Transfer of *Thiosphaera pantotropha* to *Paracoccus denitrificans*

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Comparative sequence analysis of in vitro-amplified 16S rRNA genes of *Thiosphaera pantotropha* GB17T (T = type strain) and *Paracoccus denitrificans* LMG 4218T revealed identical 16S rRNA primary structures for the two organisms. The level of overall DNA similarity of *Thiosphaera pantotropha* GB17T and *P. denitrificans* DSM 65T is 85%, as determined by quantitative DNA-DNA hybridization. Therefore, we propose the transfer of *Thiosphaera pantotropha* to *P. denitrificans*. The closest relative of *Thiosphaera pantotropha* and *P. denitrificans* is *Thiobacillus versutus*, as revealed by comparative 16S rRNA sequence analysis. These organisms are members of the alpha subclass of the Proteobacteria. Within this subclass, *Thiosphaera pantotropha*, *P. denitrificans*, and *Thiobacillus versutus* form a phylogenetic group with *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, and "*Erythrobacter lorenzii*".

*Thiosphaera pantotropha* was initially isolated by Robertson and Kuenen (28) from a denitrifying, sulfide-oxidizing, effluent treatment pilot plant. *Thiosphaera pantotropha* is a nonmotile, facultatively chemolithoautotrophic gram-negative coccus. It is able to use organic substrates and reduced sulfur compounds under aerobic and anaerobic conditions. A very versatile bacterium, it is able to grow on a wide range of carbon sources. Striking physiological similarities between *Thiosphaera pantotropha* and *Paracoccus denitrificans* have been reported (19, 24, 28, 37). *P. denitrificans*, which was first described by Beijerinck and Minkman (3), is an nonmotile, gram-negative, coccoid bacterium. Both *Thiosphaera pantotropha* and *P. denitrificans* are facultative chemolithoautotrophs and are able to grow with molecular hydrogen (18) or with thiosulfate (13, 24) as an electron donor. *Thiosphaera pantotropha* and *P. denitrificans* have almost identical DNA G+C contents of 66 to 67 mol% (19, 24). Organoaotrophic growth with formate or methanol has been described for *P. denitrificans* (7), whereas *Thiosphaera pantotropha* has been reported to be unable to utilize these substrates. These and other differing physiological characteristics induced Robertson and Kuenen (28) to propose the new genus *Thiosphaera*.

Striking similarities between *Thiosphaera pantotropha* and *P. denitrificans* have been found on the genetic level. A 13-kb DNA region responsible for thiosulfate oxidation was isolated from *Thiosphaera pantotropha*. This region was used as a probe for heterologous Southern hybridization to DNA restriction fragments from other thiobacteria. Identical hybridization patterns were obtained for *Thiosphaera pantotropha* and *P. denitrificans* (23). These data may indicate that the regions encoding the genes responsible for sulfur oxidation are identical. A high level of sequence similarity of the genes encoding the type *b* cytochrome belonging to the type *bc*1 complex has been found (37). Both *Thiosphaera pantotropha* and *P. denitrificans* contain the cytochrome *c*553 gene (27), and the organization of the DNA region around the gene is similar in the two organisms (37). To see whether the genetic and phenotypic similarities of these two organisms really do reflect phylogenetic closeness, we decided to perform a comparative 16S rRNA sequence analysis and quantitative DNA-DNA hybridizations.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *Thiosphaera pantotropha* GB17T (T = type strain) was aerobically cultivated at 30°C in Luria-Bertani medium (32). *P. denitrificans* LMG 4218T was grown aerobically at 25°C on nutrient agar CM3 (Oxoid, Welzel, Germany).

**DNA-DNA hybridization.** The DNA-DNA hybridization experiments were carried out at the facilities of the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. The DNA was purified by chromatography on hydroxyapate by using the procedure of Cashion et al. (5). DNA-DNA hybridization was performed as described by DeLey et al. (8), using a Gilford system 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogamber. Levels of DNA-DNA similarity were calculated from the renaturation rates by using the computer program TRANSFER.BAS (16).

**Sequence analysis.** DNA was purified by the method of Marrm (22). 16S rRNA genes were amplified in vitro from purified DNA or whole bacterial cells. A 16S rRNA gene fragment of *Thiosphaera pantotropha* homologous to bases 54 to 1412 of *Escherichia coli* 16S rRNA (4) was amplified in vitro by using the polymerase chain reaction technique (30) in combination with rRNA-specific oligonucleotide primers (5'-CATGCAAGTGARCG-3' and 5'-GGTGTCGAGCCG-3'). The amplified DNA fragment was cloned in the pBluescript vector (Stratagene, La Jolla, Calif.) and *Escherichia coli* JM83 as pTpan161. An alternative primer pair (5'-AGATTGGATYMTGGCTCAG-3' and 5'-AGAAAGG AGGTGATCC-3') was used for the amplification of *Thiosphaera pantotropha* and *P. denitrificans* ribosomal DNAs, and the fragments were sequenced directly. These fragments are homologous to bases 8 to 1542 of *Escherichia coli* 16S rRNA (4). The DNA sequence analysis was performed by using a Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio) and site-specific oligonucleotide primers. The oligonucleotides were synthesized with a Cyclone DNA synthesizer (Milligen Biosearch, Eschborn, Germany).

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Sequence data analysis. The 16S rRNA sequences were added to an alignment of about 900 homologous sequences. A matrix of binary phylogenetic distances (17) was established for the sequences presented here and a selection of related sequences from representatives of the alpha subclass of the Proteobacteria (36, 41). Distance matrix trees were reconstructed by using the method of Fitch and Margoliash (12), the neighbor-joining method of Saitou and Nei (31), and the FITCH and NEIGHBOR programs of Felsenstein’s (11) PHYLIP 3.4 program package. Alignment gaps and the positions of the reference sequences which had not been determined were not taken into consideration for the calculation of binary distance values.

Nucleotide sequence accession number. The 16S rRNA nucleotide sequence determined in this study was deposited in the EMBL sequence databank under accession number X69159.

RESULTS

Almost complete 16S rRNA sequences were determined for Thiosphaera pantotropha and P. denitrificans. The sequenced fragments are homologous to bases 8 to 1542 of Escherichia coli 16S rRNA (4). The sequences of the two organisms investigated in this study are identical. The 16S rRNA primary structure is shown in Fig. 1.

The nucleotide sequence was compared with all available 16S rRNA primary structures of representatives of the alpha subclass of the Proteobacteria. The 16S rRNA sequence of Escherichia coli was used as an outgroup reference. Comparison with the partial 16S rRNA sequence of Thiobacillus versutus (21) revealed that this organism is the closest relative of Thiosphaera pantotropha and P. denitrificans. The level of sequence similarity between the 16S rRNA regions which had been sequenced from Thiobacillus versutus, which is homologous to the partial 16S rRNAs of Thiosphaera pantotropha and P. denitrificans, was 97.1%. The close relationship was verified by comparison of the homologous 16S rRNA regions of the other representatives of the alpha subclass of the Proteobacteria. Because of incompleteness, the Thiobacillus versutus 16S rRNA sequence was not included in the calculation of similarity and phylogenetic distance values (Table 1) and was not used in the reconstruction of the phylogenetic tree (Fig. 2). Overall sequence similarity values for P. denitrificans (Thiosphaera pantotropha), its closest relatives, selected bacteria representing the major phylogenetic groups of the alpha subclass of the Proteobacteria, and the outgroup reference organism Escherichia coli are shown in Table 1. Similarity values of 93.3 to 93.4% were found for P. denitrificans (Thiosphaera pantotropha) and the rhodobacters. The corresponding values for the new sequences and the other reference sequences of members of the alpha subclass of the Proteobacteria were lower (80.9 to 91.3%). A phylogenetic tree based on corrected distance values is shown in Fig. 2.

An overall level of DNA-DNA similarity of 85 ± 1% for Thiosphaera pantotropha GB17 and P. denitrificans DSM 65 was determined by using the optical method for quantitative DNA-DNA hybridization (8).

DISCUSSION

Thiosphaera pantotropha was isolated and validly described by Robertson and Kuenen in 1983 (28). It shares many physiological and metabolic characteristics with P. denitrificans. Both species are metabolically versatile and use a wide range of organic substrates. Similarities in gene sequences and/or organization have been reported for cytochrome b1 and c553 genes (37) and genes involved in thiosulfate oxidation (23).

The first description of the genus Thiosphaera was based on a number of physiological characteristics that were different from characteristics of the genus Paracoccus (28). The two taxa clearly differ with respect to their rates and extents of heterotrophic nitrification and their abilities to denitrify aerobically (29). In addition, differences in substrate utilization under anaerobic respiration growth conditions have been described (28). P. denitrificans is capable of growing organoautotrophically with methanol, whereas Thiosphaera pantotropha has been described as an organism that is unable to utilize methanol (28). Later, autotrophic growth on formate by type strain Thiosphaera pantotropha GB17 was observed (6), and recently it has been shown that organoautotrophic growth of Thiosphaera pantotropha with methanol occurs after a spontaneous mutation (10). Differences in the ability to grow on various organic substrates have been described for 11 strains isolated from different habitats; all of these organisms were assigned to P. denitrificans. These substrates include alcohols, amino acids, and organic acids. However, it has been shown that utilization of several substrates occurs only after a mutational event in the type strain and new isolates (24). The physiological
TABLE 1. Overall levels of similarity and phylogenetic distances based on 16S rRNA sequence data for *P. denitrificans* (*Thiosphaera pantotropha*), selected representatives of the major phylogenetic groups of the alpha subclass of the *Proteobacteria*, and *Escherichia coli*

<table>
<thead>
<tr>
<th>Species</th>
<th>% Similarity or phylogenetic distancea</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. denitrificans</em></td>
<td>99.4 99.3 99.1 98.0 97.8 97.7 97.5 97.0 96.5</td>
</tr>
<tr>
<td><em>Rhodobacter capsulatus</em></td>
<td>0.049 0.049 0.049 0.049 0.049 0.049 0.049 0.049 0.049</td>
</tr>
<tr>
<td><em>Rhodobacter sphaeroides</em></td>
<td>0.057 0.057 0.057 0.057 0.057 0.057 0.057 0.057 0.057</td>
</tr>
<tr>
<td><em>Hirschia baltica</em></td>
<td>0.095 0.095 0.095 0.095 0.095 0.095 0.095 0.095 0.095</td>
</tr>
<tr>
<td><em>Rhodobacterium vannielii</em></td>
<td>0.091 0.091 0.091 0.091 0.091 0.091 0.091 0.091 0.091</td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em></td>
<td>0.082 0.082 0.082 0.082 0.082 0.082 0.082 0.082 0.082</td>
</tr>
<tr>
<td><em>Rhodopseudomonas palustris</em></td>
<td>0.101 0.101 0.101 0.101 0.101 0.101 0.101 0.101 0.101</td>
</tr>
<tr>
<td><em>Magnetospirillum magnetotacticum</em></td>
<td>0.099 0.099 0.099 0.099 0.099 0.099 0.099 0.099 0.099</td>
</tr>
<tr>
<td><em>Rhodospirillum rubrum</em></td>
<td>0.103 0.103 0.103 0.103 0.103 0.103 0.103 0.103 0.103</td>
</tr>
<tr>
<td><em>Holospora obtusa</em></td>
<td>0.118 0.118 0.118 0.118 0.118 0.118 0.118 0.118 0.118</td>
</tr>
<tr>
<td><em>Rickettsia rickettsiae</em></td>
<td>0.112 0.112 0.112 0.112 0.112 0.112 0.112 0.112 0.112</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>0.167 0.167 0.167 0.167 0.167 0.167 0.167 0.167 0.167</td>
</tr>
</tbody>
</table>

* The values on the upper right are levels of similarity, and the values on the lower left are phylogenetic distances (17).

See reference (17).

See reference (9).

See reference (25).

See reference (35).

See reference (42).

See reference (40).

See reference (4).

See reference (1).

See reference (41).

heterogeneity among strains of the species *P. denitrificans* diminishes the taxonomic relevance of substrate specificities for maintenance of the genus *Thiosphaera*. Both organisms harbor cryptic megaplasmids; *P. denitrificans* harbors 450- and 75-kb plasmids (15), and *Thiosphaera pantotropha* harbors 450- and 110-kb plasmids (6). These plasmids are of special interest, since one may assume that the physiological diversity of *P. denitrificans* strains and *Thiosphaera pantotropha* may result from plasmid-encoded genes.

*Thiosphaera pantotropha* GB17T and *P. denitrificans* DSM 65T differ with respect to gene transfer rates. Conjugal transfer of plasmids to *Thiosphaera pantotropha* GB17T

**FIG. 2.** Distance matrix tree, showing the phylogenetic position of *P. denitrificans* (*Thiosphaera pantotropha*) within the alpha subclass of the *Proteobacteria*. The tree is based on a data set including only sequence positions which are invariant in at least 50% of all available homologous sequences from members of the alpha subclass of the *Proteobacteria*. The tree was reconstructed by using the neighbor-joining method (31). Bar = 0.05 phylogenetic distance (17).
occurs at a frequency that is at least 2 orders of magnitude higher than the frequency observed with \textit{P. denitrificans} DSM 65. The frequency of transfer of suicide plasmid pSUP5011 carrying transposon Tn5-mob to \textit{P. denitrificans} is 1.2 \times 10^{-8} per recipient cell, and the frequency of transfer of this plasmid to \textit{Thiosphaera pantotropha} is 1.5 \times 10^{-6} per recipient cell (6).

In order to clarify the phylogenetic affiliations of \textit{Thiosphaera pantotropha} and \textit{P. denitrificans}, we analyzed the almost complete 16S RNA primary structures of these organisms. The two sequences are identical. Therefore, the optical method used in this study has been shown to be a precise procedure for determining close relationships (levels of similarity of more than 30%) among bacteria (14). The close relationship of the genera \textit{Thiosphaera} and \textit{Paracoccus} is corroborated by a high DNA-DNA similarity level (85%).

In the report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics, Wayne et al. (39) proposed that the phylogenetic definition of a species should be based on levels of DNA-DNA relatedness of 70% or more. The DNA-DNA hybridization data clearly show that \textit{Thiosphaera pantotropha} and \textit{P. denitrificans} are representatives of one genus. Some of the criteria for the differentiation of the genera \textit{Thiosphaera} and \textit{Paracoccus}, such as organoautotrophic utilization of methanol or formate, are no longer valid, and others are likely to result from mutational events (24). Therefore, we propose the transfer of \textit{Thiosphaera pantotropha} to \textit{P. denitrificans}. Following the transfer of \textit{Thiosphaera pantotropha} to \textit{P. denitrificans}, the genus \textit{Thiosphaera} and the species \textit{Thiosphaera pantotropha} no longer exist.

The phylogenetic affiliation of \textit{P. denitrificans} with the rhodobacters in the alpha subclass of the \textit{Proteobacteria} has been shown by 16S rRNA cataloging data (41). The results of a comparative analysis of the very nearly complete 16S rRNA sequence support creation of a phylogenetic cluster comprising \textit{Thiosphaera pantotropha}, \textit{P. denitrificans}, \textit{Rhodobacter capsulatus}, \textit{Rhodobacter sphaeroides}, and "\textit{Erythrobacter longus}" (Fig. 2). Interestingly, the relationship between the genus \textit{Paracoccus} and rhodobacters was also demonstrated by comparison of the presently available cytochrome c sequences of members of the alpha subclass of the \textit{Proteobacteria} (26, 27).

The emended descriptions of the genus \textit{Paracoccus} and of the species \textit{P. denitrificans} below are based on the descriptions given by Aragno and Schlegel (2), Versevel and Stouthammer (38), Kuenen and Robertson (19), and Kuenen et al. (20).

**Emended description of the genus Paracoccus.** \textit{Paracoccus} (Pa.r.a.co.c'cus. Gr. prep. \textit{para}, like, alongside of; Gr. n. \textit{coccus}, a grain, berry; M.L. masc. n. \textit{Paracoccus}, like a coccus). Spherical cells (diameter, 0.5 to 1.3 \mu m) or short rods (length, 0.9 to 1.3 \mu m) occur singly, in pairs, in chains, or in clusters. Intracellular granules of poly-\beta-hydroxybutyrate are present. No resting stages are known. Gram negative. Nonmotile. Metabolism respiratory; able to use oxygen, nitrate, or nitrogen oxide as a terminal electron acceptor. \textit{Nitrate} may be reduced to nitrous oxide, and molecular nitrogen under anaerobic conditions and by one strain under aerobic conditions. One species (\textit{P. denitrificans}) can grow either autotrophically with \textit{CO}_2 or \textit{H}_2 or heterotrophically with a wide variety of organic compounds as the sole carbon source. Grows in the presence of 0 to 5\% NaCl. Growth becomes scantly at NaCl concentrations above 5\%. A second species (\textit{Paracoccus halodenitrificans}) is not capable of autotrophic growth but is halophilic, requiring thiamine and at least 3% NaCl for growth. Oxidase and catalase positive. Occurs in soil and presumably in natural and artificial brines. The G+C content of the DNA is 64 to 67 mol\% (as determined by the melting temperature and buoyant density methods).

Type species: \textit{Paracoccus denitrificans} (Beijerinck 1910) Davis 1969, 384.  

**Emended description of Paracoccus denitrificans** (Beijerinck 1910) Davis 1969, 384\-


