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

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We determined the nucleotide sequence of a 16S rRNA gene of *Rhodococcus chlorophenolicus* PCP-I^T (= DSM 43826^T) (T = type strain). Sequence comparisons revealed that there was a close relationship between strain PCP-I^T 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-I^T. We propose that strain PCP-I^T should be transferred to the genus *Mycobacterium* and renamed *Mycobacterium chlorophenolicum* PCP-I^T 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 menaquinone 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-9(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 bronchialis*, *Rhodococcus terrae*, *Rhodococcus rubropertinctus*, and *Rhodococcus sputi*) 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-I^T (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-I^T 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 known 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-I^T 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-I^T 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-I^T 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-I^T and members of the genus *Mycobacterium*. Therefore, we propose that strain PCP-I^T 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-I^T (= DSM 43826^T) (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-I^T 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 1,660 × 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 × 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 × g for 15 min. The aqueous phase was

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TABLE 1. Comparison of the 16S rRNA gene sequence of *R. chlorophenolicus* PCP-I^T with the sequences of other bacteria^a

Organism	EMBL accession no. ^b	No. of bases ^c	% Homology
<i>Mycobacterium chubuense</i>	X55596	1,458	99.04
<i>Mycobacterium sphagni</i>	X55590	1,462	97.74
<i>Mycobacterium fortuitum</i>	X52933	1,452	97.05
<i>Mycobacterium smegmatis</i>	X52722	1,457	97.38
<i>Mycobacterium terrae</i>	X52925	1,401	97.10
<i>Mycobacterium komossense</i>	X55591	1,464	97.05
<i>Mycobacterium nonchromogenicum</i>	X52928	1,455	96.96
<i>Mycobacterium phlei</i>	M28566	1,358	97.45
<i>Mycobacterium simiae</i>	X52931	1,453	96.55
<i>Mycobacterium bovis</i>	M20940	1,538	96.96
<i>Mycobacterium genavense</i>	X60070	1,452	96.68
<i>Mycobacterium tuberculosis</i>	X58890	1,538	96.83
<i>Mycobacterium marinum</i>	X52920	1,493	96.67
<i>Mycobacterium gordonae</i>	X52923	1,464	96.13
<i>Mycobacterium szulgai</i>	X52926	1,465	95.99
<i>Mycobacterium avium</i>	X52918	1,466	95.79
<i>Mycobacterium leprae</i>	X53999	1,550	95.65
<i>Nocardia asteroides</i>	X5749	1,449	93.84
<i>Gordona terrae</i>	X53202	1,465	91.15
<i>Rhodococcus erythropolis</i> DSM 43188 ^{T d}		1,358	91.01
<i>Gordona bronchialis</i> ATCC 25592 ^{T d}		800	92.87
<i>Rhodococcus equi</i>	M29574	1,358	91.66
<i>Rhodococcus fascians</i> DSM 20131 ^{T d}		1,358	87.62
<i>Arthrobacter globiformis</i>	M23411	1,527	90.55
<i>Frankia</i> sp. strain AcN14a	M88466	1,518	90.50

^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-isoamyl alcohol (24:1) and twice with 1 volume of chloroform-isoamyl alcohol. The DNA was recovered from the aqueous phase by ethanol precipitation (30).

PCR. A standard PCR (27) was performed in a 100- μ l (total volume) solution containing 1.25 U of *Taq* polymerase (Bethesda Research Laboratories), 10 μ l of 10 $\times$ PCR buffer (500 mM KCl, 25 mM MgCl₂, 200 mM Tris-HCl [pH 8.4], 1 mg of bovine serum albumin per ml), 1 μ l of gelatin, 1 μ l of the detergent Nonidet P-40, 1 μ 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-I^T DNA. The 100- μ l mixture was covered by 20 to 30 μ 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 *Bam*HI site (5' CACGGAT CCGGACGGGTGAGTAACACG; the recognition site is in italics); and an antisense primer at position 1115, which had an additional *Hind*III site (5' GTGAAGCTTAGGGTTGCGC TCGTTG; 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

FIG. 1. Southern blot of digested total genomic DNA from strain PCP-I^T hybridized with the strain PCP-I^T 16S rRNA PCR fragment as a probe. The restriction enzymes used were *Sal*I (lane 1), *Sph*I (lane 2), and *Sst*I (lane 3). The position of the 2.1-kb fragment used for sequence analysis is indicated by an arrow.

gene from strain PCP-I^T. All of the isolation, cloning, and sequence analysis manipulations were done by using previously described procedures (30). Total DNA from strain PCP-I^T 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-I^T DNA. It was labelled by nick translation by using [α -³²P]dATP (27). A 2.1-kb *Sph*I fragment that hybridized to the probe was isolated and cloned by using pUC19 in *E. coli* TG1.

The 2.1-kb *Sph*I 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 Boehringer (Mannheim, Germany). Oligonucleotides were synthesized with a Bioscience 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 (Genofit, 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-I^T 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-I^T was aligned manually with the sequences of closely related bacteria. Evolutionary distance (K_{nuc}) 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-

A

Strain PCP-1

Fast-Growing
(*M. phlei*)

Slow-Growing
(*M. goodii*)

E. coli

451- A -482

B

Sequence identity:

1	<i>E. coli</i>	GAGGAAGG	GA	GTAAGCTTA	TACTCTTGCT	GATTGACGTT
2	Strain PCP-1	GACGAAGC	---	---	-----	-C AAGTGACGGT
3	<i>R. equi</i>	GACGAAGC	---	---	-----	-A GAGTGACGGT
4	<i>M. chubuense</i>	GACGAAGC	---	---	-----	-C AAGTGACGGT
5	<i>M. phlei</i>	GACGAAGC	---	---	-----	-T GAGTGACGGT
6	<i>M. flavescens</i>	GACGAAGC	---	---	-----	-C AAGTGACGGT
7	<i>M. thermoresist</i>	GACGAAGC	---	---	-----	-G AAGTGACGGT
8	<i>M. aurum</i>	GACGAAGC	---	---	-----	-C AAGTGACGGT
9	<i>M. chelonae</i>	GACGAAGC	---	---	-----	-C AAGTGACGGT
10	<i>M. neoaurum</i>	GACGAAGC	---	---	-----	-C AAGTGACGGT
11	<i>M. senegalensis</i>	GACGAAGC	---	---	-----	-C AAGTGACGGT
12	<i>M. triivale</i>	GACGAAGC	---	---	-----	-C AAGTGACGGT
13	<i>M. terrae</i>	GGCGAAGCTC	CGTNGTT	---	TTCTCGG	GGGTGACGGT
14	<i>M. nonchromogen</i>	GGCGAAGGTC	CAAGCTN	---	TTCTTGG	GGGTGACGGT
15	<i>M. asiaticum</i>	GACGAAGGTC	CG-GGTT	---	TTCT-CG	GATTGACGGT
16	<i>M. gordonae</i>	GACGAAGGTC	CG-GGTT	---	TTCT-CG	GGGTGACGGT
17	<i>M. bovis</i>	GACGAAGGTC	CG-GGTT	---	TTCT-CG	GATTGACGGT
18	<i>M. paratubercul</i>	GACGAAGGTC	CG-GGTT	---	TTCT-CG	GATTGACGGT
19	<i>M. kansasii</i>	GACGAAGGTC	CG-GGTT	---	TTCT-CG	GATTGACGGT

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 *E. coli* 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 (*M. phlei* is a representative of the fast-growing mycobacteria). The slowly growing mycobacteria have a long helix (*M. goodii* is a representative of the slowly growing mycobacteria). The asterisks indicate the signature sequences used, which were obtained from reference 34. Abbreviations: *M. thermoresistans*; *M. thermoresistibile*; *M. nonchromogenes*; *M. nonchromogenicum*; *M. paratuberculosis*; *M. paratuberculosis*.

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-I^T exhibited high levels of homology (95.65 to 99%) with the sequences of all of the mycobacterial species examined (Table 1). Strain PCP-I^T exhibited complete homology with sequence elements unique to the genus *Mycobacterium*. In addition, long stretches of complete sequence conservation were found only with *Mycobacterium chubuense* (from positions 234 to 420 and 440 to 845) and *Mycobacterium sphagni* (from positions 150 to 182, 203 to 259, 272 to 389, and 391 to 442).

Identification, cloning, and nucleotide sequence of the 16S rRNA gene of *R. chlorophenolicus* PCP-I^T. The 16S rRNA genes on chromosomal DNA fragments from *R. chlorophenolicus* PCP-I^T were identified by using a 16S rRNA sequence-containing PCR fragment (positions 124 to 1115, *E. coli* numbering system) as a probe and Southern blots of total strain PCP-I^T DNA (Fig. 1). Two bands were visible in the lanes containing chromosomal DNA from strain PCP-I^T digested with *SalI* and *SphI* (Fig. 1, lanes 1 and 2, respectively), and four less intense bands were visible in the lane containing DNA digested with *SstI* (Fig. 1, lane 3). It appears that two copies of the 16S rRNA gene are present in the chromosomal DNA of strain PCP-I^T and that both copies contain an *SstI* site. To sequence the 16S rRNA gene, we chose the 2.1-kb *SphI* fragment. A sequence analysis revealed that the 2.1-kb *SphI* fragment included the complete 16S rRNA gene, which consisted of 1,532 bp, and contained an *SstI* site (Fig. 2).

Analysis of phylogenetic relationships. The 16S rRNA gene sequence of *R. chlorophenolicus* PCP-1^T was compared with the

Bacteria that occupy the same phylogenetic position are characterized by one or several elements, including conserved regions and signature structures. One example of signature structures consists of the length of and sequence variation in the helix flanked by positions 451 and 482. A study of the secondary structure of the 16S rRNA gene of strain PCP-IT^T revealed that this structure is a short helix that has nucleotide sequences similar to those of the fast-growing *Mycobacterium* species (Fig. 3).

High levels of sequence homology similar to those observed between the 16S rRNA gene of strain PCP-1^T and the 16S rRNA genes of *Mycobacterium* spp. were not observed when the sequences of members of the related genera *Gordona*,

FIG. 4. Unrooted phylogenetic tree showing the relationships among *R. chlorophenolicus* PCP-I^T, selected mycobacterial species, and members of several related mycolic acid-containing actinomycete genera. 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., *Corynebacterium*; G., *Gordonia*; 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-I^T and the sequences of other rhodococci (Table 1).

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

A phylogenetic tree analysis based on corrected distance (K_{nuc}) values also revealed the close relationship between strain PCP-I^T and mycobacterial species (Fig. 4). Strain PCP-I^T 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-I^T 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-I^T 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-I^T 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-I^T is consistent with the lack of known strains that exhibit 100% homology with the strain PCP-I^T 16S rRNA gene sequence. On the basis of the 16S rRNA gene sequence, nucleic acid probes for the rapid, specific detection of strain PCP-I^T 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-I^T 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 bovis*, *Mycobacterium intracellulare*). Two copies of the 16S rRNA gene were found in strain PCP-I^T, as shown in this paper. With respect to the number of rRNA gene copies, strain PCP-I^T 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-I^T to survive and grow under stressful conditions (6, 7).

The close relationship of strain PCP-I^T 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-I^T contains mycolic acids that are typical of mycobacteria, a finding that is supported by the data of Häggblom et al. (19) in the accompanying paper. In light of these data, we proposed that pentachlorophenol-mineralizing

strain PCP-1^T should be renamed *Mycobacterium chlorophenolicum* PCP-1^T.

ACKNOWLEDGMENTS

We are grateful to Wolfgang Ludwig (Technical University of Munich) for constructing phylogenetic trees and to Ans C. M. Geerling for expert technical assistance.

Part of this work was supported by fellowships from the European Environmental Research Organization and the OECD Project on Biological Resource Management" (to M. Briglia).

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