Phylogenetic Relationships and Uncertain Taxonomy of *Pedomicrobium* Species

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The phylogenetic relationships among the species of the genus *Pedomicrobium* were studied by comparing their 16S rRNA sequences. The *Pedomicrobium* species form a coherent phylogenetic cluster within the genera of the hyphal budding bacteria in the α -Proteobacteria. The sequences of two strains of *Pedomicrobium australicum* were obtained from DNAs extracted from nonviable freeze-dried cells, which are the only source of material available, and were found to be almost identical (level of similarity, 99.9%). Overall, the *Pedomicrobium* species are closely related, with sequence similarities ranging from 96.2 to 99.9%. *Pedomicrobium manganicum* is phylogenetically the most distantly related species and exhibits the lowest similarity (96.2%) with *Pedomicrobium americanum*. Australian isolate *Pedomicrobium* sp. strain ACM 3067, *P. americanum*, and *P. australicum* are all very highly related, with similarities greater than 99%. *Pedomicrobium* sp. strain ACM 3067 is most closely related to *P. australicum* (level of similarity, 99.6%) and *P. americanum* (99.4%). These manganese-oxidizing species are more closely related to the iron-oxidizing species *Pedomicrobium ferrugineum* than to the other manganese-oxidizing species, *P. manganicum*. Taxonomic uncertainties resulting from the loss of the type culture of *P. australicum* are discussed.

In 1988, Sly et al. reported on the characteristics of strains of *Pedomicrobium manganicum* isolated from manganese-depositing biofilms in drinking water distribution systems in Australia (28). At that time, there were only two valid species of the genus *Pedomicrobium*, *Pedomicrobium ferrugineum* and *P. manganicum* (20, 27). Later, Gebers and Beese (7) described two new species, *Pedomicrobium americanum* and *Pedomicrobium australicum*. Unfortunately, the type strain and a second isolate of *P. australicum* were lost before they could be accessioned for distribution (13, 14, 32), and we were unable to compare these strains with our isolates, which appeared to be more closely related phenotypically to *P. australicum* and *P. americanum* than to *P. manganicum*.

The absence of a viable type strain of *P. australicum* is contributing to taxonomic uncertainty in the genus *Pedomicrobium*. The availability of PCR techniques provided an opportunity to amplify 16S rRNA genes from nonviable freeze-dried ampoules and to determine the 16S rRNA sequences of *P. australicum* for comparison with the sequences of the other *Pedomicrobium* species and our local isolates. Although Gebers and Beese (7) showed that *P. australicum* and *P. americanum* exhibited only 25 to 28% DNA homology and were separate species as determined by currently accepted standards for DNA-DNA homology levels (31, 35), there were no reliable phenotypic characteristics to differentiate the two species. If sufficient differences in the sequences of the 16S rRNAs of *P. australicum* and *P. americanum* can be found, then it may be possible to determine species-specific RNA sequence signatures for designating a neotype strain of *P. australicum* for further study.

MATERIALS AND METHODS

Microorganisms. Most of the strains used in the study were obtained from the Australian Collection of Microorganisms, Department of Microbiology, University of Queensland, Brisbane, Australia; the only exceptions were *P. australicum* IFAM ST-1306^T (T = type strain) and IFAM WD-1355, which were obtained as nonviable freeze-dried material from Peter Hirsch at the Institut für Allgemeine

Mikrobiologie in Kiel, Germany. Details of the strains are shown in Table 1. All viable strains were maintained on PSM agar plates (6) and were incubated at 28°C. Manganese oxidation was verified on PC agar plates (34).

Extraction and purification of genomic DNA. Bacterial growth from PSM agar plate cultures or freeze-dried material of those strains which were nonviable was collected and pelleted by centrifugation in a microcentrifuge at 3,000 rpm, and the genomic DNA was isolated by the method of Bollet et al. (2). DNA was released from cells in 1.5-ml microcentrifuge tubes (Treff Laboratories, Degerheim, Switzerland) by heating the tubes in a 650-W microwave oven for three 1-min intervals. The concentration of DNA in a sample was estimated by electrophoresing 5 µl of purified DNA with 3 µl of loading buffer (0.25% bromophenol blue and 30% glycerol in water) and a 1-kb DNA ladder (Gibco, Gaithersburg, Md.) on a 1% agarose gel containing 5 µl of a 10-mg/ml ethidium bromide solution per 100 ml.

PCR amplification of 16S ribosomal DNA (rDNA). A 1- to 3-µl portion of bacterial genomic DNA was used in each PCR mixture, as described by Bond et al. (3). The first denaturation step consisted of incubation at 98°C for 2 min. The thermal profile of the PCR apparatus (Perkin-Elmer Cetus, Norwalk, Conn.) included repeated cycles of primer annealing at 48°C for 1 min, extension at 72°C for 2 min, and denaturation at 93°C for 1 min. After 28 cycles, primer annealing was repeated, and this was followed by a final extension step at 72°C for 5 min. The primers used for amplification were primers 27f and 1525r (18). The PCR products were purified by using Wizard Mini Preps for DNA purification (Promega, Madison, Wis.) as recommended by the supplier. The concentrations of purified PCR products were estimated by electrophoresing 2 µl of each PCR product on a 1% agarose gel as described above for genomic DNA.

Sequencing of 16S rDNA. Sequencing reactions were carried out by using a PRISM Ready Reaction DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer/Applied Biosystems Div., Foster City, Calif.). A 1- to 3-µl portion of purified PCR product was used per reaction mixture (total volume, 20 µl) along with 9 µl of Terminator Premix and 1 µl of sequencing primer. Primers 27f, 357f, 530f, 803f, 1114f, 1230f, 342r, 519r, 787r, 1100r, 1392r, and 1525r (18) were used to sequence the whole 16S rRNA gene in both forward and reverse directions. Extension products were purified, and sequences were determined by automated DNA sequencing with an Applied Biosystems model 373A instrument as recommended by the manufacturer.

Phylogenetic analysis. The full-length 16S rRNA sequences of *P. ferrugineum* ACM 3037^T, *P. manganicum* ACM 3038^T, *P. americanum* ACM 3090^T, *P. australicum* IFAM ST-1306^T and IFAM WD-1355, and *Pedomicrobium* sp. strain ACM 3067 were manually aligned with sequences of eight other members of the Proteobacteria, including the nearest known relative, *Hyphomicrobium vulgare* (30), by using the *Escherichia coli* sequence as the reference sequence. The sequences of the following strains were obtained from the Ribosomal Database Project, Urbana, Ill. (19): *Hyphomicrobium vulgare* ATCC 27500 (accession no. X53182), *Hyphomonas jannaschiana* ATCC 33882 (accession no. M83806), *Hirschia baltica* ATCC 49814^T (accession no. X52909), *Rhodomicrobium vannielii* ATCC 51194 (accession no. M34127), *E. coli* (accession no. J01695), *Neisseria gonorrhoeae* ATCC 19424 (accession no. X07714), *Desulfovibrio desulfuricans* ATCC 27774 (accession no. M34113), and *Campylobacter jejuni* ATCC 43431 (accession no. Z29326).

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TABLE 1. *Pedomicrobium* strains used in this study

Microorganism	Strain ^a	Source ^a
<i>P. americanum</i>	ACM 3090 ^T	IFAM G-1381 ^T (= ATCC 43612 ^T)
<i>P. australicum</i>	IFAM ST-1306 ^T	IFAM ST-1306 ^T (= ATCC 43611 ^T)
<i>P. australicum</i>	IFAM WD-1355	IFAM WD-1355 (= ATCC 43614)
<i>P. ferruginum</i>	ACM 3037 ^T	IFAM S-1290 ^T (= ATCC 33119 ^T)
<i>P. manganicum</i>	ACM 3038 ^T	IFAM E-1129 ^T (= ATCC 33121 ^T)
<i>Pedomicrobium</i> sp.	ACM 3067	ACM 3067

^a ACM, Australian Collection of Microorganisms, Department of Microbiology, University of Queensland, Brisbane, Australia; IFAM, Institut für Allgemeine Mikrobiologie, Kiel, Germany.

The outgroup chosen for each analysis was the organism most distantly related to the genus *Pedomicrobium* on the prokaryotic phylogenetic tree (23) in each data set. Unsequenced regions and regions of alignment uncertainty due to length variations were omitted from the analyses. Levels of similarity were calculated for each pair of sequences by using the ae2 editor program (19) and a Sun workstation.

Three different methods available in the PHYLIP version 3.5c phylogeny inference package (4) were employed to infer the phylogenetic relationships. Pairwise evolutionary distances were computed with DNADIST by using the correction of Jukes and Cantor (16). Phylogenetic trees were constructed by using the neighbor-joining method of Saitou and Nei (26) in the program NEIGHBOR (4). Parsimony and maximum-likelihood analyses were carried out by using DNAPARS and DNAML (4), respectively. Bootstrap analyses that generated 100 data sets were also performed on the phylogeny derived from the neighbour-joining analysis by using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE (4) to determine the statistical level of confidence for the branch points.

Nucleotide sequence accession numbers. The sequences determined in this study for *P. australicum* IFAM ST-1306^T (accession no. X97693), *P. australicum* IFAM WD-1355 (accession no. X97694), *P. americanum* ACM 3090^T (accession no. X97692), *P. ferruginum* ACM 3037^T (accession no. X97690), *P. manganicum* ACM 3038^T (accession no. X97691), and *Pedomicrobium* sp. strain ACM 3067 (accession no. X97695) have been deposited in the EMBL database, Cambridge, United Kingdom.

RESULTS AND DISCUSSION

As the type culture of *P. australicum* had been lost since its description by Gebers and Beese (7), a complete analysis of the phylogenetic relationships among the species of the genus *Pedomicrobium* depended on the ability to extract DNA from nonviable freeze-dried material. Cells of two strains of *P. australicum* that had been freeze-dried in skim milk during the period from 1984 to 1991 were kindly provided by Peter Hirsch. Preliminary experiments (data not shown) performed with cells of *Pedomicrobium* sp. strain ACM 3067 showed that the presence of 20% skim milk in the reaction mixtures did not adversely affect extraction of DNA from the cells or interfere with subsequent PCR amplification of the 16S rDNA in the extracted DNA. Extraction of DNA from *Pedomicrobium* species has been reported to be difficult (12). However, the microwave method of Bollet et al. (2) was found to release sufficient DNA for PCR amplification.

Full 16S rRNA sequences were obtained for the type cultures of the validly published *Pedomicrobium* species (20, 27), as well as for *P. australicum* IFAM WD-1355 and *Pedomicrobium* sp. strain ACM 3067. The sequences of the *Pedomicrobium* strains were found to contain 27 of the 29 16S rRNA signature sequences for the α -Proteobacteria (36), which confirmed membership of the genus *Pedomicrobium* in the α sub-division (30).

A phylogenetic tree based on Jukes-Cantor evolutionary distances was constructed by using the neighbor-joining method for 1,201 nucleotide positions of the sequences of six *Pedomicrobium* strains and eight reference species, including four representatives of the hyphal budding bacteria in the α -Proteobacteria for which sequences are available for the type

FIG. 1. Neighbor-joining tree showing the relationships among six *Pedomicrobium* strains, four other hyphal budding bacteria, and representatives of the β -, δ -, γ -, and ϵ -Proteobacteria. *C. jejuni* was used as the outgroup. Bootstrap values from 100 analyses are shown at the branch points. Scale bar = 10 nucleotide substitutions per 100 nucleotides of 16S rRNA sequence.

strains and four representatives of the β -, γ -, δ -, and ϵ -Proteobacteria (Fig. 1). Phylogenetic trees were also constructed by using the maximum-likelihood and parsimony methods for comparison (data not shown), and the *Pedomicrobium* species formed a coherent phylogenetic group in all analyses. A bootstrap resampling analysis revealed considerable support (97 to 100%) for the branch points of the genera of the hyphal budding bacteria. Only one branch point of the species of the genus *Pedomicrobium* was doubtful (55% support for the branch point of *P. americanum* and *Pedomicrobium* sp. strain ACM 3067) (Fig. 1).

A second analysis of the sequences of the *Pedomicrobium* species over a longer stretch (1,369 nucleotide positions) was performed by using the sequence of the proposed neotype strain ATCC 27500 (= IFAM MC-750) (15) of the nearest relative, *Hyphomicrobium vulgare*, as the outgroup. The sequences of the two *P. australicum* strains were found to be almost identical (level of similarity, 99.9%). The species of the genus *Pedomicrobium* are closely related, with sequence similarities ranging from 96.2 to 99.9%. *P. manganicum* is phylogenetically the most distantly related species in the genus and exhibits the lowest level of similarity (96.2%) with *P. americanum*. *Pedomicrobium* sp. strain ACM 3067, *P. americanum*, and *P. australicum* are all very highly related, with sequence similarities of more than 99%. The Australian isolate *Pedomicro-*

bium sp. strain ACM 3067 is most similar to *P. australicum* (level of similarity, 99.6%) and *P. americanum* (99.4%). The latter manganese-oxidizing species are more closely related to the iron-oxidizing species *P. ferrugineum* (levels of similarity, 98.8 to 99%) than to the other manganese-oxidizing species, *P. manganicum* (levels of similarity, 96.2 to 96.5%).

Stackebrandt et al. (30) found in a catalog analysis of 16S rRNA oligonucleotides that the genus *Pedomicrobium* fell within the *Hyphomicrobium* cluster and questioned the validity of the genus *Pedomicrobium*. However, there was considerable diversity in the *Hyphomicrobium* strains studied, which may have been representatives of more than one genus. This view is supported by the rRNA cistron similarity data of Roggentin and Hirsch (24), which showed the phylogenetic heterogeneity of strains currently assigned to the genus *Hyphomicrobium*. Likewise, the genomic DNA base compositions (9) of the *Hyphomicrobium* strains that have been studied are very different, and two of the strains, T-854 and SW-808, are considered only *Hyphomicrobium*-like (12). This demonstrates the difficulties faced in accurately positioning these organisms, which are closely related phylogenetically and have few differential phenotypic characteristics to accurately define the genus. It is encouraging for the validity of the genus *Pedomicrobium* that all of its species grouped together to the exclusion of the other genera of hyphal budding bacteria on the basis of their 16S rRNA sequences determined in the present study. However, there is only one sequence of a *Hyphomicrobium* species available for comparison, so these results are not conclusive. A complete data set of the sequences of members of the genus *Hyphomicrobium* and other genera of the hyphal budding bacteria will be needed to clarify the validity of the genus *Pedomicrobium*, which has been questioned on the basis of morphology (1, 33) and phylogeny (30) and has been supported on the basis of DNA composition (8, 12), genome size (17), and DNA homology (10, 11).

Because of the high levels of 16S rRNA sequence similarity among the species of the genus *Pedomicrobium*, it is not possible to use this criterion to accurately assign the local isolate *Pedomicrobium* sp. strain ACM 3067 to one of the existing *Pedomicrobium* species. However, the 16S rRNA similarity between this isolate and *P. manganicum* is less than 97%, and it is therefore unlikely that strain ACM 3067 belongs to *P. manganicum*, as originally thought (28, 31). Because strain ACM 3067 exhibits 98.8 to 99.6% sequence similarity with *P. australicum*, *P. americanum*, and *P. ferrugineum*, determination of its species status will depend on supporting DNA-DNA homology data or similar evidence obtained by using techniques such as cellular protein polyacrylamide gel electrophoresis patterns. This will not be possible for *P. australicum*, whose type strain is unavailable.

The taxonomic position of *P. australicum* is problematic and uncertain. Gebers and Beese (7) showed that *P. australicum* was a distinct species on the basis of its DNA-DNA homology values of less than 28% with all other *Pedomicrobium* species. However, circumscription of a species also requires phenotypic characteristics to adequately differentiate the organism from other species (21, 35), and there are insufficient phenotypic characteristics to differentiate *P. australicum* from *P. americanum* (7). A similar situation exists in the genus *Bacillus*, in which Fox et al. (5) reported that strains which exhibited more than 99.5% 16S rRNA sequence similarity and had phenotypic similarities were convincingly established as separate species on the basis of previous DNA-DNA hybridization studies (5, 22, 25). Thus, Fox et al. (5) concluded that some species may not be recognizable from their 16S rRNA gene sequences,

even though sequences can be used to distinguish relationships between genera and some well-resolved species.

The taxonomic uncertainty of *P. australicum* is exacerbated by the additional loss of the only other strain studied by Gebers and Beese (7), strain IFAM WD-1355, so the prospect of designating a neotype strain for the species is remote in the absence of suitable differentiating phenotypic characteristics and their close phylogenetic relationships. The objective of finding a neotype strain for *P. australicum* will have to remain unresolved because it is not possible at this time to extract a sufficient quantity of DNA from the freeze-dried ampoules to carry out DNA-DNA hybridization studies.

In view of the absence of a type strain, insufficient phenotypic characteristics to differentiate isolates of closely related species, and the absence of phylogenetic 16S rRNA signature sequences, the species *P. australicum* should not be retained. The present research has demonstrated the value of using nonviable preserved specimens to investigate taxonomic issues by molecular methods, as foreshadowed by Sneath (29).

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