Phylogenetic Analysis of the Genus *Desulfotomaculum*: Evidence for the Misclassification of *Desulfotomaculum guttoideum* and Description of *Desulfotomaculum orientis* as *Desulfosporinus orientis* gen. nov., comb. nov.

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Almost complete 16S ribosomal DNA (rDNA) sequences were determined for the type strains of nine species belonging to the genus *Desulfotomaculum* and for seven strains described as strains of this genus. The sequences were compared with previously published 16S rDNA and rRNA sequences of the type strains of the other species of the genus. The majority of the species form a phylogenetically coherent cluster within the Clostridium-Bacillus subphylum of gram-positive bacteria. The cluster consists of phylogenetically well-separated lineages containing (i) *Desulfotomaculum nigrificans*, *Desulfotomaculum aeronauticum*, and *Desulfotomaculum ruminis*, (ii) *Desulfotomaculum geothermicum*, *Desulfotomaculum thermosaporovorum*, and *Desulfotomaculum sapomandens*, (iii) *Desulfotomaculum kaznetersii*, *Desulfotomaculum australicum*, and *Desulfotomaculum thermocisternum*, (iv) *Desulfotomaculum thermobenzoicum* and *Desulfotomaculum thermococoides*, and (v) *Desulfo-

The genus *Desulfotomaculum* comprises 15 validly described species, more than half of which have been described during the past 7 years. The majority of the more recently described species originate from thermal environmental sites which are presently among the prime habitats in the search for novel prokaryotic biodiversity (38).

Although several *Desulfotomaculum* species show gram-negative staining behavior, their cell walls possess the typical ultrastructure of gram-positive bacteria (29, 36). Species are defined by sulfate reduction and the presence of spores, but the shape of spores (spherical to oval) and the location of spores (central, subterminal, or terminal) vary. *Desulfotomaculum* species differ from each other in physiology; e.g., some species are autotrophic, while others grow by fermentation of glucose and other organic substrates. A few species perform homoacetogenesis by converting substrates, such as H$_2$ plus CO$_2$ and a few others, to acetate (24, 44).

Several studies involving comparative sequence analysis of 16S rRNA and 16S ribosomal DNA (rDNA) have shown that *Desulfotomaculum* species are related to members of the Clostridium-Bacillus subphylum (4, 9, 10, 16, 38). However, fine details concerning the phylogenetic coherence of this genus and the relatedness of its species have not yet been fully explored. Most previous studies included only a few species, and even the most recently published phylogenetic trees did not include more than eight or nine species (14, 18, 31). These studies showed that the majority of species cluster together to form a major subline of descent among the several clusters encompassing clostridia and their non-spor-forming and/or coccioid relatives (4). Consistently, the main *Desulfotomaculum* species cluster adjacent to members of the genera *Moorella*, *Thermoanaerobacterium*, and *Thermoanaerobacter*. In contrast, *Desulfotomaculum orientis* branched outside the main *Desulfotomaculum* cluster, showing moderate relatedness to members of the genus *Desulfotobacterium* (31, 42). However, the branching order of higher taxa within the Clostridium-Bacillus subphylum has not yet been unambiguously determined, because certain factors affect the branching pattern of lineages; these factors include the selection and number of reference organisms, the length of the sequences included in the analysis and the regions of the molecule compared, differences in the DNA base compositions of rDNA from mesophilic and thermophilic species, and the quality of the sequences in the database, which is related to the various sequencing approaches used (32). The classification of the genus *Clostridium* and its relatives (e.g., the genera *Peptococcus*, *Eubacterium*, *Ruminococcus*, and *Bacillus*) is presently undergoing a dramatic revision which is based on the results of comparative 16S rDNA sequence analyses. In this paper we describe the phylogenetic relatedness of the type strains of all available species of the genus *Desulfotomaculum* and some undescribed strains of this genus; our data led to the conclusion that, with two exceptions, the results of phylogenetic analysis match the phenotype-based classification of *Desulfotomaculum* species.

**MATERIALS AND METHODS**

**Bacterial strains.** The strains analyzed in this study, their growth media, and their growth temperatures (11, 12) are listed in Table 1. All strains were cultivated anaerobically as described previously (11, 12).

**16S rDNA sequence determination and analysis.** Genomic DNAs were extracted from the strains investigated in this study and were used for PCR-mediated amplification of 16S rDNA (34). The purified PCR products were cloned, the 16S rDNA inserts were reamplified and sequenced as described previously (33), and the sequence reaction mixtures were electrophoresed by using a model 373A automatic DNA sequencer (Applied Biosystems, Foster City, Calif.).

In order to analyze the closest relatives of *Desulfotomaculum* strains, their
phylogenetic positions were initially determined by using the database ARB (39). Fine resolution of the relatedness between Desulfotomaculum strains and their closest relatives was obtained by using the a2e editor (26). Phylogenetic dendrograms were constructed by using treeing algorithms contained in the PHYLIP package (15). The G+C contents of rDNA genes were calculated and a transversion analysis was done as described previously (32). Bootstrap values were determined by using the PHYLIP package (15). The accession numbers for the 16S rDNA sequences of reference organisms were as follows: *Ammonifex deflia*, U34975; *Clostridium spiroides*, X73449; *Clostridium celercrecens*, X71848; *Clostridium aerotolerans*, X76163; *Clostridium sphenoides*, X71855; *Desulfitobacterium dehalogenans*, L29946; *Desulfotomaculum hafniense*, X94795; *Desulfotomaculum aurantiacum*, X98407; *Desulfotomaculum australicum*, X98408; *Desulfotomaculum nigricans*, X62176; *Desulfotomaculum thermocysteinum*, U33455; *Heliolobacter chlorum*, M11212; *Moorella thermoacetica*, X78749; *Peptococcus niger*, X57597; *Thermoanaerobacter ethanolicus*, L91162; *Thermoanaerobacterium sp.*, L91167; and *Thermoanaerobacterium saccharolyticum*, L91169. The sequences of *Desulfotomaculum* strains and their respective strains was determined by parsimony analysis (38).

**RESULTS AND DISCUSSION**

All presently available type strains of *Desulfotomaculum* species were included in this study. The type strain of *Desulfotomaculum antarcticum* (3) was originally deposited as strain IAM 64 in the culture collection of the Institute of Applied Microbiology, Tokyo, Japan. According to the curator of this collection, the strain deposited under this designation is not a *Desulfotomaculum* strain, and as we are not aware that the type strain has been deposited in any other collection, it must be considered lost.

**16S rDNA sequence analysis.** It became apparent during the sequence analysis of the 16S rDNAs of *Desulfotomaculum* species that the sequence of the 5'-terminal 110 nucleotides (*Escherichia coli* nomenclature [1]) could not be resolved, irrespective of whether primer 27f or primer 343r (33) was used in the cycle sequencing reaction. In order to obtain unambiguous sequences, the products of PCR amplification of 16S rDNAs of 10 strains were cloned. Between three and eight cloned 16S rDNA inserts per strain were sequenced. A sequence analysis of the cloned genes allowed clear resolution of the primary structure of the clone inserts. Several strains possessed large inserts in helical region 73-82/87-97 of the rDNA (47). The primary structure of these intervening sequences and their fate in transcription of rRNA genes will be discussed elsewhere (37). Because of this strain-specific sequence heterogeneity of operons and the fact that many 16S rDNA reference sequences deposited in the data banks (26, 39) have high degrees of sequence ambiguity (denoted by Ns in the sequences), the region between positions 80 and 100 was omitted from the phylogenetic analysis.

**Phylogenetic analysis.** The lengths of the 16S rDNA sequences of the *Desulfotomaculum* strains analyzed ranged between 1,350 and 1,450 nucleotides. These sequences were compared to the data set containing clostridial 16S rDNA sequences, and the tentative phylogenetic position of *Desulfotomaculum* strains was determined by parsimony analysis (38). Subsequent analyses, in which we used the a2e editor (26) and the programs contained in the PHYLIP package (15), included neighbor-joining (35) and maximum-parsimony analyses and an analysis with the distance matrix algorithm of De Soete (8).

After inclusion of reference sequences of strains of neighboring taxa and omission of the 73-97 region, a stretch of 1,125 nucleotides, ranging from position 65 to position 1450, was used to determine sequence similarities. All of the analyses produced very similar patterns of relatedness for the *Desulfotomaculum* strains, which were found in three distinct lineages, designated clusters I to III below. These clusters were recovered in a high proportion of the trees generated, as demonstrated by the bootstrap values for the groups.

The base compositions of the 16S rDNA stretch sequenced indicated that certain thermophilic representatives of the strains analyzed had G+C contents that were about 3 to 5 mol% higher than the G+C contents of mesophilic strains and certain other thermophilic strains (Table 1). In order to exclude a G+C bias that distorts the relatedness determination, a transversion data set was analyzed by using the method of De Soete (8) and the neighbor-joining method (15). The lengths of the 16S rDNA sequences determined by parsimony analysis (38).

**TABLE 1. Strains investigated in this study, growth media, growth temperatures, G+C contents of rDNAs, and references**

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Strain</th>
<th>Growth medium</th>
<th>Growth temp (°C)</th>
<th>G+C content of rDNA (mol%)</th>
<th>Reference</th>
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* Growth media are described in references 11 and 12.
strains on all dendrograms (with bootstrap values of less than 10% [data not shown], on the neighbor-joining tree A. degensii was rooted deeply with members of the genus Moorella. As the intrageneric clustering of Desulfotomaculum strains was not affected by this switch in branching order, we decided to report the phylogenetic clustering of Desulfotomaculum strains as a result of the transversion neighbor-joining analysis (Fig. 1). The basis for this dendrogram was the similarity values for the transversed sequences, which were transformed to evolutionary distance values (23).

Similarity values of 83 and 90% separate members of the three clusters. These values are similar to those found to separate other phylogenetically well-separated lineages from each other, including the Selenomonas-Sporomusa group, the Thermoaerobacter group, and the several lineages composed of clostridia and their relatives (Fig. 1) (4).

Cluster I. Cluster I includes the majority of the Desulfotomaculum species. This cluster is characterized by the presence of five subclusters (subclusters Ia through Id), most of which are well-separated by similarity values between 89 and 93%. Within the multistrain subclusters the similarity values were greater than 95.5%. The phylogenetic depth of the individual subclusters are different; while subclusters Ia and Ie contain one to several slightly deeply branching strains (separated by similarity values of 94 to 96%), subclusters Ib, Ic, and Id are more shallow (with strains having similarity values greater than 98%). Subcluster Ia contains the type species of the genus, Desulfotomaculum nigrificans (3), as well as Desulfotomaculum ruminis (3), Desulfotomaculum aeronauticum (18), and strain DSM 7440 (12); these organisms have rather low rDNA G+C contents (54.5 to 55.5 mol%). Subcluster Ib contains the type strains of Desulfotomaculum sapomandens (5) and Desulfotomaculum thermosapovorans (14) and strain 7213 (12). Desulfotomaculum geothermicum (6) branches slightly deeper than these three strains; members of this subcluster are defined by rDNA G+C contents of 55.5 to 58.0 mol%. Subcluster Ic contains Desulfotomaculum australicum (25), Desulfotomaculum thermocistentum (31), and Desulfotomaculum kuznetsovii (29), while Desulfotomaculum thermacoxtodans (27), Desulfotomaculum thermodenitrificans (41), and strains DSM 7474, DSM 7475, and DSM 7476 (40) constitute subcluster Id. The latter two subclusters appear to be closely related (similarity values, more than 97%), and all members have G+C contents of 58.5 to 59.5 mol%. So far, subcluster Ie is defined solely by Desulfotomaculum acetoxidans (45), which has an rDNA G+C content of 53.5 mol%.

In order to support the affiliation of strains with the individual subclusters, the 16S rDNA data set was searched for the presence of subcluster-specific signature nucleotides (Table 2). These signature nucleotides are derivatives of the clustering process; e.g., signatures are determined for those organisms that are contained in a particular data set. Subclusters Ia, Ib, and Ic can be defined by a significant number of signature nucleotides, while subclusters Ic and Id have a high number of common signature nucleotides (29 of 33 signature nucleotides). These two subclusters could therefore be regarded as a single subcluster. It is also obvious that Desulfotomaculum geothermicum should be considered a member of subcluster Id, as this organism has 28 of the 33 signature nucleotides of this subcluster. Compared to the sequences of members of the other subclusters, the sequence of Desulfotomaculum acetoxidans is also characterized by longer stems in regions 200-217 (an additional 12 bp) and 455-477 (an additional 6 bp). These sequence idiosyncrasies are also present in most members of the sister taxon of Desulfotomaculum cluster I, which contains the genera Desulfotobacterium, Moorella, Ammonifex, and Selenomonas and their relatives. The structural peculiarities may be an indication that Desulfotomaculum acetoxidans could be considered a descendant of the most ancient Desulfotomaculum species that originated from a non-sulfate-reducing ancestor; this possibility is supported by the position of this organism on the phylogenetic tree, on which Desulfotomaculum acetoxidans appears to branch earlier than the other Desulfotomaculum species.

Cluster II. Cluster II contains two strains of Desulfotomaculum orientis which show 98.8% 16S rDNA sequence similarity to each other. The DNA reassociation value for type strain DSM 765 (3) and DSM 8344 (43) is 39%, which clearly indicates the presence of two genomospecies. At present, the lack of distinguishing phenotypic properties prevents us from describing a new species for Desulfotomaculum orientis DSM 8344. Strains of Desulfotomaculum orientis are moderately related to members of the genus Desulfotobacterium (42), with which they exhibit between 95.0 and 95.7% sequence similarity. Desulfotomaculum orientis and the genus Desulfotobacterium form a separate subline of descent within the Clostridium-Bacillus subphylum (20). Reinvestigation of physiological properties of the type strain of Desulfotomaculum orientis (24) led to the unexpected finding that this species, in contrast to the other Desulfotomaculum strains investigated in the study of Klopse et al. (24), was able to grow chemoautotrophically with hydrogen, carbon dioxide, and sulfate. It was also able to grow in the absence of sulfate with formate, methanol, ethanol.
lactate, pyruvate, or trimethoxybenzoate. It was concluded on the basis of the formation of acetate that this species can be considered a homooacetogenic bacterium.

Cluster III. Cluster III is composed of Desulfotomaculum guttoideum DSM 4024T (17). This species is highly related to certain members of Clostridium cluster XIVa, as defined by Collins and coworkers (4). When we used the truncated 16S rDNA sequences that were used to determine the relatedness shown in Fig. 1, the similarity values found for Desulfotomaculum guttoideum and C. sphenoides, C. celerecrescents, C. aerotolerans, and C. xylanolyticum were greater than 98.5%. When almost complete sequences, including the variable regions, were compared, the similarity values decreased slightly. The levels of 16S rDNA sequence similarity between the type strain of Desulfotomaculum guttoideum and the type strains of C. celerecrescents and C. sphenoides are each 99.0%. The DNA base compositions of these three strains ranged between 73 and 78%. In order to verify the authenticity of the type strain of Desulfotomaculum guttoideum, a new culture (strain VKM B-1591) was obtained from the original depositor, M. B. Vainshtein of Moscow, Russia, and a partial analysis of the 16S rDNA sequence of this organism was performed. Absolute sequence identity was found for the 5′-terminal 350 bases, and strains DSM 4024T and VKM B-1591 should be considered identical.

Reinvestigation of some phenotypic properties revealed differences from the properties published in the original description of strain VKM B-1591 (17). In our experience, strain DSM 4024T and newly received strain VKM B-1591 are fermentative and saccharolytic and reduce sulfite and thiosulfate, but they do not reduce sulfate. Desulfotomaculum guttoideum resembles C. sphenoides and C. celerecrescents in cell morphology (drop-shaped versus wedge-shaped sporulating cells [unpublished data for the type strain of the latter species]), but the DNA base compositions differ by about 10 mol%. Reinvestigation of the DNA base compositions of the type strains of the three species by high-performance liquid chromatography revealed that these strains have similar DNA G+C contents (42.7 mol% for Desulfotomaculum guttoideum, 42.8 mol% for C. celerecrescents, and 44.2 mol% for C. sphenoides).

Taxonomic conclusions. The majority of Desulfotomaculum species form a phylogenetically homogeneous cluster that can be interpreted in terms of a genomically well-defined genus. On the other hand, the intersubcluster I 16S rDNA similarity values are as low as the levels of similarity found between several neighboring genera, some of which are shown in Fig. 1 (e.g., the genera Moorella and Thermoaerobacterium, as well as the genera Selenomonas, Syntrophospora, Helio bacterium, and Desulfotibacterium). This raises the question of whether the subclusters could be treated taxonomically as individual genera. However, despite the differences in properties at the 16S rDNA level (levels of similarity, G+C contents, structural features, signature nucleotides), very little phenotypic and molecular evidence is available (2, 3, 24, 30, 44, 46) that supports the dissection of the genus at this time. The phylogenetic
clustering should encourage microbiologists to search for the presence of subcluster-specific properties that have diagnostic value and could be used in a future revision of the genus.

As judged from the 16S rDNA similarity values, some of the undescribed *Desulfotomaculum* strains most likely represent novel species; two examples are strain DSM 7213 from freshwater mud and strain DSM 7440 from cooling tower water. The question of whether strain DSM 7574 and strains DSM 7475 and DSM 7476 (all from thermophilic fermentor sludge) are strains of *Desulfotomaculum thermobenzoicum* and *Desulfotomaculum thermooceanoxydans*, respectively, should be decided on the basis of the results of DNA-DNA hybridization studies. Physiological data are not available for these strains and are needed before a decision about taxon affiliation can be made.

Based on phylogenetic evidence, the following two *Desulfotomaculum* species should be excluded from the genus: *Desulfotomaculum guttoides*, which shows high levels of relatedness to a cluster of *Clostridium* species (*C. sphenoides*, *C. celerecrescens*, *C. aerotolerns*, and *C. xylanolyticum*), and *Desulfotomaculum orientis*, which clusters as a phylogenetic neighbor of the genus *Desulfitobacterium*. The observed phenotypic differences between the original description of *Desulfotomaculum guttoides* and the results of tests performed with the deposited type strain of the species indicate that a careful reinvestigation of the taxonomic status of this organism must be performed. Because of the information available at this time, we do not present a formal proposal to reclassify *Desulfotomaculum guttoides* as a species of the genus *Clostridium*. The taxonomic distinctness of *Desulfotomaculum orientis* led us to remove this species from the genus *Desulfotomaculum* and propose a new genus, the genus *Desulfosporosinus*, for this organism. The description of this genus below is based on previously published data (2, 3, 19, 24) and our own data.

**Description of Desulfosporosinus gen. nov. Desulfosporosinus** (Des.sulfo.sporo.sinus. L. pref. de; from: L. n. sulfur, sulfur; M. L. n. spora, spore; L. n. sinus, bend; N. L. masc. n. Desulfosporosinus, a spore-forming curved [organism] that reduces sulfur compounds). Gram-negative, curved rod that has a multilayered cell wall structure. Endospores are produced; they are oval and subterminal and slightly swell the cells. Motile, with peritrichous flagella. Strictly anaerobic. Desulfovibrio and cytochrome c₃ are absent; bisulfite reductase F₃₅S is present. Sulfate and thiosulfate are reduced to sulfide in the presence of hydrogen, and further physiological characteristics of this organism are given in the DSMZ catalogue (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen).

**REFERENCES**


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