16S rRNA Gene Sequence of *Rubrobacter radiotolerans* and Its Phylogenetic Alignment with Members of the Genus *Arthrobacter*, Gram-Positive Bacteria, and Members of the Family *Deinococcaceae*

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The nearly complete sequence of the 16S rRNA gene of an extremely highly radiotolerant bacterium, *Rubrobacter radiotolerans* (reclassified from *Arthrobacter radiotolerans* based on chemical characteristics), was determined by PCR amplification of the genomic DNA followed by cloning of the amplified gene and sequencing by the dideoxynucleotide method. The sequence was aligned with the sequences of members of the genus *Arthrobacter* and also with the sequences of representatives of the gram-positive bacteria having high G+C contents and the family *Deinococcaceae* (radioresistant micrococci and their relatives). The results of our phylogenetic analysis confirmed that *R. radiotolerans* is not a member of the *Arthrobacter* group and thus supported the previous reclassification. Moreover, although it is radioresistant and has a high G+C content, *R. radiotolerans* is more closely related to the gram-positive bacteria with high G+C contents than to the radioresistant members of the *Deinococcaceae*.

Arthrobacter radiotolerans, an extremely highly radiotolerant bacterium, was isolated by Yoshinaka et al. (21) from a radon-containing hot spring in Misasa, Tottori, Japan. On the basis of its chemical characteristics, this organism was reclassified in a new genus as *Rubrobacter radiotolerans* (16). *Rubrobacter radiotolerans* is gram positive and forms short, pleomorphic, rod-shaped cells. It has an optimum growth temperature of about 48°C. This bacterium is extremely radioresistant; it has an inactivation dose (D_0 value) of 10,000 Gy, which is 2,000 times greater than the D_0 value of human cells (5 Gy) and also is much greater than the D_0 values of other known radioresistant bacteria (12).

Uncertainty concerning the phylogenetic position of *Rubrobacter radiotolerans* (formerly *Arthrobacter radiotolerans*) in relation to members of the genus *Arthrobacter* prompted us to study the phylogenetic and evolutionary relationships of this bacterium. In addition, since this organism is radioresistant and has a high G+C content, we were also interested in determining its relationships to bacteria that are gram positive and have high G+C contents and to members of the family *Deinococcaceae* (radioresistant micrococci and their relatives) (19).

In contrast to traditional bacterial classification, which is based on phenotypic characteristics and principally involves analyses of differential metabolic properties, 16S rRNA similarities are now generally used to deduce phylogenetic and evolutionary relationships among bacteria, archaeobacteria, and eukaryotic organisms. 16S rRNA sequences are obtained by various methods, including oligonucleotide cataloging (6), sequencing of clones, direct sequencing of RNA with reverse transcriptase (9), and sequencing of cloned genes obtained by the PCR (2). Approximately 3,500 complete 16S rRNA sequences have been determined (3).

In this study, the 16S rRNA gene of *Rubrobacter radiotolerans* was amplified by PCR and was ligated into the pUC19 vector. The purified plasmid was sequenced by the dideoxynucleotide method (15). The resulting sequence was aligned with the sequences of representatives of the genus *Arthrobacter*, gram-positive bacteria having high G+C contents, and members of the *Deinococcaceae*. Our results indicate that *Rubrobacter radiotolerans* is not a member of the *Arthrobacter* group. In addition, *Rubrobacter radiotolerans* is more closely related to gram-positive bacteria with high G+C contents than to the radioresistant members of the *Deinococcaceae*.

MATERIALS AND METHODS

Culture of *Rubrobacter radiotolerans*. *Rubrobacter radiotolerans* JCM 2153^T (= IAM 12072^T) was obtained from the Japan Collection of Microorganisms. R broth (20) was used to culture the organism. Cells were grown at 48°C for 2 days. The exponentially growing cells were harvested by centrifugation at 5,000 × g for 10 min at 4°C. The bacterial cells were washed twice with TNE buffer (0.01 M Tris-HCl, 0.01 M NaCl, 0.01 M EDTA; pH 8.0) and were stored by freezing at –20°C.

DNA extraction. DNA was extracted by the modified method of Head et al. (7). A 0.1-g portion of biomass was transferred to a microcentrifuge tube and washed three times with 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). The cell pellet was resuspended in 100 µl of lysis buffer (TE buffer containing 1% [vol/vol] Tween 80). The cell suspension was placed in boiling water for 1 min and then snap frozen in dry ice. This procedure was repeated three times, and the cell lysate was extracted with chloroform. The aqueous layer, which was separated during centrifugation at 5,000 × g for 5 min, was used as a source of DNA for PCR amplification.

PCR amplification of the 16S rRNA gene. The 16S rRNA gene was amplified by using primers fd1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGG CTCAG-3') and rd1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAG CC-3') as forward and reverse primers, respectively (18); these primers include *SalI* and *BamHI* restriction sites (rarely found internally in 16S ribosomal DNA), respectively, to facilitate cloning into compatible vectors. The PCR was performed by the procedure of Nazaret et al. (11) for 30 cycles (with each cycle consisting of 1 min at 93°C, 1 min at 57°C, and 1 min of extension at 70°C) with a thermocycler (model 2400 Gene Amp PCR System; Perkin-Elmer Co., Norwalk, Conn.). Each reaction mixture (100 µl) contained 2 to 5 µl of DNA solution, the primers (each at a concentration of 1 µM), a mixture of deoxynucleoside triphosphates (each at a concentration of 200 µM), and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Co.) in buffer supplied by the manufacturer. A 5-µl aliquot of the reaction mixture was examined visually on a 1.5% (wt/vol) agarose gel stained with ethidium bromide. A single amplified product

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FIG. 1. Phylogenetic tree for *Rubrobacter radiotolerans* and seven *Arthrobacter* species. Bar = 0.1 K_{nuc} . The numbers at the major branches are bootstrap values determined from 1,000 trials. Abbreviations: Ar., *Arthrobacter*; R., *Rubrobacter*.

that was approximately 1.5 kb long was observed. The PCR product was purified from a 1% (wt/vol) low-melting-temperature agarose gel (Nippon Gene Co., Ltd, Tokyo, Japan) by the method of Sambrook et al. (14), and the resulting product was suspended in TE buffer.

Isolation and cloning of the amplified 16S rRNA gene. The PCR product was digested with restriction enzymes *Sal*I and *Bam*HI as recommended by the manufacturer (Boehringer Mannheim GmbH, Mannheim, Germany). The digests were extracted with phenol-chloroform and precipitated with ethanol. The pUC19 vector was digested with *Sal*I and *Bam*HI and purified from a 1% (wt/vol) low-melting-temperature agarose gel by the method of Sambrook et al. (14). The digested vector and PCR product were resuspended in TE buffer and ligated with T4 ligase (Takara Biomedicals, Shiga, Japan). Since *Sal*I and *Bam*HI produce compatible ends after digestion, the PCR products were cloned into pUC19 in known orientations. After ligation at 16°C for 30 min, *Escherichia coli* XL1-blue cells were transformed with the plasmid containing the 16S rRNA gene (1).

Sequencing of the 16S rRNA gene. Plasmids containing the 16S rRNA gene were isolated by the standard purification method (14). Purified plasmids were sequenced by using a PRISM ReadyReaction DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer Co.) with AmpliTaq DNA polymerase. Sequencing gel electrophoresis was performed and nucleotide sequences were obtained automatically by using an Applied Biosystems model 373A DNA sequencer. The protocol and software recommended by the manufacturer were used. The primers used for sequencing were forward primer P7 (5'-CGCCAGGGTTTCCCA GTCACGAC-3') and reverse primer P5 (5'-CAGGAAACAGCTATGAC-3').

Phylogenetic comparison. A phylogenetic analysis was performed by using the package program CLUSTAL W (17). Alignment of 16S rRNA sequences, estimation of genetic distances (K_{nuc} values) from the alignment by Kimura's two-parameter method (8), tree construction from the distances by the neighbor-joining method (13), and evaluation of the confidence intervals with bootstrap values (5) were performed by using the programs ALIGN, DNADIST, PHYLIP, and PHYLIPBOOT in CLUSTAL W, respectively. Only data for homologous sites at which alignment of the 16S rRNA gene sequences was unambiguous were included in the phylogenetic analysis. The sequences used to construct the phylogenetic tree in Fig. 1 included the sequences of *Arthrobacter ureafaciens*, *Arthrobacter globiformis*, *Arthrobacter nicotianae*, *Arthrobacter ilicis*, *Arthrobacter protophormiae*, *Arthrobacter pascens*, and *Arthrobacter uratoxydans*. The phylogenetic tree in Fig. 2 includes *Rubrobacter xylanophilus*, *Actinomyces bovis*, *Propionibacterium freudenreichii*, *Mycobacterium tuberculosis*, *Rhodococcus rhodochrous*, strain TH3, *Acidothermus cellulolyticus*, *Deinococcus radiodurans*, *Deinococcus* sp., *Thermus aquaticus*, *Thermus ruber*, *Thermus* sp. strain YS38, *Thermus* sp. strain Vil7, and *Thermus* sp. strain SPS14.

Nucleotide sequence accession numbers. The sequence of the 16S rRNA gene of *Rubrobacter radiotolerans* has been deposited in the GenBank data library under accession no. U65647. The accession numbers for the other sequences used to construct the phylogenetic trees are given in Table 1.

RESULTS

Double-stranded amplification with primers fD1 and rD1 (4, 18) of the gene encoding the 16S rRNA of *Rubrobacter radiotolerans* resulted in a single amplified product that was approximately 1.5 kb long. This PCR product was purified and used as the sequencing template. The amount of DNA solution used for PCR amplification was 2 to 5 μ l. If this amount was decreased or increased, the amplification efficiency decreased.

In our experiment a sequence that was 1,550 bases long was obtained after eight repetitions of the experiment with differ-

FIG. 2. Phylogenetic tree for *Rubrobacter radiotolerans*, gram-positive bacteria with high G+C contents, and members of the *Deinococcaceae*. Bar = 0.1 K_{nuc} . The numbers at the major branches are bootstrap values determined from 1,000 trials. Abbreviations: Acido., *Acidothermus*; Actino., *Actinomyces*; D., *Deinococcus*; M., *Mycobacterium*; P., *Propionibacterium*; R., *Rubrobacter*; T., *Thermus*; Rhodo., *Rhodococcus*.

ent clones, and this sequence was aligned with the 16S rRNA gene sequences of seven species of the genus *Arthrobacter*, including *Arthrobacter nicotianae*, *Arthrobacter protophormiae*, *Arthrobacter ilicis*, *Arthrobacter pascens*, *Arthrobacter ureafaciens*, *Arthrobacter globiformis*, and *Arthrobacter uratoxydans*, whose 16S rRNA sequences were available and sufficient to establish the phylogenetic standing of the organisms. The phylogenetic analysis data are summarized in Fig. 1. The tree that was constructed indicates that all seven *Arthrobacter* species are closely related to each other but not to *Rubrobacter radiotolerans*.

Figure 2 shows another phylogenetic tree that was obtained by the method described above, and on this tree *Rubrobacter*

TABLE 1. Strains and accession numbers of the 16S ribosomal DNA sequences used in this study

Taxon	Nucleotide sequence accession no.
<i>Arthrobacter ureafaciens</i>	X80744
<i>Arthrobacter globiformis</i>	X80736
<i>Arthrobacter nicotianae</i>	X80739
<i>Arthrobacter ilicis</i>	X83407
<i>Arthrobacter protophormiae</i>	X80745
<i>Arthrobacter pascens</i>	X80740
<i>Arthrobacter uratoxydans</i>	X83410
<i>Rubrobacter xylanophilus</i>	X87135
<i>Actinomyces bovis</i>	X53224
<i>Propionibacterium freudenreichii</i>	X53217
<i>Mycobacterium tuberculosis</i>	X52917
<i>Rhodococcus rhodochrous</i>	X79288
Strain TH3.....	M79434
<i>Actinothermus cellulolyticus</i>	X70635
<i>Deinococcus radiodurans</i>	M21413
<i>Deinococcus</i> sp.	D78365
<i>Thermus aquaticus</i>	L09663
<i>Thermus ruber</i>	Z15059
<i>Thermus</i> sp. strain YS38.....	Z15062
<i>Thermus</i> sp. strain Vil7.....	Z15061
<i>Thermus</i> sp. strain SPS14.....	Z15060

radiotolerans is compared with gram-positive bacteria having high G+C contents and members of the *Deinococcaceae*. The tree in Fig. 2 indicates that *Rubrobacter radiotolerans* is related to the gram-positive bacteria having high G+C contents but not to members of the *Deinococcaceae* (radioresistant micrococci and their relatives) despite the fact that both *Rubrobacter radiotolerans* and members of the *Deinococcaceae* are highly radiotolerant.

DISCUSSION

An investigation of the chemotaxonomic characteristics of *Arthrobacter radiotolerans* was performed by Suzuki et al. (16). This organism has a peptidoglycan based on L-lysine (variation A3 α) and a DNA base composition of 67.9 mol% G+C. The cellular fatty acids are primarily methyl-branched fatty acids, and 12-methylhexadecanoic acid is the predominant component. The major isoprenoid quinone is menaquinone with eight isoprene units, and the polar lipids comprise four phospholipids, one phosphoglycolipid, and one glycolipid.

When Suzuki et al. (16) suggested that *Arthrobacter radiotolerans* should be reclassified in the new genus *Rubrobacter* as *Rubrobacter radiotolerans* on the basis of the characteristics mentioned above, the 16S rRNA gene sequence analysis technique had not been developed. However, this technique has since become invaluable for taxonomic assessments. The RNA sequence is considered a reliable phylogenetic indicator since the variability in this conserved molecule reflects mutations that occur at a relatively regular pace over the course of organism evolution. We analyzed the 16S rRNA gene of *Rubrobacter radiotolerans* to elucidate the development of radiotolerance in this organism in an evolutionary context.

On the basis of the 1,550-bp sequence determined, the phylogenetic relationship of *Rubrobacter radiotolerans* to members of the genus *Arthrobacter* with whom it was grouped previously was determined (Fig. 1). Since *Rubrobacter radiotolerans* is a gram-positive bacterium with a high G+C content and exhibits radioresistance, we also determined its phylogenetic relationships to representative gram-positive bacteria with high G+C contents (19) and to members of the *Deinococcaceae* (radioresistant micrococci and their relatives) (19) (Fig. 2). Figure 1 shows that all seven members of the genus *Arthrobacter* are phylogenetically very closely related to one another. *Rubrobacter radiotolerans* is, however, phylogenetically very distantly related to these organisms. Thus, we concluded that the decision to transfer *Arthrobacter radiotolerans* to the new genus *Rubrobacter* as *Rubrobacter radiotolerans* is reasonable in light of the results of our phylogenetic analysis of the 16S rRNA gene, as well as the chemical characteristics of this bacterium determined previously (16).

The phylogenetic tree in Fig. 2 shows that *Rubrobacter radiotolerans* is a member of the group that contains the gram-positive bacteria with high G+C contents but is not a member of the *Deinococcaceae* (radioresistant micrococci and their relatives). Thus, while both *Rubrobacter radiotolerans* and members of the genus *Deinococcus* exhibit radioresistance, *Rubrobacter radiotolerans* is located some distance from the *Deinococcaceae* on the phylogenetic tree. On the basis of 16S rRNA similarity values, *Rubrobacter radiotolerans* is more closely related to gram-positive bacteria with high G+C contents. Moreover, *Rubrobacter radiotolerans* is very closely related to *Rubrobacter xylanophilus*, which has recently been iso-

lated from thermally polluted industrial runoff (10); this finding supports our view that despite the radiotolerance of *Rubrobacter radiotolerans*, the correct position of this bacterium is among the gram-positive bacteria with high G+C contents rather than among the members of the *Deinococcaceae*.

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