lacey, A1905	AF012806
K185	<i>Saccharomonospora viridis</i>	E. Greiner-Mai, Llv	AF012807
K191	<i>Saccharomonospora viridis</i>	E. Greiner-Mai, R25	AF012808

* Abbreviations: KCTC, Korean Collection for Type Cultures; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK.

bikiniensis (GenBank accession no. M64290), since the genera *Saccharomonospora* and *Streptomyces* are actinomycetes. The sequences of the primers RNP1 and RNP2 were 5' GGGGATCCGAGGAAMGTCCGGGCTC 3' (M = A or G) and 5' CGGAATTCTAMGCCGRTTCTGT 3' (M = A or C, R = A or G), respectively. Amplification conditions were the same as those described by Yoon *et al.* (1997a) and the PCR products were cloned into the *Bam*HI–*Eco*RI sites of either pGEM3zf or pGEM7zf (Promega). General techniques for DNA manipulation were as described by Sambrook *et al.* (1989).

Nucleotide sequence determination. Nucleotide sequences were determined using the T7 Sequencing kit of Pharmacia Biotech or the Sequenase version 2.0 kit from United States Biochemicals with the T7 (5'-TAATACGACTCACTATAGGCGCA-3') or SP6 primers (5'-ATTTAGGTGACACT-ATAGAATACT-3'). Two primers (RNP3, 5'-GTGTAAG-AGACCACCAGCGC-3' and RNP4, 5'-CCTTGCTCCG-GGTGGGGTTTACC-3') were additionally designed based on the results of sequencing and were further used for sequencing of the complementary strands.

Data analysis. The sequences of the RNase P RNA gene of the genus *Saccharomonospora* were aligned with those of other Gram-positive bacteria using CLUSTAL W software (Thompson *et al.*, 1994) followed by manual corrections. GenBank accession numbers and retrieved nucleotide (nt) positions for aligning the sequences are as follows: '*Streptomyces lividans*' (M64552, nt 1436–1780), *Streptomyces bikiniensis* (M64290, nt 136–477), *Bacillus brevis* (M19023, nt 53–394), *Bacillus stearothermophilus* (M19021, nt 44–400), *Bacillus megaterium* (M19022, nt 40–391), *Bacillus subtilis* (M13175, nt 43–384), *Mycoplasma hyopneumoniae* (X69982, nt 119–422), *Mycoplasma pneumoniae* (RNase P RNA database nt 31–326), *Mycoplasma flocculare* (X69983, nt 116–426), *Mycoplasma*

capricolum (D13066, nt 98–397), *Mycoplasma genitalium* (RNase P RNA database nt 41–338), *Helicobacterium mobilis* (U64882, nt 1–342), *Lactobacillus acidophilus* (U64883, nt 1–328), *Streptococcus faecalis* (U64887, nt 1–331), *Saccharomonospora faecium* (U64886, nt 1–331). Trees were constructed using the neighbour-joining method (Saitou & Nei, 1987) from a distance matrix calculated by the CLUSTAL W software. The topologies of the trees were evaluated by performing a bootstrap analysis (1000 replications) of the sequence data with CLUSTAL W software.

RESULTS

Phylogenetic analysis using the RNase P RNA gene within the genus *Saccharomonospora*

The genes encoding the RNase P RNA in the genus *Saccharomonospora* were amplified by PCR using primers designed based on the comparative analysis of sequences reported from other organisms. The sizes of the PCR products generated by the primers RNP1 and RNP2 were approximately 340 bp in all cases in the genus *Saccharomonospora*. This size represented over 85% of the known size of RNase P RNA. The PCR products corresponded to the sequences of the same gene from *Streptomyces bikiniensis* or '*Streptomyces lividans*' though lacking the first 40 bases (Brown, 1997; Morse & Schmidt, 1992; Shareck *et al.*, 1995). Thus this size was believed to contain the most sequence information of this gene and was deemed suitable for testing as a molecular phyletic marker. Nucleotide sequences were determined after the PCR products had been cloned into the cloning vector. Thus

Table 2. Levels of nucleotide similarity with or without gaps included for the RNase P RNA genes in the representative strains of the genus *Saccharomonospora* and other Gram-positive bacteria

The values on the lower left are levels of nucleotide similarity, and the values on the upper right are those with gaps included.

Strains	Percentage similarity to:												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1 <i>Saccharomonospora azurea</i> K161 ^T *		99.7	95.6	95.3	95.6	95.9	94.4	94.1	80.3	75.3	50.9	41.6	50.7
2 ' <i>Saccharomonospora caesia</i> ' SB-22	99.7		95.3	95.0	95.3	95.6	94.1	93.8	80.6	75.3	51.2	40.9	50.5
3 <i>Saccharomonospora glauca</i> K169 ^{T†}	95.9	95.6		99.7	95.3	94.7	93.3	93.3	80.4	75.1	52.1	42.5	49.9
4 <i>Saccharomonospora glauca</i> K194	95.6	95.3	99.7		95.3	94.4	93.0	93.0	80.7	74.9	51.9	42.2	49.9
5 <i>Saccharomonospora</i> sp. K180	96.1	95.8	96.1	96.1		95.3	93.5	93.3	81.7	75.0	51.6	42.8	48.8
6 <i>Saccharomonospora cyanea</i> K168 ^T	95.9	95.6	95.0	94.7	95.8		92.4	92.1	80.2	75.9	52.7	43.3	49.3
7 <i>Saccharomonospora viridis</i> K73 ^{T‡}	95.0	94.7	94.1	93.8	94.7	92.9		99.7	78.6	75.1	51.7	42.0	51.5
8 <i>Saccharomonospora viridis</i> K185§	94.7	94.4	94.1	93.8	94.3	92.6	99.7		78.6	75.4	51.5	42.2	51.5
9 <i>Streptomyces bikiniensis</i>	82.2	83.3	83.3	83.6	84.4	83.3	80.7	80.7		71.3	49.3	42.1	51.2
10 <i>Micrococcus luteus</i>	80.9	80.9	81.5	81.2	81.1	82.1	81.3	81.6	76.4		48.7	42.9	51.3
11 <i>Bacillus subtilis</i>	61.0	61.1	65.2	64.3	60.4	61.3	62.1	61.9	57.1	56.6		57.0	67.3
12 <i>Mycoplasma hyopneumoniae</i>	54.3	53.4	55.8	55.0	55.2	56.1	56.2	55.8	53.1	53.8	64.1		42.2
13 <i>Lactobacillus acidophilus</i>	62.1	61.5	60.2	60.8	58.6	61.8	63.3	65.1	60.4	58.3	74.3	55.4	

* Identical results were obtained for *Saccharomonospora azurea* K161^T, '*Saccharomonospora caesia*' K76^T, K163, K182, K200 and SB-01.

† Identical results were obtained for *Saccharomonospora glauca* K169^T, K202 and K195.

‡ Identical results were obtained for *Saccharomonospora viridis* K73^T and K197.

§ Identical results were obtained for *Saccharomonospora viridis* K185 and K191.

the 17 bp sequence at the 5' end and the 15 bp sequence at the 3' end may differ from the original sequences.

The sequences determined from the six representative strains of the genus *Saccharomonospora* were aligned and those alignments were sufficient to show the interspecific variations. It was of interest that the gene also contained conserved and variable domains. The regions at nucleotides (nt) 94–105, nt 190–210 and nt 300–315 displayed sequence variation between representative strains of the genus *Saccharomonospora*. In particular, six bases around nt 94 showed strong divergence between species, indicating that sequence determination of this variable region may be sufficient for differentiating these species. In addition, sequences between nt 120–190 showed strong conservation between species. The sequences from *Saccharomonospora azurea* K161^T and '*Saccharomonospora caesia*' K76^T were found to be identical agreeing with similar findings with respect to the data regarding 16S rDNA and ITS sequences in these strains. This result may verify the speculation that these two species are actually a single species (Kim *et al.*, 1995; Yoon *et al.*, 1997a). Also, *Saccharomonospora* sp. K180 exhibited sequences in the variable domains distinct from other validly described species while still showing strong similarity in the conserved domains. Together with the results from the 16S rDNA and ITS regions (Kim *et al.*, 1995; Yoon *et al.*, 1997a), this species should be assigned as a unique species within this genus.

Similarity between or within species of the genus *Saccharomonospora* was calculated with or without gaps included in the alignment (Table 2). The similarity values between the four validly described species were from 92.9% (*Saccharomonospora viridis* K73^T vs *Saccharomonospora cyanea* K168^T) to 96.1% (*Saccharomonospora azurea* K161^T, '*Saccharomonospora caesia*' K76^T or *Saccharomonospora glauca* K169^T vs *Saccharomonospora* sp. K180) when gaps were excluded for calculation of the similarity (Table 2). When gaps were included, they were from 92.4% (*Saccharomonospora viridis* K73^T vs *Saccharomonospora cyanea* K168^T) to 95.9% (*Saccharomonospora azurea* K161^T or '*Saccharomonospora caesia*' K76^T vs *Saccharomonospora cyanea* K168^T) (Table 2). The strain *Saccharomonospora viridis* K73^T displayed the lowest level of similarity to other representative strains including *Saccharomonospora* sp. K180. *Saccharomonospora* sp. K180 exhibited a mean similarity value of $95.4 \pm 0.7\%$ with the four validly described species.

Intraspecific divergence was examined using six strains of '*Saccharomonospora caesia*', four strains of *Saccharomonospora glauca* and four strains of *Saccharomonospora viridis* listed in Table 1. '*Saccharomonospora caesia*' strain SB-22 showed one nucleotide alteration at nt 195 while the other four '*Saccharomonospora caesia*' strains displayed identical sequences to that of '*Saccharomonospora caesia*' K76^T. The nucleotide A at this position was replaced

Fig. 1. Phylogenetic tree showing the genetic relationships of the genus *Saccharomonospora* with other Gram-positive bacteria. The GenBank accession numbers and sequence position applied for alignments are listed in Methods. The scale bar indicates 5 nucleotide substitutions per 100 nucleotides. Bootstrap confidence values by 1000 trials are shown at branching points.

with G in strain SB-22. In the case of *Saccharomonospora glauca*, the nucleotide T at nt 244 was substituted with C in strain K194, while the other two strains, K202 and K195, showed no sequence differences to *Saccharomonospora glauca* K169^T. In the case of *Saccharomonospora viridis*, strains K185 and K191 had base A at nt 199. This result represented 99.7–100% intraspecific sequence similarities. Taken together, the data derived from sequence analysis strongly suggest that the sequence of the RNase P RNA gene in this single genus showed divergence between species with a range of variation sufficient to detect interspecific differences, while containing 99.7–100% intraspecific sequence similarities (Table 2).

The phylogenetic tree generated with these sequence data is shown in Fig. 1. Each species formed a distinct genetic lineage and showed clear inter- or intra-specific relationships. As in the case of 16S rDNA sequences, *Saccharomonospora viridis* strains appeared to be the most distantly related species and *Saccharomonospora* sp. K180 showed a clearer branching point in this tree than was the case with respect to 16S rDNA sequences (Kim *et al.*, 1995). This strain also formed a separate lineage in the tree generated by ITS sequences (Yoon *et al.*, 1997a).

Phylogenetic analysis using the RNase P RNA gene with other Gram-positive bacteria

In an attempt to determine whether the genus *Saccharomonospora* can form a distinct genetic cluster among prokaryotic taxa using the sequence of the RNase P RNA gene, the phylogenetic relationship was examined by aligning the sequences of members of this genus with those from other Gram-positive bacterial taxa. Thus far, the sequences of this gene have only been reported for five genera of high G + C containing bacteria and about twenty genera of low G + C

containing bacteria (Brown, 1997). The sequences corresponding to the same region as determined in the genus *Saccharomonospora* in this study were retrieved from the GenBank database and appropriately aligned. The nucleotide positions and their GenBank accession numbers are listed in Methods. The level of nucleotide similarity between the sequences of these representative reference strains and those of the *Saccharomonospora* strains are also summarized in Table 2. Aligning these sequences required the introduction of several gaps due to the high divergence in the sequences between variable bacterial taxa. Thus, nucleotide similarity rates were calculated with or without gaps included. As expected, the highest nucleotide similarities were observed when compared to those of *Streptomyces bikiniensis* or '*Streptomyces lividans*', which belong to the same order, *Actinomycetales*. The mean nucleotide similarity value between *Streptomyces bikiniensis* and representative strains of the *Saccharomonospora* genus was $82.6 \pm 1.9\%$ and $80.15 \pm 1.5\%$ with the gaps excluded or included, respectively. With other high-G + C bacteria, *Micrococcus luteus*, gave a value of approximately $81.5 \pm 0.6\%$ and $75.5 \pm 0.5\%$ similarity to those genes in the genus *Saccharomonospora* with gaps excluded or included, respectively. However, the bacteria belonging to the low-G + C subdivisions exhibited quite a low level of similarity (Table 2). The mean nucleotide similarity values with (or without) gaps between *Bacillus subtilis*, *Mycoplasma hyopneumoniae* and *Lactobacillus acidophilus* and the representative strains in the genus *Saccharomonospora* were $51.8 \pm 0.9\%$ ($62.8 \pm 2.4\%$), $43 \pm 0.9\%$ ($55.3 \pm 1.0\%$) and $50.15 \pm 1.4\%$ ($61.0 \pm 2.4\%$), respectively.

The phylogenetic tree was generated with the sequences of RNase P RNA from six representative strains of the genus *Saccharomonospora*, three high-

G+C-and 13 low-G+C-content bacterial species (Fig. 1). The genus *Saccharomonospora* formed a single genetic lineage among the Gram-positive bacterial taxa tested. The branching pattern displayed by the tree in Fig. 1 is quite similar to that derived from 16S rDNA sequences (Maidak *et al.*, 1997) or RNase P Database (Brown, 1997). Altogether, the RNase P RNA gene performed well as a functional marker for discriminating individual species and for determining the taxonomic relationship within the genus *Saccharomonospora* and provides the possibility of its application to other taxa.

DISCUSSION

In this study, we evaluated the RNase P RNA gene as a marker for the discrimination or classification of prokaryotic taxa, using the genus *Saccharomonospora*, and have demonstrated its usefulness. Since we have performed molecular phylogenetic studies on the genus *Saccharomonospora* using the 16S rDNA and ITS sequences (Kim *et al.*, 1995; Yoon *et al.*, 1997a), the evaluation or comparison of data obtained with different markers was possible.

In the sequence alignments, six representative strains of the genus *Saccharomonospora* exhibited sequence variation at distinct variable domains. Using the RNase P RNA gene, the mean nucleotide similarity was $94.5 \pm 1.6\%$ among the representative strains of the genus *Saccharomonospora*. This value was intermediate between those obtained using sequences of the 16S rDNA ($97.6 \pm 1.3\%$) (Kim *et al.*, 1995) and ITS regions ($87.6 \pm 4.0\%$) (Yoon *et al.*, 1997a), with this genus (Table 2). As described above, the sequences at nt 90 showed strong variation in all the validly described *Saccharomonospora* species in this genus. The intraspecific variations in all tested strains were 99.7–100%. These results strongly suggest that sequence variation in the RNase P gene shows significant variation between species with negligible variation within species. The phylogenetic tree generated with the sequences, although only limited numbers of sequences were available, clearly demonstrates that the genus *Saccharomonospora* forms a single branching point with a reasonable degree of relatedness to other taxa (Fig. 1). Also, this study provides further evidence supporting previous speculations (Kim *et al.*, 1995; Yoon *et al.*, 1997a). These are (i) that *Saccharomonospora azurea* and '*Saccharomonospora caesia*' may be an identical species and (ii) that *Saccharomonospora* K180 is a distinct species within this genus. *Saccharomonospora azurea* K161^T and '*Saccharomonospora caesia*' K76^T had identical RNase P RNA sequences at the determined region and *Saccharomonospora* sp. K180 showed a sequence distinct from other validly described species with a $94.6 \pm 1.0\%$ similarity value. DNA–DNA hybridization experiments, as a final validating criterion, are under progress to confirm our findings.

RNase P is regarded as an interesting molecule since it

is a holoenzyme consisting of protein and RNA components (Pace & Brown, 1995). In addition to its unique biochemical character as a ribozyme *in vitro*, the conserved structure and universal function of the RNA component during the evolutionary pathway has made it an important molecule for researchers investigating molecular evolution (Haas *et al.*, 1994; Pace & Brown, 1995). In addition to its evolutionary significance, this molecule provides other attractive features for use as a phylogenetic marker. First, the size of the gene is less than 400 bp. In *Escherichia coli*, whose RNase P RNA has been most thoroughly characterized, it is known to be 377 bp (Lawrence *et al.*, 1987). Although the full size of this gene in most prokaryotes is known to be about 400 bp, a 340 bp portion that was amplified in this study turned out to be sufficient to display the sequence variations. Thus, sequence determination can be accomplished in less time and allow rapid identification or classification results. Although the sequences in this study were determined after the gene had been cloned into a vector, direct sequencing with internal primers would also be possible. This is thought to be highly advantageous since sequence determination of the 1500 bp of 16S rDNA requires considerably more time, labour and expense. Although partial sequences of the variable region in the 16S rDNA have been used in some cases, it should be noted that these sequences should not be used as conclusive data for determination of species identity. Second, there is only a single locus coding this gene in a genome. In contrast to the multiple presence of rRNA operons in most bacterial genome, it is known that a single *rnpB* gene encodes the RNase P RNA. While the multiple presence of the rRNA operons in a single genome generates diversity in length and sequence between species, such multiple numbers often cause serious problems for strain identification purposes, particularly in the analysis of ITS regions (Hain *et al.*, 1997). When ITS regions are amplified by primers designed using conserved regions of rDNA sequences, either multiple numbers of products or a single-sized product composed of mixed bands can be generated causing difficulty in alignment and comparison of such sequences for classification. Such problems have been alleviated by high resolution electrophoresis and GENESCAN 672 software (Hain *et al.*, 1997). Thus, the homogeneity of the amplified product for the RNase P RNA gene was recognized as advantageous for simple interpretation of the data obtained.

Although we have tested the application of the RNase P gene with only one genus in this study, we believe that the same approach can be extended to other genera and we are currently performing such experiments with other taxa, especially with actinomycetes whose RNase P RNA gene sequences have not been determined. In particular, it would be worthwhile investigating those species or strains having higher 16S rDNA or rRNA similarities with complex pattern of ITS sequences. As discussed above, the variation rate

in the RNase P RNA sequence was higher than that of 16S rDNA or rRNA sequences in spite of its smaller size. In the genus *Streptomyces*, the RNase P sequences of *Streptomyces bikiniensis* and '*Streptomyces lividans*' showed 88.4% sequence identity, while these two species had 96.8% sequence identity with respect to 16S rDNA. Thus it is believed that extending the database of this gene would be very helpful for the discrimination of bacterial taxa as well as for our understanding of evolutionary pathways.

It is not expected that a single macromolecule can provide all information about rate, tempo, mode and distance of evolution. Depending on the marker applied, the knowledge we can obtain will be different. The more information that is obtained, the more our understanding of molecular evolution will be enhanced and we believe that the use of the RNase P RNA gene will contribute further to this end.

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