International Journal of Systematic and Evolutionary Microbiology (2000), **50**, 909–915 Printed in Great Britain

Differentiation of newly described Antarctic bacterial isolates related to *Roseobacter* species based on 16S–23S rDNA internal transcribed spacer sequences

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The 16S–23S rDNA internal transcribed spacer (ITS) of *Roseobacter denitrificans*, *Roseobacter litoralis*, *Ruegeria algicola* and strains of the recently described species *Antarctobacter heliothermus* and *Roseovarius tolerans* were analysed in order to examine DNA sequence variations and to draw conclusions about inter- and intraspecific relationships. *A. heliothermus* included four strains with an ITS fragment length of 1092 bp. *Roseovarius tolerans* was described on the basis of eight strains. Five of these harboured two ITS fragments of different lengths (959 and about 1100 bp), while the others had one fragment of either 1083 bp (two strains) or 1165 bp (one strain). ITS lengths of the related species *Roseobacter denitrificans*, *Roseobacter litoralis* and *Ruegeria algicola* were found to be 980, 984 and 1158 bp, respectively. Phylogenetic analyses of the DNA sequences allowed species affiliation of strains with sequence length differences of >200 bp and recognition of relationships based on a well-supported ITS tree. The strains of *A. heliothermus* and *Roseovarius tolerans* each formed a monophyletic branch and they were separated from each other by *Ruegeria algicola.* This species was now clearly separated from *Roseobacter denitrificans* and *Roseobacter litoralis*, which corresponded to the new genus affiliation of *Ruegeria algicola*. These data were additionally supported by analyses of the structure, relative position and order of genes for $tRNA_{Ile}$ and $tRNA_{Ala}$ found within the ITS of each strain. Comparative DNA sequence analyses of ITS and 16S rDNA revealed limitations, on species and strain levels, with respect to the phylogenetic resolution of the 16S rDNA due to the limited number of informative (variable) sites, while ITS sequence analyses provided more variable and sufficiently conserved positions to discriminate between strains and to reconstruct their taxonomic relationships.

Keywords: *Roseobacter*, *Roseovarius*, *Antarctobacter*, *Ruegeria*, 16S–23S rDNA ITS

INTRODUCTION

Length and sequence polymorphisms found in the 16S–23S rDNA internal transcribed spacer (ITS) have been used for the discrimination of bacterial species (Barry *et al*., 1991; Gürtler & Stanisich, 1996; Lu *et al*., 1997). Even strains of one species have been differentiated (Hain *et al*., 1997; Vinuesa *et al*., 1998). The construction of specific primer pairs for the whole or parts of the rDNA spacer region has made it possible to identify species or genera by the length of PCR products (Forsman *et al*., 1997; Gu *et al*., 1998). This tool for fast species identification is of growing interest, especially for fast detection of pathogenic micro-organisms. However, the discrimination of strains has often proved impossible when ITS lengths alone were compared (Centurion-Lara *et al*., 1996; Mendoza *et al*., 1998), although inter-strain discrimination was successful with *Legionella* spp. when PCR-RFLP

Abbreviations: ASW, artificial sea water; ITS, internal transcribed spacer; MP, maximum parsimony; NJ, neighbour joining.

The EMBL accession numbers for the 16S–23S ITS sequences reported in this paper are AJ012700–AJ012707.

analysis of ITS sequences was applied (Riffard *et al.*, 1998). Resolution of species and strains was achieved more precisely with DNA sequence analyses of the ITS (Leblond-Bourget *et al.*, 1996; Yoon *et al.*, 1997, 1998; Roth *et al.*, 1998). In comparison with a discrimination of strains by PCR-RFLP, an alignment of orthologous ITS sequences yields the exact length of PCR products and would also allow the estimation of pairwise evolutionary distances, the resolution of inter-strain relationships and the fine-tuning of phylogenetic trees derived from 16S rDNA.

Antarctobacter heliothermus (Labrenz *et al.*, 1998) was recently described on the basis of four strains with nearly identical morphological and physiological characteristics, while *Roseovarius tolerans* (Labrenz *et al.*, 1999) comprised eight strains with different colony colours, variable bacteriochlorophyll *a* content and other minor phenotypic heterogeneities. However, 16S rDNA sequence analysis, DNA–DNA hybridizations and very homogeneous chemotaxonomic data suggested the affiliation of all eight strains of *Roseovarius* with one species (Labrenz *et al.*, 1999).

This study was aimed at the differentiation of strains of the genera and species of *A. heliothermus*, *Roseovarius tolerans* and other closely related representatives of the *Roseobacter* group belonging to the *α-Proteobacteria*. In order to gain additional support for the affiliation of strains of both new genera, their 16S–23S ribosomal ITS was analysed for length of PCR products, DNA sequence variability and structural features of tRNA genes. Also, their relationships to *Ruegeria algicola* (Uchino *et al.*, 1998), originally described as *Roseobacter algicola* (Lafay *et al.*, 1995), *Roseobacter litoralis* and *Roseobacter denitrificans* needed to be clarified with these methods. The ITS-derived affiliation was furthermore compared with the phylogenetic relationships reconstructed from the 16S rDNA sequence comparison.

METHODS

Bacterial strains and culture conditions. The following cultures were used: *Roseobacter denitrificans* (DSM 7001^T), *Roseobacter litoralis* (DSM 6996^T), *Ruegeria algicola* (DSM 10251^T), *Antarctobacter heliothermus* strains EL-219^T (=DSM 11445^T), EL-165 (=DSM 11440), EL-54 and EL-185 and *Roseovarius tolerans* strains EL-172^T (=DSM 11457^T), EL-222 (=DSM 11463), EL-52, EL-78, EL-83, EL-90, EL-164 and EL-171. All strains were cultivated at 20 °C in oligotrophic medium PYGV prepared with 25‰ artificial sea water (or with 40‰ artificial sea water for EL-83 and EL-54) as described previously (Labrenz *et al.*, 1998, 1999).

DNA extraction. Wet cells (200 mg) were suspended in 1 ml saline EDTA (150 mM NaCl, 100 mM EDTA, pH 8) and centrifuged and the pellet was resuspended in 1 ml proteinase K buffer (0·01 M Tris/HCl, 0·005 M EDTA, 0·50% SDS, pH 7·8). Cell lysis and DNA extraction were achieved by adding 15 µl proteinase K (20 mg ml^{-1}) and 5·5 µl 22·7% (w/v) SDS and incubating at 55 °C for 1 h. Next, 55 µl 22·7% SDS and 40 µl 5 M NaCl were added, followed by phenol extraction and ethanol precipitation.

PCR amplification and direct DNA sequencing of the 16S–23S rDNA ITS. Amplification of the ITS was performed according to Scheinert *et al.* (1996) in a reaction volume of 50 µl, which contained 150–250 ng genomic DNA, 50 pmol each of primers 5′-AAGTCGTAACAAGGTARC-3′ (region 3 on the basis of the criteria given by Gürtler & Stanisich, 1996; positions 1492–1509 of 16S rDNA) and 5′-GGTTBCCCCATTCRG-3′ (region 6; Gürtler & Stanisich, 1996;positions 115–130 of 23S rDNA), 0·2 mM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), 1 U *Taq* polymerase (Appligene) and the recommended PCR buffer (10 × concentrated, Appligene). The reaction mixture was covered with 40 µl Chill out wax (MJ Research). Nucleotide positions for the primers are according to the *Escherichia coli* numbering for the respective ribosomal genes of the *rrnB* operon (Brosius *et al.*, 1981), and primers were manufactured by Pharmacia.

Thermal cycling (Mastercycler 5330; Eppendorf) was started hot (heated to 94 °C, cooled to 82 °C after 5 min, held until polymerase addition), followed by 24 cycles of 94 °C denaturation (30 s), 48 °C annealing (30 s) and 72 °C elongation (20 s). The final 72 °C elongation step was prolonged for 7 min and terminated by cooling to 4 °C. PCR products were analysed with a 0·8% (w/v) agarose gel (analytical grade, Promega) in TBE buffer (Sambrook *et al.*, 1989). PCR products were excised from the agarose gel and recovered using the QiaEx kit (Qiagen) according to the manufacturer's instructions, except that, for improved recovery, DNA was eluted twice at 50 °C with 40 µl 1 mM Tris/HCl, pH 8·0. In order to allow rapid strain discrimination, only the shortest PCR product was prepared in those cases where two PCR bands were amplified, as with strain EL-172^T. The amount of eluted DNA was estimated visually after running 1/10 of the recovered volume on a 1% (w/v) agarose gel and comparing band intensities to a DNA mass ladder (Gibco-BRL).

DNA sequence analyses. DNA sequencing reactions were set up using the PRISM Ready Reaction dye deoxy terminator Ampli*Taq* FS kit (PE-Applied Biosystems) for direct PCR fragment sequencing according to the manufacturer's recommendations, except that the total reaction volume was reduced to 10 µl, which contained the eluted DNA (200 ng), the sequencing primer (2·5 pmol; one of the PCR primers or a specific internal primer) and 2 µl of the Ready Reaction mix including Ampli*Taq* FS polymerase. Cycle sequencing was done on a Trio-Block thermal cycler (Biometra) with 25 cycles of 96 °C for 15 s, 50 °C for 15 s (but varying between 45 and 60 °C, depending on the primer melting points) and 60 °C for 4 min. Excess dye terminators were removed by ethanol precipitation. Electrophoretic separation and on-line analysis of the sequence reactions were performed on an ABI 377 automated sequencer running a 5% (w/v) polyacrylamide gel (Long Ranger; FMC BioProducts).

The use of both PCR primers as sequencing primers resulted in single-stranded sequences of at least 700 bp reading length. The following walking primers were designed to obtain complete double-stranded DNA sequences: (i) forward primer (5′–3′), *A. heliothermus* CATAAGTGTT-TGGCAATCTCG, *Roseovarius tolerans* and *Roseobacter* species including *Ruegeria algicola* GATTTGCATTCA-GGAGGTCA; (ii) reverse primer (5′–3′), *A. heliothermus* GCCTTGCAGCGATCCTTTAT, *Roseovarius tolerans* TATCTCTCTAWACGATGTCAA and *Roseobacter* species including *Ruegeria algicola* GTTAGTGTACTTG-ACTTGGAC. DNA sequences were inspected visually using

the program SEQUENCE NAVIGATOR (PE-Applied Biosystems), ensuring reliable sequence data. DNA sequences were deposited in the EMBL database (for accession numbers see Table 1).

tRNA genes. Scanning for tRNA genes in ITS sequences was done with the program tRNASCAN-SE (version 1.11; Lowe & Eddy, 1997), which provided cloverleaf structures of the sequences identified. Phylogenetic analysis of tRNA features was done using common cladistic rules for traditional character analyses (Hennig, 1966).

Sequence alignment and phylogenetic analyses. The full-length ITS sequences were pre-aligned using the CLUSTAL option of the program MEGALIGN (Lasergene; DNASTAR) and checked by eye. Sequence statistics and phylogenetic analyses were performed using the program PAUP (test version 4.0d64, with the author's permission; Swofford, 1998) and checked in MEGA (version 1.01; Kumar *et al.*, 1993). Trees were computed either in both programs [neighbour-joining (NJ) trees; Saitou & Nei, 1987] or in PAUP only [maximum parsimony (MP) trees]. The alignment of ITS sequences is available by anonymous FTP from the EMBL FTP server (ftp://ftp.ebi.ac.uk/pub/databases/embl/align/) under accession number ds36433.

The 16S rRNA gene sequences used for phylogenetic tree construction (Fig. 2b) were received as a sub-alignment from the universal SSU alignment of the ribosomal database (RDP database, Maidak *et al.*, 1997), omitting common gaps. Sequences not included in the RDP database were added manually. The final alignment of both ITS and 16S rDNA sequences included the following taxa (accession numbers in parentheses): *A. heliothermus* EL-219^T (Y11552), *Roseovarius tolerans* EL-172^T (Y11551), *Ruegeria algicola* ATCC 51440^T (X78315), *Roseobacter denitrificans* ATCC 33942^T (M96746) and *Roseobacter litoralis* ATCC 49566^T (X78312).

RESULTS

Approximations of ITS lengths were obtained from an agarose gel (Fig. 1). Strain EL-219^T (~ 1100 bp) represented the *A. heliothermus* strains with equal ITS lengths. EL-172^T and four strains of *Roseovarius tolerans* (EL-52, EL-78, EL-83 and EL-90) had two ITS fragments of different lengths (~ 1000 bp and ~ 1200 bp), whereas three other *Roseovarius tolerans* strains had one ITS of either ~ 1100 bp (EL-171, EL-222) or ~ 1200 bp (EL-164). The ITS of *Ruegeria algicola* (~ 1200 bp), *Roseobacter litoralis* (~ 1000 bp) and *Roseobacter denitrificans* (~ 1000 bp) showed lengths similar to those of the two ITS bands of *Roseovarius tolerans*. Thus, a clear species or strain differentiation could not be obtained in this way. More accurate and discriminating ITS lengths were obtained from DNA sequencing (Table 1), including approximately 150 bp of the 16S and 23S rDNA, which revealed only a few differences in nucleotide sequences. Strains EL-171 and EL-222 of *Roseovarius tolerans* were identical with respect to both ITS length and DNA sequence and hence represented one branch in the phylogenetic tree (Fig. 2a).

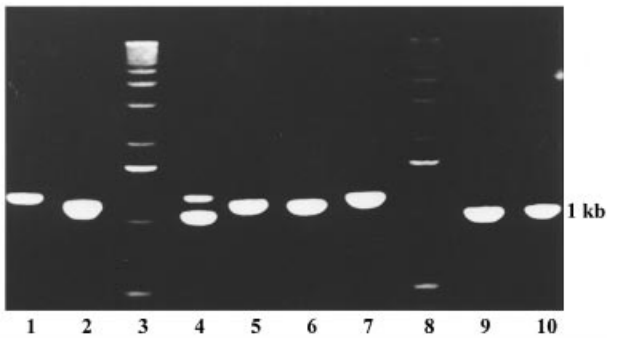

Fig. 1. Ethidium bromide-stained agarose gel displaying length variations of 16S–23S rDNA ITS PCR fragments. Lanes: 1, *Roseovarius tolerans* EL-164 (~ 1200 bp); 2, *Roseovarius tolerans* EL-171 (~ 1100 bp); 3, 1 kb ladder; 4, *Roseovarius tolerans* EL-172^T (~ 1000 bp plus ~ 1200 bp); 5, *A. heliothermus* EL-219^T (~ 1100 bp); 6, *Roseovarius tolerans* EL-222 (~ 1100 bp); 7, *Ruegeria algicola* DSM 10218^T (~ 1200 bp); 8, 1 kb ladder; 9, *Roseobacter litoralis* DSM 6996^T (~ 1000 bp); and 10, *Roseobacter denitrificans* DSM 7001^T (~ 1000 bp).

A search for tRNAs within the ITS resulted in a set of two tRNA genes found in each strain, $tRNA_{Ala}$ and $tRNA_{Ile}$. The $tRNA_{Ile}$ consisted of 77 bp (identical in all strains) and the $tRNA_{Ala}$ was 76 bp. According to tRNA consensus structure numbering, the length difference between the tRNAs was caused by an additional U in a variable position (20a) of the $tRNA_{Ile}$ D loop. Structural differences and the relative positions and order of tRNA genes identified are shown in Table 1. Sequence differences in the conserved base-paired positions 51/63 and 52/62 in the $tRNA_{Ala}$ TΨC stem allowed the differentiation of two groups: (i) *Roseobacter denitrificans* and *Roseobacter litoralis* and (ii) *Roseovarius tolerans*, *Ruegeria algicola* and *A. heliothermus*. The latter species was separated from the other two species as a single line of descent by an inverse order of the tRNA genes (Table 1). Strains of *Roseovarius tolerans* and *Ruegeria algicola* contained the same set of tRNA genes with respect to structure and order, but in different relative locations with respect to flanking rRNA genes. Considering the tRNA genes only, further discrimination of the *Roseovarius tolerans* strains was not possible (Table 1). All tRNA features described supported individual branches of the ITS tree derived from the phylogenetic analyses of total ITS sequences (Fig. 2a).

The overall presence of a phylogenetic signal within the ITS sequences was demonstrated by a left-skewed distribution (data not shown) of 10^6 randomly sampled trees (parsimony analyses), according to Hillis & Huelsenbeck (1992). Data sets containing phylogenetic signals result in a significantly left-skewed distribution, while those containing random noise result in symmetrical distributions. The g1 statistic is a measure of the skewness of a distribution and critical values of g1 for tree length distributions of data sets containing different taxa, sequence lengths and variabilities are given by Hillis & Huelsenbeck (1992). The calculated value for g1 of −1·40 is considerably less than the 95% and even the 99% critical value (between −0·39 and

Table 1. Features of 16S–23S rDNA ITS sequences

Sequences include 32 nucleotides from the 3′ end of the 16S rDNA and approximately 120 nucleotides from the 5′ end of the 23S rDNA.

Species	Strain	Length (nt)	$tDNA_{Ile}$ location (nt)	$tDNA_{Ala}$ location (nt)	Base substitution in the TψC arm of $tRNA_{Ala}$*	Accession number
Roseovarius tolerans	EL-164	1165	207–283	425–500	AGGAGUUCGAUCCUCCU‡ >>>>.....<<<<	AJ012701
	EL-171	1083	207–283	425–500		AJ012702
	EL-172^T†	959	170–246	346–421		AJ012700
	EL-222	1083	207–283	425–500		AJ012703
Ruegeria algicola	DSM 10251^T	1158	239–315	492–567	AGGAGUUCGAUCCUCCU >>>>.....<<<<	AJ012705
A. heliothermus	EL-219^T	1092	350–426	250–325	AGGAGUUCGAUCCUCCU >>>>.....<<<<	AJ012704
Roseobacter denitrificans	DSM 7001^T	980	243–319	338–413	AGCGGUUCGAUCCCGCU >>>>.....<<<<	AJ012706
Roseobacter litoralis	DSM 6996^T	984	243–319	338–413	AGCGGUUCGAUCCCGCU >>>>.....<<<<	AJ012707

* Base positions 49–65 according to tRNA consensus numbering; >>, << and dots indicate bases involved in stem–loop structures.
† ITS sequence from lower band (Fig. 1).
‡ Identical in all *Roseovarius tolerans* strains.

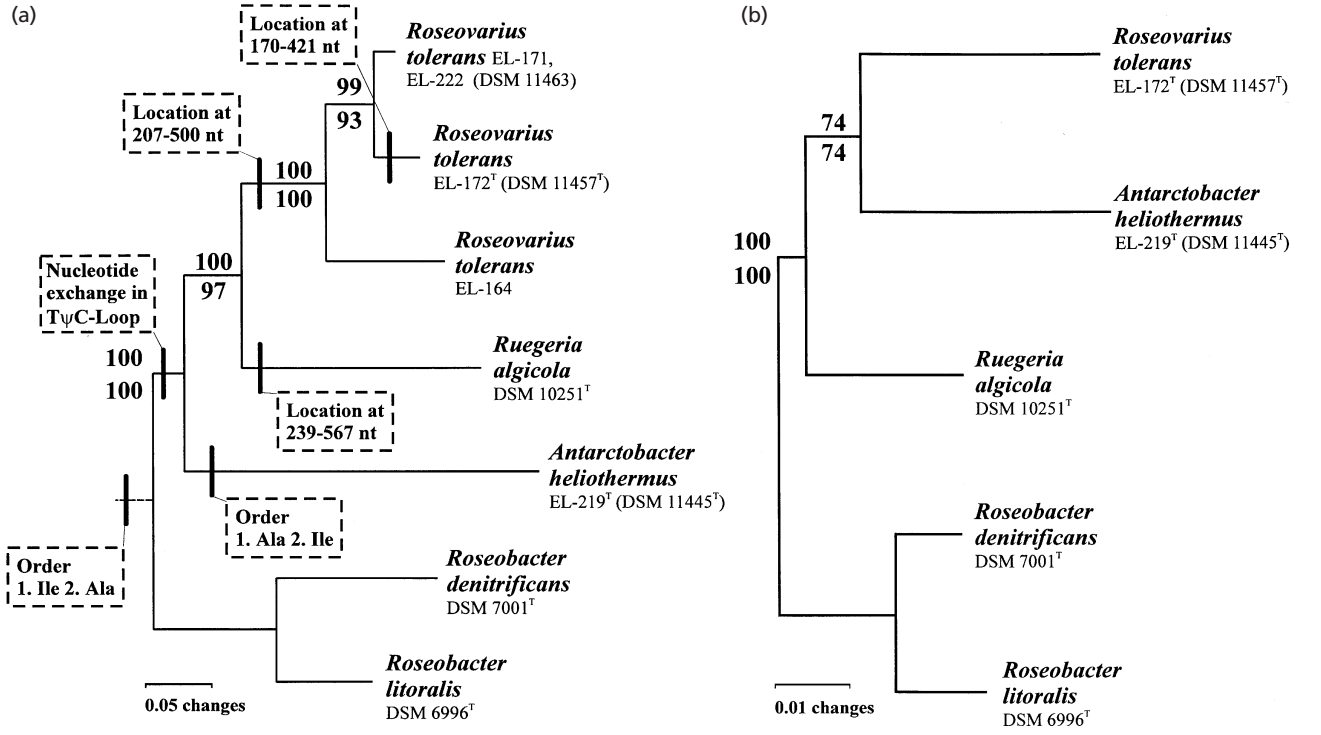

Fig. 2. Phylogenetic trees reconstructed from ITS (a) and 16S rDNA (b) sequence alignments (1279 and 1278 positions, respectively) using PAUP. Rooting was done using the sequences of *Roseobacter litoralis* and *Roseobacter denitrificans* as a monophyletic outgroup. Bootstrap values (%) obtained from resampling of 1000 replications are indicated above (NJ) and below (MP) branches. NJ trees were calculated using pairwise distances (Table 2); the scale bar indicates base changes. MP trees were based on 390 (ITS) or 60 (16S rDNA) parsimony-informative positions (Branch and Bound search, unweighted). MPT step-length (ITS/16S rDNA) = 1329/187, consistency index = 0·877/0·888. 'Heuristic search' was applied for computation of bootstrap values. Additional support of individual branches (ITS tree only) by tRNA characters is indicated.

Table 2. Pairwise computed distances between ITS and 16S rRNA gene sequences

Values shown are the percentages of nucleotide positions where the two sequences differ. The sequences compared are those of the ITS (below the diagonal) and 16S rRNA genes (above the diagonal). ND, Not determined.

Taxon	1	2	3	4	5	6	7
1. *Roseovarius tolerans* EL-171, EL-222	–	0*	0*	ND	ND	ND	ND
2. *Roseovarius tolerans* EL-172^T	5·10	–	0*	5·51	6·50	7·30	7·33
3. *Roseovarius tolerans* EL-164	12·26	17·86	–	ND	ND	ND	ND
4. *Ruegeria algicola* DSM 10251^T	32·33	28·46	33·64	–	6·54	5·23	4·79
5. *A. heliothermus* EL-219^T	43·22	43·78	46·75	48·12	–	6·81	6·28
6. *Roseobacter denitrificans* DSM 7001^T	40·91	40·98	41·62	47·79	48·94	–	2·09
7. *Roseobacter litoralis* DSM 6996^T	36·24	35·32	37·47	40·11	46·50	19·24	–

* The 16S rRNA genes of *Roseovarius tolerans* non-type strains were partially sequenced (approx. 800 bp) and revealed 100% identity to *Roseovarius tolerans* EL-172^T (Labrenz *et al.*, 1999).

−0·49 for seven taxa and 250–500 parsimony-informative characters). This suggests that the ITS sequence alignment contains a significant phylogenetic signal.

Genetic distances of both ITS and 16S rDNA sequences are shown in Table 2. Pairwise computed distances of ITS sequences were approximately 5- to 9-fold larger than 16S rDNA distances. Phylogenetic trees reconstructed from ITS sequences using the neighbour-joining (NJ) or maximum-parsimony (MP) methods are shown in Fig. 2(a). The two tree reconstruction methods resulted in trees of identical topologies and were supported by high bootstrap values. Individual branches of the trees were additionally supported by the results of the comparative structural analysis and relative gene positions of tRNAs within the ITS (Table 1), as indicated on the phylogenetic tree (Fig. 2a). The fact that the two tree reconstruction methods produced identical trees indicated that the phylogenetic relationships presented here were probably not affected by the program algorithms applied. The species, as well as most strains, were clearly separated and strains of a species clustered together. All strains of *Roseovarius tolerans* formed a monophyletic branch separated from *A. heliothermus* EL-219^T by *Ruegeria algicola*. However, this did not agree with the tree reconstructed from 16S rDNA sequences (Fig. 2b).

Again, the two phylogenetic trees reconstructed from 16S rDNA sequences using the NJ and MP algorithms showed consistent topologies (Fig. 2b), contradicting the close relationship of *Ruegeria algicola* and *Roseovarius tolerans* suggested by the ITS tree. Instead, *Roseovarius tolerans* and *A. heliothermus* appeared as sibling species, but less well supported by bootstrapping than the ITS tree.

DISCUSSION

ITS length differences of about 200 bp were detected between EL-164 and the lower band of *Roseovarius tolerans* EL-172^T. Species- and even strain-level relationships were consistently resolved in a phylogenetic analysis of the ITS sequences independently of the method of tree construction (NJ or MP). The positions of individual branches were strongly supported by bootstrap values and by structural peculiarities of tRNA genes. Differences found between strains of *Roseovarius tolerans* in the relative position of tRNAs corresponded to phenotypic heterogeneities. Previous studies have revealed strong resemblances between the strains with respect to chemotaxonomical, biochemical and physiological data (Labrenz *et al.*, 1999), but also a few minor heterogeneities. However, no further correlations between ITS sequence data and phenotypic heterogeneities could be found.

Compared with the position and clustering of the genera *Antarctobacter* and *Roseovarius* in the 16S rDNA tree of Labrenz *et al.* (1998) and a corresponding sub-tree including only the strains of this present study (Fig. 2b), a better-supported resolution of relationships based on ITS sequences is shown in Fig. 2(a). In contrast, phylogenetic trees obtained from the 16S rRNA alignment were less well supported by bootstrap values (Fig. 2b), especially if one considers relationships within the cluster formed by *Ruegeria algicola*, *Roseovarius* and *Antarctobacter*. A questionable reliability was already apparent from the clearly poor g1 value ($g1 = -0{\cdot}622$). While critical values for five taxa and 60 parsimony-informative characters range between −0·78 and −0·93 (Hillis & Huelsenbeck, 1992), g1 values from data that are significantly more structured than random should be more negative. This was probably due to the strikingly smaller sequence distances compared with the ITS sequences (Table 2). Greater distances would be needed for reliable resolution of interspecific and interstrain relationships in phylogenetic reconstructions. Consequently, the strongly supported relationship of *Roseovarius* and *Ruegeria algicola* in the ITS tree (Fig. 2a, bootstrap values 100 or 97%) was more probable

than the less well supported relationship of *Roseovarius* and *Antarctobacter* in the 16S rDNA tree (Fig. 2b, bootstrap value 74%). This conclusion was additionally supported by the inverse order of tRNA genes in the ITS of *A. heliothermus* (Fig. 2b), separating it from *Ruegeria algicola* and *Roseovarius tolerans*. The separation of *Ruegeria algicola* from the outgroup *Roseobacter denitrificans* and *Roseobacter litoralis* was strongly supported by sequence differences in the conserved stem of the TΨC arm.

Thus, although the ITS and 16S rDNA trees do not agree concerning the phylogenetic relationships of *Ruegeria algicola*, the ITS tree presented here is in good agreement with the 16S rDNA tree of Labrenz *et al*. (1998), which included more species. Similarly, the addition of more ITS sequences from isolates in the phylogenetic vicinity of *Ruegeria algicola* would lead to a more profound view. The use of further chemotaxonomic data is recommended in order to gain additional support.

Generally, the presence of multiple rDNA operons in certain species (Hain *et al*., 1997) may cause problems in a rapid ITS analysis when PCR fragments are sequenced directly. In these cases, a preparation of only the smallest ITS band would give enough information for well-supported phylogenetic relationships, as was shown with *Roseovarius tolerans* EL-172^T (Fig. 2a). However, the analysis of multiple ITS sequences of one strain could complicate resolution of inter-strain relationships, as was reported for two *Bacillus subtilis* strains (Nagpal *et al*., 1998).

In conclusion, the application of rapid, direct ITS sequence analyses and reconstruction of phylogenetic relationships (which were reported here for a particular group of the α-*Proteobacteria*) has become increasingly important when focusing on the species level of biodiversity. Small subunit rDNA analyses (prokaryotes and eukaryotes) or mitochondrial sequence analysis (eukaryotes) would not cope with the demand for the phylogenetic resolution that is needed to describe the notable inter- and intraspecific biodiversity that is expected (Pace, 1997; Stork, 1997).

ACKNOWLEDGEMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft to P. H. (Hi 68/25-1 to -3).

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