Phylogenetic Evidence for Transfer of Pentachlorophenol-
Mineralizing Rhodococcus chlorophenolicus PCP-1T to the Genus Mycobacterium

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We determined the nucleotide sequence of a 16S rRNA gene of Rhodococcus chlorophenolicus PCP-1T (= DSM 43826T) (T = type strain). Sequence comparisons revealed that there was a close relationship between strain PCP-1T and strains belonging to the genus Mycobacterium. The sequence data were used to construct a phylogenetic tree, which showed that Mycobacterium chubuense is the closest relative of strain PCP-1T. We propose that strain PCP-1T should be transferred to the genus Mycobacterium and renamed Mycobacterium chlorophenolicum PCP-1T comb. nov.

Historically, the genus Rhodococcus has encompassed a group of aerobic, nonmotile, gram-positive actinomycetes that exhibit considerable morphological diversity, contain mycolic acids, and can be acid fast (18). This genus used to be heterogeneous and included species that contain menaquinone MK-8(H2) and MK-9(H2) with eight and nine isoprenoid units, respectively, and one hydrogenated double bond and also contain long-chain mycolic acids with 48 to 66 C atoms. In a subsequent phylogenetic study of this genus in which 16S rRNA sequence data were used, Stackebrandt et al. showed that the species containing menaquinone MK-8(H2) and long-chain mycolic acids with 63 to 69 C atoms clustered together on a phylogenetic tree on a branch that was separate from the branch that contained the other rhodococci (31). Therefore, these species (Rhodococcus bronchiialis, Rhodococcus terrae, Rhodococcus rubropertinctus, and Rhodococcus spuiti) appeared not to belong to the genus Rhodococcus. The transfer of these taxa to the revived genus Gordona (31) made the genus Rhodococcus more homogeneous chemotaxonomically. Most members of the genus Rhodococcus have type IV cell walls and contain menaquinone MK-8(H2) and mycolic acids with 34 to 64 C atoms. The only exception is the pentachlorophenol-mineralizing actinomycete Rhodococcus chlorophenolicus PCP-1T (T = type strain), which was isolated from lake sediment (1-3). This strain contains predominantly menaquinone MK-9(H2), which is found in strains belonging to the genera Gordona, Tsukamurella, and Mycobacterium, and mycolic acids that have 33 to 43 C atoms, which is typical of the genus Rhodococcus. R. chlorophenolicus PCP-1T has been shown to mineralize pentachlorophenol in liquid cultures, as well as in polluted soils (3, 6, 23, 29). Moreover, this pentachlorophenol-degrading bacterium has been found to survive longer than the other pentachlorophenol-mineralizing microorganism, Flavobacterium sp., when it is introduced into natural soil; the reasons for this are not known (7). Determining the phylogenetic location of strain PCP-1T in more detail could provide new insights into the physiological properties of this organism. This could lead to a better understanding of the behavior of strain PCP-1T in environments that are more complex than liquid culture (soil, sediment, sludge, etc.), and it could result in more efficient use of this bacterium in bioremediation processes (6, 23, 29, 36, 37).

To obtain more information about the phylogenetic position of R. chlorophenolicus PCP-1T within the Actinomycetes, we cloned one of the two copies of the 16S rRNA genes and determined its nucleotide sequence. Our results revealed that there is a close relationship between strain PCP-1T and members of the genus Mycobacterium. Therefore, we propose that strain PCP-1T should be removed from the genus Rhodococcus and transferred to the genus Mycobacterium.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strain used in this study was R. chlorophenolicus PCP-1T (= DSM 43826T) (1), which was grown aerobically in the dark in a medium containing (per liter) 4 g of glucose, 4 g of yeast extract, and 4 g of malt extract at 28°C on an orbital shaker (140 rpm). Escherichia coli TG1 (17) was used as a host in cloning experiments; this organism was grown as described previously (30).

Extraction of DNA. Cells of R. chlorophenolicus PCP-1T were harvested at the late logarithmic phase. Since lysis of these cells by lysozyme appeared to be inefficient, a modified procedure that involved treatment with sodium dodecyl sulfate (SDS) and heat shock was used (11). In this procedure 1 ml of a cell suspension was centrifuged, and the resulting pellet was resuspended in a solution containing 900 µl of 0.1 M Na2HPO4,NaH2PO4 (pH 7.4) and 100 µl of 10% SDS. The sample was heated at 70°C for 30 min and then centrifuged at 1,600 x g for 10 min at 20°C to separate the cell debris from the supernatant. The nucleic acids present in the supernatant were concentrated by precipitation with polyethylene glycol 6000. Polyethylene glycol 6000 precipitation was performed by adding NaCl to a final concentration of 0.5 M and then adding 0.5 volume of 50% polyethylene glycol 6000. The resulting mixture was kept at 4°C overnight and then centrifuged at 6,500 x g for 2 min. The supernatant was removed and discarded. The loose pellet was resuspended in 0.5 ml of TE buffer (0.010 M Tris, 0.001 M EDTA; pH 8.0). The polyethylene glycol 6000 was removed by extraction with TE buffer-saturated phenol; 0.5 volume of TE buffer-saturated phenol was added, and the preparation was stirred for 3 min and centrifuged at 6,000 x g for 15 min. The aqueous phase was
TABLE 1. Comparison of the 16S rRNA gene sequence of R. chlorophenolicus PCP-1\(^I\) with the sequences of other bacteria\(^a\)

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<th>Organism</th>
<th>EMBL accession no.</th>
<th>No. of bases(^c)</th>
<th>% Homology</th>
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\(^a\) The values are from a reduced data set.
\(^b\) EMBL accession numbers of (partial) 16S rRNA sequences.
\(^c\) Number of bases that were unambiguously determined.
\(^d\) Data from reference 33.

similarly extracted once with 1 volume of a mixture containing equal amounts of phenol and chloroform-isooamyl alcohol (24:1) and twice with 1 volume of chloroform-isooamyl alcohol. The DNA was recovered from the aqueous phase by ethanol precipitation (30).

**PCR.** A standard PCR (27) was performed in a 100-\(\mu\)l (total volume) solution containing 1.25 U of Taq polymerase (Beethesda Research Laboratories), 10 \(\mu\)l of 10 \(\times\) PCR buffer (300 mM KCl, 25 mM MgCl\(_2\), 200 mM Tris-HCl [pH 8.4], 1 mg of bovine serum albumin per ml), 1 \(\mu\)l of gelatin, 1 \(\mu\)l of the detergent Nonidet P-40, 1 \(\mu\)l of a deoxynucleoside triphosphate mixture (10 mM each in 10 mM Tris-HCl [pH 7.5]), 100 ng of primer, and 5 to 10 ng of \(R.\) chlorophenolicus PCP-1\(^I\) DNA. The 100-\(\mu\)l mixture was covered by 20 to 30 \(\mu\)l of light mineral oil (Sigma, St. Louis, Mo.). Most of the 16S rRNA gene was amplified by using the following two universal bacterial 16S rRNA primers (14): a sense primer at position 124 (\(E.\) coli numbering system of Brosius et al. [8]), which was equipped with an additional BamHI site (5’ CACGGAT CCGGACGCGGTTGAAGTACACG; the recognition site is in italics); and an antisense primer at position 1115, which had an additional HindIII site (5’ GTTTAGTGTTTGGTCTTGGGCG TCCTTG; the recognition site is in italics). The PCR involved 35 amplification cycles (1 min at 94°C, 2 min at 48°C, 3 min at 72°C), and the last cycle was followed by a chase (7 min at 72°C). The amplified fragment obtained from the PCR was about 1 kb long and was used as a probe to identify 16S rRNA gene-containing DNA fragments.

Isolation, cloning, and sequence analysis of a 16S rRNA gene from strain PCP-1\(^I\). All of the isolation, cloning, and sequence analysis manipulations were done by using previously described procedures (30). Total DNA from strain PCP-1\(^I\) cells was digested with restriction enzymes, and the resulting fragments were separated by agarose gel electrophoresis and transferred to Hybond N\(^+\) membranes (Amersham, Buckinghamshire, England). The probe used for Southern hybridization of these membranes was the 16S rRNA gene fragment (positions 124 to 1115) obtained by PCR amplification of strain PCP-1\(^I\) DNA. It was labelled by nick translation by using \([\alpha-^32P]dATP\) (27). A 2.1-kb SalI fragment that hybridized to the probe was cloned and sequenced by using pUC19 in \(E.\) coli TG1.

The 2.1-kb SalI fragment was subcloned in M13mp18 and M13mp19 (40) and was used to sequence the 16S rRNA gene with both forward-sequencing primer M13 and 16S rRNA gene-specific primers. DNA sequencing was performed by using the dideoxy chain termination method (31). All enzymes were purchased from Life Technologies, Inc. (Gaithersburg, Md.), Pharmacia LKB Biotechnologies (Uppsala, Sweden), or Boeringer (Mannheim, Germany). Oligonucleotides were synthesized with a Biosearch Cyclone DNA synthesizer (New Brunswick Scientific Corp.) at the Netherlands Institute for Dairy Research, Ede, The Netherlands. A computer analysis of the sequence data was performed by using PC/Gene program version 5.01 (Genoff, Geneva, Switzerland) and the Genetics Computer Group package (version 6.0) (12). The levels of similarity between the 16S rRNA gene sequence of strain PCP-1\(^I\) and the sequences of other strains were calculated by using the BestFit computer program. The 16S rRNA gene sequences used for comparative analyses and their sources are shown in Table 1.

**Sequence alignment and phylogenetic tree construction.** The nucleotide sequence of the 16S rRNA gene of strain PCP-1\(^I\) was aligned manually with the sequences of closely related bacteria. Evolutionary distance (\(K_{\text{nucl}}\)) values were determined by using the neighbor-joining method of Saitou and Nei (28), as implemented in the NEIGHBOR program in the PHYLIP version 3.4 program package developed by Felsenstein (15). The topology of the tree was evaluated by performing a bootstrapped parsimony analysis, using the SNAPARS and DNABOOT programs of Felsenstein (15).

**Nucleotide sequence accession number.** The nucleotide se-
sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession number X79094.

RESULTS

Identification, cloning, and nucleotide sequence of the 16S rRNA gene of <i>R. chlorophenolicus</i> PCP-IT. The 16S rRNA genes on chromosomal DNA fragments from <i>R. chlorophenolicus</i> PCP-IT were identified by using a 16s rRNA sequence of genes on chromosomal DNA fragments from M. chubuense and M. sphagni (Fig. 1, lanes 1 and 2, respectively), DNA of strain PCP-IT and that both copies contain an SstI site. DNA digested with SstI (Fig. 1, lane 3). It appears that containing PCR fragment (positions 124 to 1115, and four less intense bands were visible in the lane containing numbering system) as a probe and Southern blots of total DNA of strain PCP-IT. Relevant restriction sites are indicated.

FIG. 2. Complete sequence of the 16s rRNA gene of <i>R. chlorophenolicus</i> PCP-IT. Relevant restriction sites are indicated.

Analysis of phylogenetic relationships. The 16S rRNA gene sequence of <i>R. chlorophenolicus</i> PCP-IT was compared with the 16S rRNA sequences of a wide variety of strains (Table 1) available in previously published papers and in the EMBL RNA data base. The 16S rRNA sequence of strain PCP-IT exhibited high levels of homology (95.65 to 99%) with the sequences of all of the mycobacterial species examined (Table 1). Strain PCP-IT exhibited complete homology with sequence elements unique to the genus <i>Mycobacterium</i>. In addition, long stretches of complete sequence conservation were found only with <i>Mycobacterium chimaera</i> (from positions 234 to 420 and 241 to 477). The helix present in the strain PCP-IT 16s rRNA gene sequence resembles the helix which is typical of fast-growing mycobacteria (<i>M. phlei</i> is a representative of the fast-growing mycobacteria). The slowly growing mycobacteria have a long helix (<i>M. gordoneae</i> is a representative of the slowly growing mycobacteria). The asterisks indicate the signature sequences used, which were obtained from reference 34. Abbreviations: M. thermoresistibile; M. nonchromogen, M. nonchromogenicum; M. paraaurubal, M. paraaureus.

FIG. 3. Helix (A) and signature sequence (B) of strain PCP-IT compared with the helices and sequences of fast-growing and slowly growing mycobacteria. There was length variation in mycobacterial helices homologous to the helix in <i>E. coli</i> bounded by positions 455 and 477. The helix present in the strain PCP-IT 16S rRNA gene sequence resembles the helix which is typical of fast-growing mycobacteria (<i>M. phlei</i> is a representative of the fast-growing mycobacteria). The slowly growing mycobacteria have a long helix (<i>M. gordoneae</i> is a representative of the slowly growing mycobacteria). The asterisks indicate the signature sequences used, which were obtained from reference 34. Abbreviations: M. thermoresistibile; M. nonchromogen, M. nonchromogenicum; M. paraaureus; M. paraaureal. The helix which is typical of fast-growing mycobacteria (<i>M. phlei</i> is a representative of the fast-growing mycobacteria). The slowly growing mycobacteria have a long helix (<i>M. gordoneae</i> is a representative of the slowly growing mycobacteria). The asterisks indicate the signature sequences used, which were obtained from reference 34. Abbreviations: M. thermoresistibile; M. nonchromogen, M. nonchromogenicum; M. paraaureus; M. paraaureal.

Analysis of phylogenetic relationships. The 16S rRNA gene sequence of <i>R. chlorophenolicus</i> PCP-IT was compared with the
Rhodococcus, Mycobacterium, Nocardia M., Mycobacterium; N., Nocardia; R., Rhodococcus; were examined. Our results showed that the domain containing the fast-growing mycobacterial species and the slowly growing species. Strain PCP-IT is located on two main bifurcations which correspond to the rhodococcal species.

The tree is based on the results of a distance matrix analysis (neighbor joining method) of the complete high-G+C-content data set. Only positions which have the same composition in at least 50% of the sequences in the data set used were included. The tree was evaluated by performing parsimony analyses of the complete data set and maximum-likelihood analyses of subsets. Multifurcations indicate that relative branching orders could not be determined unambiguously. Bar = 10% estimated divergence. Abbreviations: C., Corynebacteriun; G., Gordona; M., Mycobacterium; N., Nocardia; R., Rhodococcus; F and S, fast-growing and slowly growing mycobacterial species, respectively.

Nocardia, and Rhodococcus were examined. Remarkably low levels of homology (87.6 to 91.0%) were observed between the 16S rRNA gene sequence of strain PCP-IT and the sequences of other rhodococci (Table 1).

Multiple alignments of the 16S rRNA gene sequence of strain PCP-IT with the sequences of a great number of species were examined. Our results showed that the domain containing stretches with nucleotide sequences specific for strain PCP-IT is in variable regions V2 and V3 (38, 39).

A phylogenetic tree analysis based on corrected distance (K_{cor}) values also revealed the close relationship between strain PCP-IT and mycobacterial species (Fig. 4). Strain PCP-IT clearly clustered within the confines of the genus Mycobacterium, and it was well removed from the genus Rhodococcus and other mycolic acid-containing taxa. Moreover, in the tree shown in Fig. 4 the mycobacterial species are located on two main bifurcations which correspond to the major subdivisions of the genus Mycobacterium, the fast-growing species and the slowly growing species. Strain PCP-IT is located on the bifurcation containing the fast-growing mycobacterial species.

On the basis of the results obtained in this study, it appears that strain PCP-IT is not a rhodococcal species but is a mycobacterial species.

**DISCUSSION**

Comparative analysis of 16S rRNA gene sequences has been used to examine the phylogenetic relationships among many organisms. Sometimes this has led to the transfer of species from one genus, where they had been placed on the basis of the results of classical taxonomy, to another genus (9, 10, 13, 22, 33).

Sequence divergence among 16S rRNAs is not random but is confined to certain areas, as comparative analyses of 16S rRNAs have revealed regions of highly conserved primary sequences and other regions with considerable amounts of variability. The variable regions characterize taxa, groups, or species (38).

In this study the close relationship of *R. chlorophenolicus* PCP-IT to all of the species belonging to the genus *Mycobacterium* examined was indicated by average similarity values which were greater than 95%. Stahl and Urbance (34), who also used 16S rRNA sequencing to infer phylogenetic relationships among selected species of mycobacteria and related organisms, reported the same average similarity values. Rogall et al. (26) found similar values in a study of the phylogenetic relationships of mycobacterial species, which allowed these authors to differentiate the organisms into fast-growing species and slowly growing species. Pitulle et al. (25) confirmed this distinction by constructing a detailed phylogenetic tree in which all known fast-growing mycobacteria were grouped. A similar differentiation was found in the phylogenetic tree shown in Fig. 4.

The occurrence of a specific domain in the 16S rRNA gene of strain PCP-IT is consistent with the lack of known strains that exhibit 100% homology with the strain PCP-IT 16S rRNA gene sequence. On the basis of the 16S rRNA gene sequence, nucleic acid probes for the rapid, specific detection of strain PCP-IT in environmental samples have been designed.

Natural relationships are also reflected in the common sequence patterns or structures found in members of a common line of descent (16). The sequence of strain PCP-IT was found to contain a short helix flanked by positions 451 and 482 which is comparable to the helix found in *Mycobacterium phlei* (34), which was used as a representative fast-growing mycobacterium by Stahl and Urbance (34). Similarly, Stackebrandt and Smida found that there is a substantial difference between fast-growing and slowly growing mycobacteria (32); the former mainly form a 21-base loop, while the latter form a longer version in which a 15-base loop is interrupted by a 7-bp stem and a 4-base loop.

The division between fast-growing and slowly growing mycobacteria also has been reported to be reflected by rRNA gene copy number (5, 35); two genomic copies occur in the fast-growing species (*M. phlei, Mycobacterium smegmatis*), and one copy occurs in the slowly growing species (*Mycobacterium tuberculosis, Mycobacterium avium, Mycobacterium intracellulare*). Two copies of the 16S rRNA gene were found in strain PCP-IT, as shown in this paper. With respect to the number of rRNA gene copies, strain PCP-IT has features distinct from those of *E. coli*, which harbors seven rRNA gene copies (21), and those of *Streptomyces coelicolor* and *Streptomyces lividans*, each of which has six rRNA copies per chromosome (4). Thus, it appears that among the actinomycetes the slowly growing organisms, as has been reported previously, have fewer rRNA genes, supporting the suggestion that the number of copies of rRNA genes is proportional to the growth rate (24). This is advantageous for bacteria growing under unfavorable conditions and could explain the ability of strain PCP-IT to survive and grow under stressful conditions (6, 7).

The close relationship of strain PCP-IT to mycobacteria inferred from the rRNA data is supported by data for other properties, such as the resistance of the cell wall to lysozyme and the presence of menaquinone MK-9(H2) (1), which is similar to the menaquinone present in mycobacteria. Moreover, Hamid et al. (20) have recently reported that *R. chlorophenolicus* PCP-IT contains mycolic acids that are typical of mycobacteria, a finding that is supported by the data of Haggblom et al. (19) in the accompanying paper. In light of these data, we proposed that pentachlorophenol-mineralizing
ACKNOWLEDGMENTS

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REFERENCES


