Microbiology, Vol. 70, No. 6, 2001, pp. 678–684. Translated from Mikrobiologiya, Vol. 70, No. 6, 2001, pp. 788–795.

EXPERIMENTAL ARTICLES

Heterogeneity of the Nucleotide Sequences of the 16S rRNA Genes of the Type Strain of *Desulfotomaculum kuznetsovii*

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Received June 28, 2001

Abstract—Two copies of the 16S rRNA gene, *rrnA* and *rrnB*, of the type strain 17^T of the thermophilic sulfate-reducing bacterium *Desulfotomaculum kuznetsovii* were cloned and completely sequenced. The comparison of the determined sequences revealed considerable heterogeneity (8.3%) of the two genes, *rrnA* and *rrnB*. The main differences were associated with superlong inserts located in the variable 5'- and 3'-terminal regions of the 16S rRNA genes. Comparative analysis that involved analogous genes from the phylogenetically closest representatives of the genus *Desulfotomaculum* showed that disregard of the heterogeneity of the two gene copies distorts the position of the bacterium studied in the phylogenetic tree.

Key words: Desulfotomaculum kuznetsovii, 16S rRNA genes, *rrnA* and *rrnB*, phylogenetic analysis.

Comparative analysis of the genes coding for the RNA of the small ribosomal subunit (16S rRNA) is currently widely used to determine phylogenetic relationships of prokaryotes and is virtually obligatory for the determination of the taxonomic position of newly isolated organisms. In addition, the analysis of 16S rRNA genes is the main instrument of molecular ecology, which allows researchers to obtain data on the diversity of prokaryotes in various natural ecosystems. As distinct from traditional microbiological approaches, this method does not require isolation of pure cultures of the individual members of a microbial community but is based on the estimation of the diversity of 16S rRNA genes present in the total DNA of the community studied. It is thus assumed that each strain (cultured organism) and each community member (uncultured organism) is represented by a unique sequence of the 16S rRNA gene (phylotype). This assumption is undoubtedly valid if each organism contains in its genome a single copy of the 16S rRNA gene. This is indeed so for the majority of prokaryotes [1]. However, in many prokaryotes, the 16S rRNA gene is present in several copies. For example, there exist microorganisms that contain in their genomes three (*Rhodobacter sphaeroides*) [2], four (*Streptococcus gordonii*) [3], six (*Streptomyces coelicolor*) [4], seven (*Escherichia coli* and *Salmonella typhimurium*) [5], ten (*Bacillus subtilis*) [6], and even fifteen (*Clostridium paradoxum*) [7] copies of the 16S rRNA gene.

The discovery of the occurrence of multiple copies of 16S rRNA genes in the genomes of some prokaryotes necessitates determination of the degree of heterogeneity of the copies. Comparative analysis of sequences available from GenBank revealed a certain level of intrastrain distinctions that could not be explained solely by experimental errors [8]. However, the level of distinctions usually did not exceed 2%; this confirmed the common opinion that different copies of 16S rRNA genes present in the genome of one organism are virtually identical and allowed the analysis of 16S rRNA gene sequences to be widely used in phylogenetic studies.

At the same time, there are data on a considerable heterogeneity of individual copies of the 16S rRNA gene in some prokaryotes. Thus, the 15 copies of the 16S rRNA gene of *Clostridium paradoxum* vary considerably in the structure of intervening sequences (IVS) present in the hypervariable region corresponding to positions 73–97 in *E. coli* numbering [7]. Considerable heterogeneity of the two copies of the 16S rRNA gene was revealed in *Haloarcula marismortui* (74 nucleotide substitutions, or 5%) [9, 10] and *Thermobispora bispora* (98 nucleotide substitutions, or 6.4%) [11]. Meanwhile, a 2.5–3.0% distinction (about 50 nucleotide substitutions in the gene) is considered

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0026-2617/01/7006-0678$25.00 © 2001 MAIK "Nauka / Interperiodica"

sufficient to claim that two compared prokaryotes belong to different species [12].

The aim of the present study was to verify the assumption that the genome of the type strain 17 of *Desulfotomaculum kuznetsovii* contains several copies of the 16S rRNA gene and to determine and compare their complete nucleotide sequences.

MATERIALS AND METHODS

Bacterial strain. This work used the type strain *D. kuznetsovii* 17^T (VKM B-1805, DSM 6115) isolated [13] and stored at the Laboratory of Oil Microbiology, Institute of Microbiology, Russian Academy of Sciences. Single bacterial colonies were obtained on agarized Widdel and Pfennig's medium [14] supplemented with sodium propionate and reduced with 360 mg/l of $Na_2S \cdot 9H_2$ and dithionite.

Isolation and characterization of total DNA. The isolation of DNA for the polymerase chain reaction (PCR) was carried out according to the Miniprep (Promega, United States) protocol with minor modifications. The concentration of the DNA preparations obtained was 5–7 μg/ml; RNA was present in trace amounts (less than 1% as determined electrophoretically).

PCR amplification. The genes studied were amplified by PCR with universal primers corresponding to the conserved site at the 3' and 5' ends of the gene [15]. PCR was conducted on a Cetus 480 (Perkin Elmers, Sweden) thermocycler using the BioTaq (Dialat LTD, Moscow) thermostable polymerase and according to the recommendations of the polymerase manufacturer. The volume of the reaction mixture was 20 μl. The temperature cycling was as follows: first cycle, 3 min at 94°C, 3 min at 50°C, and 3 min at 72°C; then 30 cycles of 30 s at 94°C, 30 s at 40°C, and 30 s at 72°C; and then final polymerization for 7 min at 72°C. The PCR products were analyzed by electrophoresis in 1% agarose gel. The bands were visualized in UV after staining the gels with ethidium bromide and photographed using a BioKom (Moscow) transilluminator. The isolation and purification of fragments from low-gelling-temperature agarose were performed using a Wizard PCR Preps kit (Promega, United States) according the manufacturer's recommendations.

Cloning and restriction analysis. Amplified fragments of the 16S rRNA genes were cloned in the Sma I site of the vector pGEM-3Z(f+) polylinker. To obtain recombinants, competent cells of *E. coli* DH5α were used. Screening for recombinants was performed after the treatment of plasmids isolated from single colonies with *Pvu*II restriction endonuclease in a reaction mixture containing 1 μg of plasmid DNA, 3 units of *Pvu*II restriction endonuclease, 10 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT. The mixture was incubated for 1 h at 37°C. Electrophoretic separation of the restriction products was accomplished in 1% agarose gel at an electric field intensity of 6 V/cm. As a molecular mass standard, Gene Ruler 1000 bp Fermentas (Sm#0321) was used.

Sequencing of the 16S rRNA genes. Sequencing of cloned 16S rRNA genes was performed on a DNA Sequencer 373A automatic sequencer using Applied Biosystems N 402080 Ready Reaction Dye Terminator Sequencing Kit with AmpliTaq Polymerase, FS, according to the manufacturer's recommendations. The primers used for sequencing were universal plasmid primers (SP6 and T7) and universal terminal and internal primers [15].

Phylogenetic analysis. The nucleotide sequences of the 16S rRNA genes were analyzed using the Ribosomal Database Project CHECK_CHIMERA facility. For the comparative analysis, the nucleotide sequences were aligned with the 16S rRNA gene sequences of related *Desulfotomaculum* species using a CLUSTALW program. Phylogenetic trees based on the 16S rRNA gene sequences were constructed using the neighbor-joining method implemented in the TREECONW software package [16]. The statistical significance of the branching order was assessed by bootstrap analysis of 100 alternative trees.

Deposition of sequences. The nucleotide sequences of the cloned 16S rRNA genes determined in this work were submitted to GenBank (accession numbers, AY036903 and AY036904).

RESULTS

Currently, the commonly accepted method for the determination of the primary structure of the 16S rRNA genes involves direct sequencing of the products of amplification performed with universal primers corresponding to the most rigorously conserved sites of the gene [15]. When universal primers are used, all copies of the 16S rRNA gene that are present in the genome of a given organism are amplified; therefore, direct sequencing of the amplificate is only possible if the gene is present in a single copy or if the copies are homogenous.

When sequencing by the above-mentioned method the 16S rRNA gene of strain *D. kuznetsovii* 17^T [17], we found superlong inserts (each about 120 nucleotides long) in the terminal regions of the gene corresponding to *E. coli* positions 68–101 and 1445–1457, which is unusual for the majority of such genes. Direct sequencing of these inserts of the 16S rRNA gene of *D. kuznetsovii* 17^T produced ambiguous results varying from experiment to experiment; the determined sequences failed to form an adequate secondary structure. On the contrary, the distinctions between the results of sequencing of the central part of the gene were insignificant and did not exceed the experimental error. Stackebrandt *et al.* [18] faced an analogous problem when performing a phylogenetic analysis of the genus *Desulfotomaculum.* Therefore, comparative phylogenetic

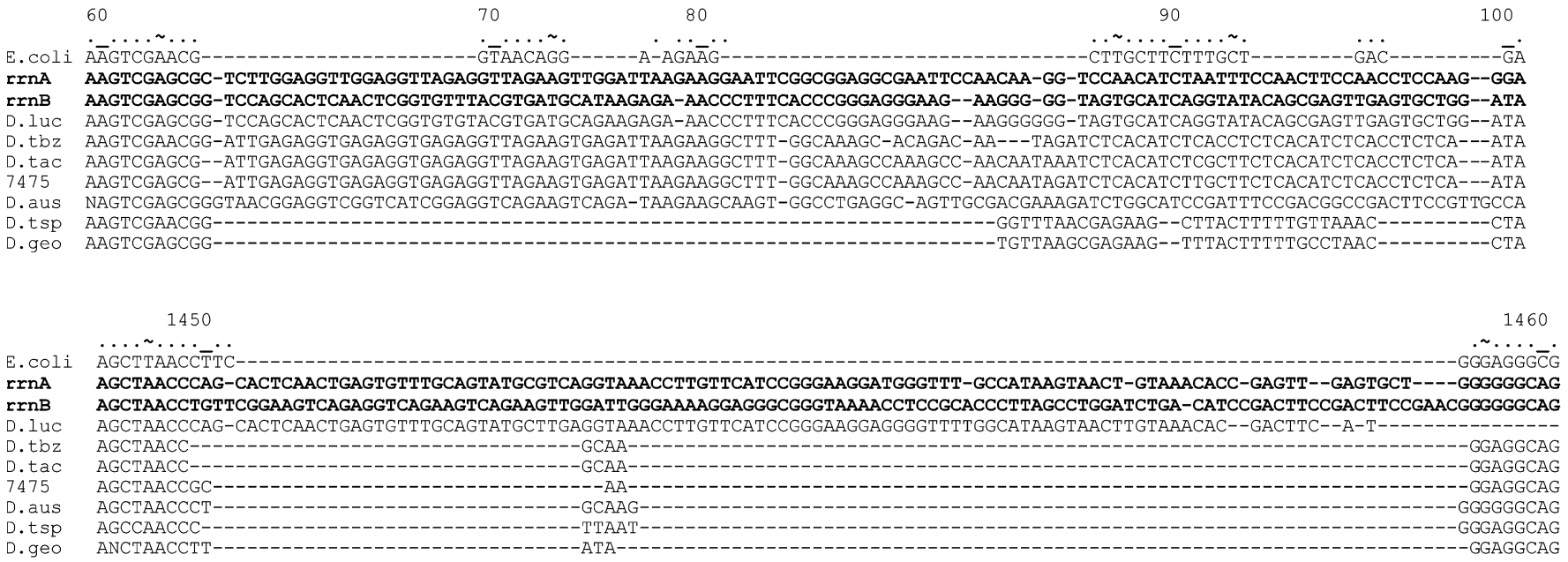

Fig. 1. Alignment of the 5'- and 3'-terminal variable regions of the two copies of the 16S rRNA gene of *D. kuznetsovii* 17^T with analogous regions of the 16S rRNA genes of related species of *Desulfotomaculum.* The designations are as follows: rrnA and rrnB, *D. kuznetsovii* 17^T; D. luc, *D. luciae* SLT^T; D. tbz, *D. thermobenzoicum* DSM 6193^T; D. tac, *D. thermoacetoxidans* DSM 5813^T; 7475, *Desulfotomaculum* sp. DSM 7475; D. aus, *D. australicum* ACM 3917^T; D. tsp, *D. thermosapovorans* DSM 6562^T; D. geo, *D. geothermicum* DSM 3669^T. Nucleotide positions are given in *E. coli* numbering.

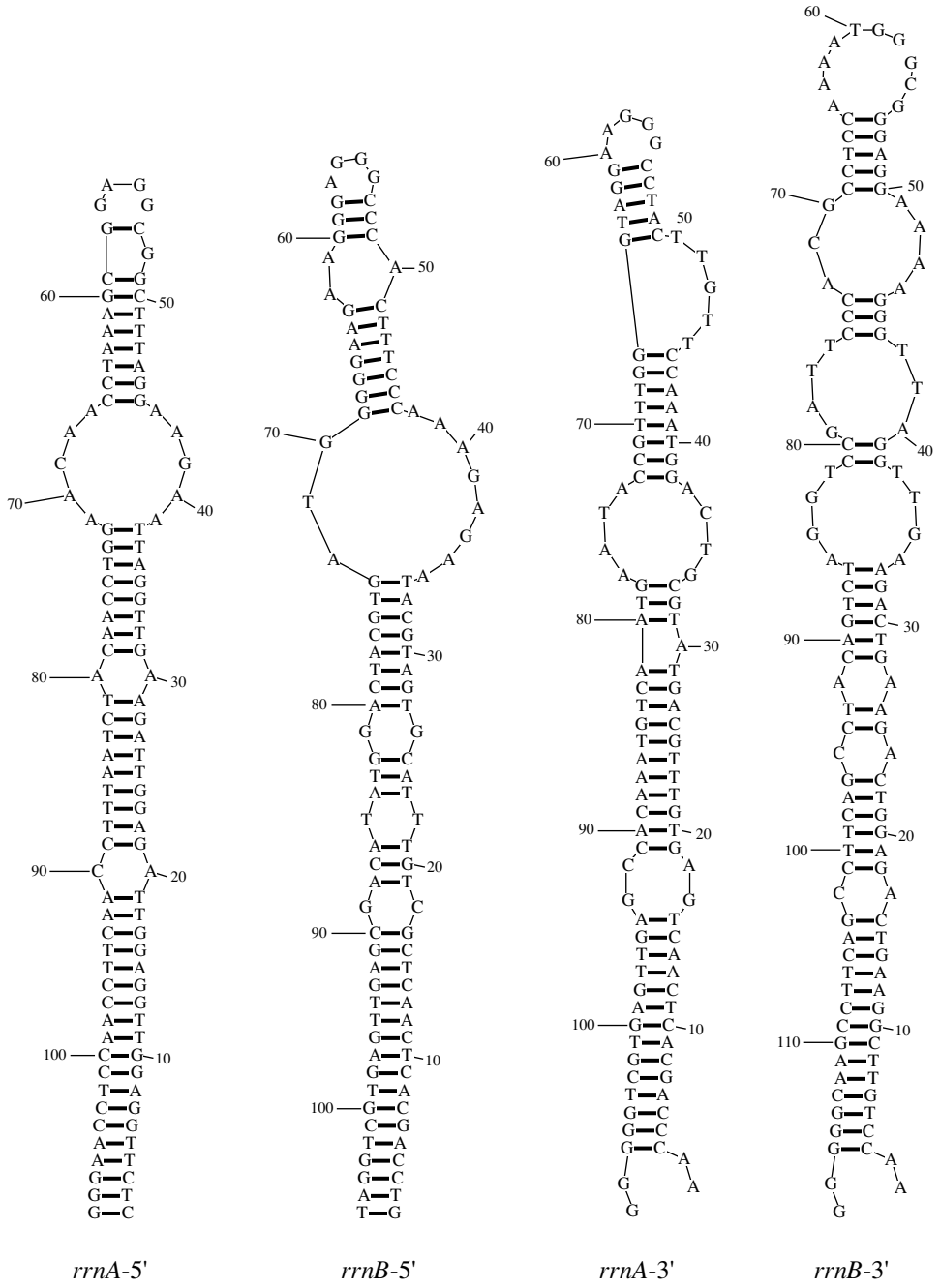

Fig. 2. Secondary structure of superlong helixes in the two copies of the *D. kuznetsovii* 17^T 16S rRNA gene. The numbering shows the positions of nucleotides in relation to the beginning of a helix.

analysis in this group of bacteria was carried out after the exclusion from the analysis of terminal regions beyond *E. coli* positions 100 and 1440 [17, 18].

Analogous inserts in the same regions were detected in *C. paradoxum* [7] and some thermophilic bacteria [19, 20]. In the genus *Desulfotomaculum*, such an insert was first found in *D. australicum* [21].

To verify the assumption of the existence in the genome of *D. kuznetsovii* 17^T of more than one copy of the 16S rRNA gene, we isolated DNA from a single colony, obtained (with the use of terminal universal primers) 1700-bp-long amplificates, and used them to produce a clone library. Restriction analysis of the clone library revealed 57 clones that carried the plasmid with an insert of the required size and subdivided these clones into two subgroups of 31 and 26 clones. One representative of each of the groups was taken for complete sequencing of the 16S rRNA genes.

As a result, complete nucleotide sequences of two copies of the 16S rRNA gene of *D. kuznetsovii* 17^T

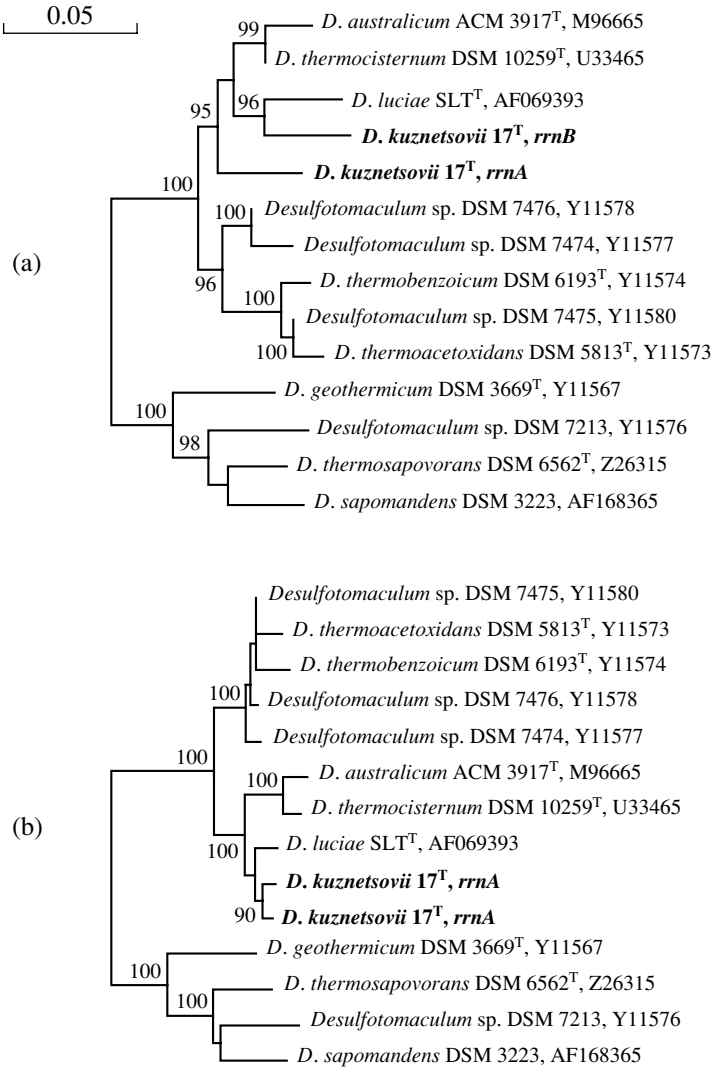

Fig. 3. Phylogenetic position of the *rrnA* and *rrnB* genes of *D. kuznetsovii* 17T. (a) The tree constructed based on the comparison of the complete sequences of the 16S rRNA genes. (b) The tree constructed upon discarding the variable 5'- and 3'-terminal regions of 16S rRNA genes. The bar corresponds to 5 nucleotide substitutions per 100 nucleotides. The figures at the branching points show the significance of the branching order as determined by bootstrap analysis (values higher than 90 were considered significant).

(*rrnA* and *rrnB*) were determined (1709 and 1715 nucleotides, corresponding to *E. coli* positions 1–1542). So far, none of the sequences of the 16S rRNA gene of *D. kuznetsovii* 17T available from GenBank (accession numbers AJ294427, AF009646, Y11569) is complete. Comparative analysis of the two gene copies sequenced in the present work revealed their considerable heterogeneity. The main distinctions were concentrated in the variable 5'- and 3'-terminal regions corresponding to *E. coli* positions 68–101 and 1445–1457. In these regions, we revealed inserts that differed considerably in the two gene copies by their length and primary structure. The length of the 5'-terminal inserts was 109 and 106 nucleotides for the *rrnA* and *rrnB* genes, respectively, and the length of the 3'-terminal inserts was 109 and 118 nucleotides. Less significant distinctions between the genes were found in the variable region corresponding to *E. coli* positions 178–198. The remaining parts of the sequences differed only in *E. coli* position 264 (T or C). The total level of the divergence of the sequences of the *D. kuznetsovii* 17T 16S rRNA gene copies was as high as 8.3%.

On the comparison of the sequences of the *D. kuznetsovii* 17T *rrnA* and *rrnB* genes with 16S rRNA genes of related *Desulfotomaculum* species (Fig. 1), it turned out that the 5'-terminal insert of the *rrnA* gene exhibits a 70% similarity with analogous inserts of the 16S rRNA genes of strains *D. thermobenzoicum* DSM 6193T, *D. thermoacetoxidans* DSM 5813T, and *Desulfotomaculum* sp. DSM 7475 and the 5'-terminal insert of the *rrnB* gene is identical to an analogous insert of *D. luciae* SLTT. The 3'-terminal insert of the *rrnA* gene displayed a 95% similarity with an analogous insert of *D. luciae* SLTT, and the 3'-terminal insert

of the *rrnB* gene proved to be unique and had no homologs.

Analysis of the secondary structure. The determination of the complete sequences of two copies of the *D. kuznetsovii* 17^T 16S rRNA gene, which we were the first to perform, allowed us to analyze the secondary structure of the superlong inserts. All four inserts formed adequate secondary structures in the form of helixes with minimum free energy dG = –64.5…–68.8 kcal/mol for the 5'-terminal helix and dG = –63.3…–64.0 kcal/mol for the 3'-terminal helix (Fig. 2). A similar superlong helix is formed by the insert found in the first hypervariable region (*E. coli* positions 68–101) of the 16S rRNA gene of *D. australicum* [21], an organism closely related to *D. kuznetsovii*. The deduced secondary structure of the superlong helixes considerably differs from the secondary structure usual for these rRNA regions; e.g., in the 16S rRNA of *E. coli* the corresponding 5'-terminal region forms a hairpin consisting of only 34 nucleotides (from position 68 to position 101), and the corresponding 3'-terminal region is a 13-nucleotide loop of a hairpin located between positions 1435 and 1466.

The effect of the heterogeneity of the 16S rRNA gene copies on phylogenetic constructs. Based on comparative analysis, two phylogenetic trees were constructed for several closely related thermophilic species of the genus *Desulfotomaculum*. For the construction of the first tree (Fig. 3a), complete sequences of 16S rRNA genes were used, including those of the two copies of this gene present in *D. kuznetsovii* 17^T. The second tree (Fig. 3b) was constructed based on the analysis that neglected the 5'- and 3'-terminal superlong inserts. In tree A, the *D. kuznetsovii* 17^T *rrnA* gene forms a separate line, whereas its *rrnB* forms a monophyletic cluster with the gene of *D. luciae* SLT^T. In tree B, the two genes of *D. kuznetsovii* 17^T are located close to each other, forming a monophyletic cluster; the difference between their reduced sequences is 0.8%, which does not exceed the experimental error.

DISCUSSION

The results obtained in this work demonstrate the occurrence of two heterogenous (more than 8% distinct) nucleotide sequences of 16S rRNA genes in the genome of *D. kuznetsovii* 17^T. The main distinctions are concentrated in two superlong inserts at the 5' and 3' termini of the 16S rRNA gene; these inserts are analogous to those discovered earlier in other representatives of *Desulfotomaculum* [21] and in some other genera [7, 19, 20]. The macroheterogeneity of the copies of the 16S rRNA gene that we discovered may significantly hinder taxonomic interpretation of phylogenetic data. These data may themselves be misleading if only one copy of a 16S rRNA gene is used in the analysis. The phylogenetic analyses of pure microbial cultures often use cloning of rDNA amplified by PCR instead of direct sequencing of PCR products. Further analysis uses a few clones or even a single clone. Thus, the existence of several 16S rRNA gene copies may be overlooked.

The microheterogeneity of 16S rRNA gene copies seems to be a usual phenomenon [8]; however, the really important question is whether macroheterogeneity (such as discovered in this and some other works [7, 9–11]) is a widely occurring phenomenon. This question, which requires further studies involving organisms from various phylogenetic groups, acquires utmost importance with the intensification of studies in the field of molecular ecology: so far it remains unclear whether the phylotypes revealed in these studies represent individual organisms or some of these phylotypes represent copies of 16S rRNA genes present in the same organisms.

It may be recommended that, to decrease distortion of phylogenentic data, hypervariable regions of 16S rRNA genes, especially those containing long inserts, should be excluded from the analyses.

According to the data of phylogenetic analysis, the organism most close to *D. kuznetsovii* 17^T is *D. luciae* SLT^T. It has been suggested that DNA–DNA hybridization should be performed to test the possible affiliation of *D. luciae* SLT^T to the species *D. kuznetsovii* [22]. The results of the comparison of the superlong inserts present in the 16S rRNA genes of *D. kuznetsovii* 17^T and *D. luciae* SLT^T can be interpreted in different ways. First, it cannot be excluded that the nucleotide sequence of the 16S rRNA gene of *D. luciae* SLT^T is a chimeric artifact composed of parts of *rrnA* and *rrnB* genes; then, strain SLT^T is a representative of the species *D. kuznetsovii*. On the other hand, it is possible that in *D. luciae* SLT^T the 16S rRNA gene is present in one copy or in several homogenous copies. In this case, the specific combination of the inserts provide grounds for retaining the independent species *D. luciae*, the more so because the insert in the variable 198–219 region of its 16S rRNA gene is unique. If the latter interpretation is valid, then a conclusion suggests itself that particular combinations of inserts in the hypervariable regions of the 16S rRNA gene may be species-specific in the genus *Desulfotomaculum*, as they are currently believed to be in the genus *Thermoanaerobacter* [20].

So far, the question about the origin of superlong inserts in the 16S rRNA genes can be discussed only hypothetically. Proceeding from the existence of identical inserts in genes of different organisms, the involvement of lateral transfer in the acquisition of the inserts can be suggested. On the other hand, in some cases homologous inserts could have been inherited from a common ancestor.

The investigation of the macroheterogeneity of the 15 copies of the 16S rRNA gene in *C. paradoxum* showed that genes containing superlong inserts are silent (not expressed) in this organism [7]. However, *D. kuznetsovii* 17^T, as distinct from *C. paradoxum*, was not found to contain genes devoid of superlong inserts.

In addition, the expression of the 16S rRNA gene having a superlong insert in the 5'-terminal region has been shown in *D. australicum*, a species closely related to *D. kuznetsovii* [21]. Therefore, it can be assumed that at least one of the insert-containing 16S rRNA genes of *D. kuznetsovii* 17^T is not silent. Since superlong variants of some helixes have been found only in thermophilic prokaryotes, it can be assumed that they are somehow involved in the operation of ribosomes at high temperatures.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, project nos. 99-04-48360 and 00-04-55000.

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