Rhodococcus zopfii sp. nov., a Toxicant-Degrading Bacterium

MATTHEW A. STOECKER,* RUSSELL P. HERWIG, AND JAMES T. STALEY

Department of Microbiology, University of Washington, Seattle, Washington 98105

A toluene-degrading bacterial strain isolated from bioreactors was identified as a member of the genus Rhodococcus on the basis of the following characteristics: meso-diaminopimelic acid, arabinose, galactose, and glucose are the diagnostic cellular sugars; the mycolic acids contain 33 to 36 carbon atoms; and the formation of a branching mycelium is followed by marked fragmentation of the mycelium into irregular rod-shaped to coccoid units. DNA-DNA hybridization analyses performed with type strains of Rhodococcus species showed that this strain is less than 70% related to other species that have similar phenotypic characteristics. On the basis of these findings, we propose that this strain should be described as a new species, Rhodococcus zopfii, in honor of Wilhelm Friedrich Zopf. The type strain is strain T1.

Actinomycetes are a diverse group of bacteria that are found in a variety of habitats. The development of chemotaxonomy has revolutionized the field of actinomycete classification and has provided tests for the identification of these bacteria (14). The characteristics of the sugars and diamino acids found in cell walls have proved to be important in determining actinomycete genera, as is lipid analysis (8, 15). Once these determinations are made, a tentative genus classification is often possible on the basis of morphological characteristics.

The members of the genus Rhodococcus exhibit a diverse range of morphological characteristics. The division cycles of these organisms range from a rod-coccus growth phase (as seen in Rhodococcus chlorophrénolicus) to the formation of a branching mycelium which fragments into irregular rod-shaped to coccus-shaped units (as seen in Rhodococcus coprophilus and Rhodococcus ruber) (7). The genus Rhodococcus is a member of the nocardioforms actinomycete group and can be distinguished from other genera in this group by the number of carbon atoms in the mycolic acids; the mycolic acids of Rhodococcus spp. have shorter carbon chains than the mycolic acids of members of other genera in this group (8). Rhodococcus spp. can also be distinguished from coryneform bacteria that have short-chain mycolic acids by the presence of tuberculostearic acid and by the G+C contents of the organisms. The levels of intraspecific relatedness of strains can be determined by DNA-DNA hybridization. Two strains are said to be members of the same species if their DNAs are >70% complementary (23).

The genus Rhodococcus may be an important genus from the standpoint of bioremediation. Utilization of aromatic rings as carbon sources is a common motif found in many Rhodococcus isolates (19), and workers have described Rhodococcus strains that degrade such toxic aromatic compounds as chlorinated phenols (1, 2), dinitrophenol (16), and naphthalene (10). Many other examples of the metabolic versatility of Rhodococcus strains have been described previously (5).

In this paper, we describe a novel species of the genus Rhodococcus. The type and only strain, strain T1, utilizes phenol, toluene, and biphenyl as sole sources of carbon and degrades several toxic compounds, including chlorinated benzenes (13).

MATERIALS AND METHODS

Chemicals. Unless specified otherwise, all chemicals used were reagent grade.

Cultures. The strains which we used are listed in Table 1. The original isolate, strain T1T (T = type strain), was obtained from a toluene-phenol bioreactor operated by the Department of Civil Engineering, University of Washington, as previously described (13). Unless indicated otherwise, strain T1T and other Rhodococcus strains were grown on glucose-yeast extract agar, which contained (per liter) 10 g of glucose, 10 g of yeast extract, and 15 g of Bacto agar (Difco).

Gram staining, catalase test, and oxidase test. Gram staining, the catalase test, and the oxidase test were performed as described previously (6).

Sole carbon sources. The growth of strain T1T on 1% inositol, 1% maltose, 1% D-mannitol, 1% L-rhamnose, 1% sorbitol, 0.1% sodium benzoate, 0.1% sodium citrate, 1% ethanol, 1% glucose, 1% sucrose, 1% D-trehalose, 0.1% sebacic acid, 0.1% sodium pyruvate, 0.1% sodium succinate, and 0.02% phenol was tested at 30°C on solid media by comparison with a control that contained no added carbon source. Plates were incubated for 7 days. Growth on solid basal medium in atmospheres saturated with toluene and biphenyl as sole carbon sources was also tested. The basal medium used was ATCC 1306 (4) supplemented with 0.5 g of NH4Cl per liter and solidified with 8.5 g of Gelrite per liter.

Cell wall diamino acids and sugars. Cell wall preparations were obtained and diamino acids and sugars were determined as described by Hasegawa et al. (11). Strains containing L,L-diaminopimelic acid (Streptoverticillium reticulum) and meso-diaminopimelic acid (Rhodococcus rhodochrous), as well as strains containing a mixture of L,L-diaminopimelic acid, meso-diaminopimelic acid, and D,D-diaminopimelic acid, were used as reference strains for the diamino acids analysis, and preparations containing galactose, arabinose, glucose, xylose, rhamnose, maltose, and ribose were used as reference preparations for the sugar analysis.

Lipids. Simple fatty acids were extracted and methylated by whole-cell methanolysis of wet organisms (20). Methyl esters of simple fatty acids were identified by gas chromatography by using Microbial Identification System software version 3.2 (Microbial ID, Inc., Newark, Del.). A Hewlett-Packard model 5890 gas chromatograph and a Hewlett-Packard methyl phenyl silicone fused-silica capillary column (25 m by 0.2 mm) were used for gas chromatography. The cells used for fatty acids analysis were grown on Trypticase soy broth agar.

* Corresponding author. Phone: (206) 685-1783. Fax: (206) 543-8297. Electronic mail address: quinn@u.washington.edu.
Mycolic acids. Mycolic acids were extracted and methylated as described by Minnikin et al. (17). Mycolic acid esters were purified by preparative thin-layer chromatography (3) and were analyzed by mass spectroscopy. A Kratos Profile model HV-4 mass spectrometer (direct inlet; ionization voltage, 70 eV; temperature, 160 to 200°C) was used to determine the numbers of carbon atoms in the mycolic esters (3).

Isolation of DNA. We developed a procedure to isolate DNA from *Rhodococcus* species that was based on the method described by Wilson (24). Cells were grown to the stationary phase on glucose-yeast extract agar and then scraped from the agar surface and suspended in 3.0 ml of a solution containing 10 mg lysozyme per ml in TE buffer (10 mM Tris, 1 mM EDTA; pH 7.6). The resulting preparation was incubated for 24 h at room temperature. A 200-μl aliquot of pronase (20 mg/ml) was added, and then 0.8 ml of 10% sodium dodecyl sulfate in TE buffer was added. This preparation was incubated for 1 h at 37°C. An 800-μl aliquot of 5 M NaCl was added, and then 640 μl of 10% cetyltrimethylammonium bromide in 0.7 M NaCl was added and the preparation was mixed well. The preparation was incubated for 10 min at 65°C, extracted with 1 volume of chloroform-isooamyl alcohol (24:1), and centrifuged in a Sorvall model M-40 for 10 min at 65°C, extracted with 1 volume of chloroform-isooamyl alcohol (24:1), and centrifuged in a Sorvall model M-40 mass spectrometer (direct inlet; ionization voltage, 70 eV; temperature, 160 to 200°C) was used to determine the numbers of carbon atoms in the mycolic esters (3).

Isolation of DNA. We developed a procedure to isolate DNA from *Rhodococcus* species that was based on the method described by Wilson (24). Cells were grown to the stationary phase on glucose-yeast extract agar and then scraped from the agar surface and suspended in 3.0 ml of a solution containing 10 mg lysozyme per ml in TE buffer (10 mM Tris, 1 mM EDTA; pH 7.6). The resulting preparation was incubated for 24 h at room temperature. A 200-μl aliquot of pronase (20 mg/ml) was added, and then 0.8 ml of 10% sodium dodecyl sulfate in TE buffer was added. This preparation was incubated for 1 h at 37°C. An 800-μl aliquot of 5 M NaCl was added, and then 640 μl of 10% cetyltrimethylammonium bromide in 0.7 M NaCl was added and the preparation was mixed well. The preparation was incubated for 10 min at 65°C, extracted with 1 volume of chloroform-isooamyl alcohol (24:1), and centrifuged in a Sorvall model RC-2B centrifuge at 7,700 × g for 4 min. The supernatant was collected, 0.6 volume of isopropanol was added, and the DNA precipitate was collected on a clean glass hook, rinsed in 95% ethanol, air dried, and redissolved overnight at 4°C in 500 μl of 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate). A 10-μl aliquot of an RNase solution (10 pg/ml) was added, and the preparation was incubated for 1 h at 37°C. A 10-μl portion of pronase was then added, and the preparation was incubated for 1 h at 37°C. Samples were extracted with an equal volume of a solution containing phenol (ultrapure; Bethesda Research Laboratories), chloroform, and isoamyl alcohol (24:1), and centrifuged in a Sorvall model W-4 mass spectrometer (direct inlet; ionization voltage, 70 eV; temperature, 160 to 200°C) was used to determine the numbers of carbon atoms in the mycolic esters (3).

Isolation of DNA. We developed a procedure to isolate DNA from *Rhodococcus* species that was based on the method described by Wilson (24). Cells were grown to the stationary phase on glucose-yeast extract agar and then scraped from the agar surface and suspended in 3.0 ml of a solution containing 10 mg lysozyme per ml in TE buffer (10 mM Tris, 1 mM EDTA; pH 7.6). The resulting preparation was incubated for 24 h at room temperature. A 200-μl aliquot of pronase (20 mg/ml) was added, and then 0.8 ml of 10% sodium dodecyl sulfate in TE buffer was added. This preparation was incubated for 1 h at 37°C. An 800-μl aliquot of 5 M NaCl was added, and then 640 μl of 10% cetyltrimethylammonium bromide in 0.7 M NaCl was added and the preparation was mixed well. The preparation was incubated for 10 min at 65°C, extracted with 1 volume of chloroform-isooamyl alcohol (24:1), and centrifuged in a Sorvall model RC-2B centrifuge at 7,700 × g for 4 min. The supernatant was collected, 0.6 volume of isopropanol was added, and the DNA precipitate was collected on a clean glass hook, rinsed in 95% ethanol, air dried, and redissolved overnight at 4°C in 500 μl of 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate). A 10-μl aliquot of an RNase solution (10 pg/ml) was added, and the preparation was incubated for 1 h at 37°C. A 10-μl portion of pronase was then added, and the preparation was incubated for 1 h at 37°C. Samples were extracted with an equal volume of a solution containing phenol (ultrapure; Bethesda Research Laboratories), chloroform, and isoamyl alcohol (25:24:1) and centrifuged for 4 min in a Beckman Microfuge E apparatus. The supernatant was collected, and the extraction was repeated until the interface was clear. Samples were then extracted with an equal volume of chloroform-isooamyl alcohol (24:1) and centrifuged for 4 min in a microcentrifuge. The supernatant was collected, 0.16 volume of 5.0 M NaCl was added, and then 0.6 volume of isopropanol was added. DNA was collected onto a clean glass hook, rinsed in 95% ethanol, air dried, and redissolved overnight at 4°C in 250 μl of 1× SSC. Optical densities were measured at 230, 260, 270, and 280 nm to determine purity. Measurements were repeated three times.

Determination of G+C content. The G+C content of the DNA was determined by the thermal denaturation method as described by Zakrzewska-Czerwinska et al. (25). Experiments were repeated three times.

DNA-DNA hybridization. Levels of DNA-DNA hybridization were determined by the thermal reannealing method described by Seidler and Mandel (22). Samples were sheared by sonication for 3 min in a Cole-Parmer model 4710 sonicator by using a microtip at setting five (21). Reannealing was performed with a Gilford Response spectrophotometer by using 250-μl cuvettes capped with Teflon stoppers. Samples of DNA (typically 60 to 75 μg/ml) were dissolved in 5× SSC containing 20% dimethyl sulfoxide. The samples were kept at 97°C for 10 min in order to denature the DNA, and then the temperature was decreased to 65°C and the preparations were kept at this temperature for 6 h. Levels of relatedness were calculated by using the formula (3) of Seidler and Mandel (22). Hybridization experiments were repeated twice, and the averages are reported below.

RESULTS

Strain TIT was isolated from a phenol-toluene bioreactor operated by the Department of Civil Engineering, University of Washington. This strain can degrade chlorinated benzenes and propenes, as well as other chlorinated compounds (13).

Morphological sequence. Strain TIT produces red to pink, wrinkled colonies on glucose-yeast extract agar. The morphological sequence during colonial development is shown in Fig. 1. After 48 h (Fig. 1A), the colonies begin to produce a branching mycelium. After 96 h (Fig. 1B), the mycelium fragments into irregular rod-shaped to coccoid units upon mechanical agitation.

Gram staining, oxidase test, and catalase test. Strain TIT was gram positive to gram variable, oxidase negative, and catalase positive.

Cell wall diamino acid and whole-cell sugars. The cell wall of strain TIT contains meso-diaminopimelic acid as the only cell wall diamino acid. The whole-cell sugars are arabinose, galactose, and glucose.

Simple fatty acids. The following simple fatty acids are released by acid methanolsysis (relative abundance values are indicated in parentheses): C14:0 (4.3), C16:0 (100), C16:1 trans 9 (27.1), C16:1 trans 9(C15:0 H) (20.4), C17:0 (3.4), C18:0 (25.5), C18:1 cis 9 (81.7), 10CH3 C18:0 (tuberculostearic acid) (17.8), and C18:3 (3.4). C16:1 trans 9 and C15:0 H are indistinguishable in the analysis system that was used.

Fatty acid acyl sequences. Purified mycolic acids were examined by mass spectrometry. The locations of the prominent anhydro-mycolate peaks (3) in the mass spectrum ranged from m/z 504 to 544, corresponding to carbon chain lengths of 33 to 36 atoms.

DNA-DNA hybridization. Strain TIT was examined for DNA-DNA hybridization to the type strains of *R. rhodochrous*, *R. coprophilus*, *R. ruber*, *Rhodococcus fascians*, *Rhodococcus erythropolis*, *Rhodococcus globenratis*, and *R. chlorophenolicus*. These *Rhodococcus* species were chosen because of their morphological similarity and chemotaxonomic characteristics (reviewed in reference 7) or, in the case of *R. chlorophenolicus*, similarity in biodegradative activity (1). Levels of hybridization ranged from 58% in the case of *R. fascians* to 4% in the case of *R. chlorophenolicus*. The levels of hybridization between strain TIT and other species are shown in Table 2.

G+C content. The G+C content of the DNA strain TIT was found to be 70 mol%.

Sole carbon sources. Strain TIT grew on 1.0% maltose, 0.1% sodium benzoate, 1.0% ethanol, 1.0% glycerol, 1.0% sebacic acid, 1.0% sodium pyruvate, 1.0% sodium succinate, 0.02% phenol, toluene vapor, and biphenyl vapor as sole sources of carbon, but not on 1.0% inositol, 1.0% D-mannit...
FIG. 1. (A) *R. zopfi* at 48 h postinoculation. Bar = 12.5 μm. Note the formation of a branching filaments. (B) *R. zopfi* at 96 h postinoculation after mechanical agitation. Bar = 12.5 μm. Note the appearance of irregular rod-shaped and coccoid units.
tol, 1.0% L-rhamnose, 1.0% sorbitol, 0.1% sodium citrate, 1.0% sucrose, or 1.0% D-trehalose.

**DISCUSSION**

The genus *Rhodococcus* is a genus whose members exhibit considerable morphological and chemotaxonomic heterogeneity. When grown on solid media, strain T1 poses a hyphal collection of branching filaments which fragment over time into irregular rod-shaped and coccoid units. Strain T1 has all of the major characteristics of the genus *Rhodococcus*. Furthermore, the presence of tuberculostearic acid and mycolic acids also distinguish strain T1 from previously described species without the need for DNA-DNA hybridization. However, while strain T1 and other *Rhodococcus* species share many characteristics, DNA-DNA hybridization data suggest that no species is very closely related to strain T1 at the molecular level. Even though the level of hybridization between strain T1 and *R. fascians* is 58%, these two organisms are easily distinguishable on the basis of phenotypic characteristics, such as pigmentation, carbon sources, and G+C content. Also, while the carbon source profile of strain T1 is very similar to that of *R. coprophilus*, these two organisms are clearly differentiated by DNA-DNA hybridization data and by mycolic acid size. Table 2 shows differential properties for strain T1 and seven other *Rhodococcus* species.

Strain T1 has all of the major characteristics of the genus *Rhodococcus*, but is not a member of any previously described species. Therefore, we propose the following species description.

**Description of Rhodococcus zopfii sp. nov.** *Rhodococcus zopfii* (zop'fii.i M.L. adj. zopfii, in honor of Wilhelm Friedrich Zopf, who first described the bacterium *R. rhodochrous*) cells are gram positive to gram variable, oxidase negative, and catalase positive and grow filamentously, exhibiting extensive branching and hyphal growth before fragmenting into irregular rod-shaped to coccoid units.

On glucose-yeast extract agar, colonies are red to orange and wrinkled.

After 7 days of incubation at 30°C in a mineral salts medium supplemented with ammonia and trace elements, growth is produced with 1% (wt/vol) maltose, 0.1% sodium benzoate, sebacic acid, sodium pyruvate, sodium succinate,
1% (vol/vol) ethanol, and glycerol, but not with 1% inositol, mannitol, rhamnose, sorbitol, sucrose, trehalose, or 0.1% sodium citrate.

The cell wall contains meso-diaminopimelic acid, arabinose, galactose, and glucose. The following simple fatty acids are released by acid methanolyisates (relative abundance values are indicated in parentheses): C14:0 (4.3), C16:0 (100), C16:1 cis 9 (27.1), C16:1 trans 9/15/20H (20.4) C17:0 (3.4), C18:0 (25.5), C18:1 cis 9 (81.7), 10CH3 C18:0 (tuberculostearic acid) (17.8), and C20:0 (3.4).

Mycolic acids are present and contain between 33 and 36 carbon atoms.

The type strain, strain T1, was isolated from a toluene-phenol bioreactor operated by the Department of Civil Engineering, University of Washington. This strain degrades chlorobenzene, 1,2 dichlorobenzene, 1,3 dichlorobenzene, and 1,4 dichlorobenzene and uses phenol and toluene as sole sources of carbon.

The G+C content of the DNA is 70 mol%.

Strain T1T has been deposited in the American Type Culture Collection as strain ATCC 51349T.

ACKNOWLEDGMENTS

This work was supported by grant ESO-4696 from the NIEHS and by grant GM 08437 from the NIH.

We thank Marie Coyle, Dave Nowawieski, and Brian Shin for their excellent technical assistance.

REFERENCES

21. Seidler, R. J. Personal communication.