African Journal of Biotechnology Vol. 4 (7), pp. 639-645, July 2005
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2005 Academic Journals

Full Length Research Paper

Rhodococcus opacus strain RW, a resorcinol-degrading bacterium from the gut of *Macrotermes michaelseni*

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Accepted 14 May, 2005

The population of resorcinol degrading bacteria in the intestinal tract of fungi-cultivating termite, *Macrotermes michaelseni*, was estimated to be 6.8 × 10^2 cells/ml. A gram-positive bacterium designated RW, capable of degrading resorcinol, was isolated from the highest positive dilution. Isolate RW could also degrade phenol and benzoic acid aerobically, and anaerobically using nitrate as an electron acceptor. The isolate is a rod-shaped bacterium that exhibited evolutionary relatedness with the genus *Rhodococcus*, as determined by phenotypic traits and physiological tests, and a 16S rRNA gene sequence similarity value of 99.6% to the closest *Rhodococcus opacus* strain. On the basis of these results isolate RW is proposed as a new strain in the species *R. opacus*. The ability of the isolate to degrade resorcinol, phenol and benzoic acid makes it a potential candidate for use in bioremediation of environments contaminated by such or related compounds.

Key words: *Rhodococcus opacus,* resorcinol, *Macrotermes michaelseni*, termites, bioremediation, monoaromatic compounds.

INTRODUCTION

Resorcinol is a natural as well as man-made phenolic compound (Hans, 1994). Natural phenolic compounds and their derivatives are present everywhere in the environment (Paula et al., 1998). Plant roots exude a variety of phenolic compounds including 4-hydroxybenzoate, ferulic, *p*-coumaric, vanilic, cinnamic and syringic acids (Harborne and Moss**,** 1993). Phenolic compound enter the environment during the biodegradation of natural polymers containing aromatic rings (Paula et al., 1998) such as lignin and tannins, and from aromatic amino acid precursors. Resorcinol is introduced into the environment through pulp mills, refineries, wood preservation plants and various chemical industries, as well as their wastewaters (Suomi and Vitaniemi, 1999).

The degradation of resorcinol has been demonstrated in a number of screening studies (Larway and Evans, 1965; Chapman and Ribbons, 1976; Gorny et al., 1992; Heider and Fuchs, 1997; Philipp and Schink, 1998). Despite the fact that phenolic compounds are present in most soils and sediments, only a few aerobic resorcinol-degrading microorganisms have been isolated and characterized (Maeda and Massey, 1993). Microorganisms capable of anaerobic degradation were described as early as 1976 (Chapman and Ribbons, 1976), pertaining to a bacterium of the genus *Pseudomonas*. Tschech and Schink (1985) documented the resorcinol degradation pathway of a fermenting bacterium of the genus *Clostridium*. Cell-free extracts of this bacterium converted resorcinol to dihydroresorcinol (Kluge et al., 1990) to form cyclohexanedione, which is further hydroxylated to 5-oxohexanoate by nucleophilic attack on one of the carbonyl carbon atoms (Schink et al., 2000). Recently a new species of the genus *Azoarcus* sp. was described by Philipp and Schink (1998) as an obligate nitrate - reducing bacterium that converts resorcinol completely to CO_2 and N_2, using an entirely

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different strategy.

Termites are considered ecosystem engineers because of their numerical and ecological significance (Brune and Fredrich, 2000). However, most studies on termite gut microbiota have focused on wood-feeders; analogous studies on other feeding guilds, especially soil-feeders and fungi-cultivators, remain sparse (Kane et al., 2001), owing to their typically remote habitats, delicate nature, and the difficulty of establishing permanent laboratory cultures (Bignell et al., 1980; Rouland et al., 1993). Species of the genus *Macrotermes* that construct large epigeal nests and extensive underground gallery systems have major effects on soil chemical and physical properties throughout the tropics and subtropics. An improved understanding of the role of termite gut microbial biota in the degradation of organic compounds especially xenobiotics in the tropical forests and savannahs, is therefore essential. Moreover, the overall understanding of invertebrate gut microbiota that catalyze degradation of aromatic compounds is still meager (Brune et al., 1995b).

Studies done by Brune et al. (1995a) focusing on the metabolism of monoaromatic compounds such as benzoic acid, cinnamic acid, ferrulic acid and phenylpropanoic acid by gut homogenates of *Nasutitermes lujae* have demonstrated that oxygen-mediated ring cleavage might occur in otherwise anoxic habitats. Thus, free monoaromatic compounds present in plant residues and monoaromatic acids and phenolic compounds are probably prime candidates for such dissimilatory activity. In this study, the mineralization of resorcinol, which is a key intermediate in aerobic microbial degradation of aromatic compounds, was investigated. The assumption was that microbes in the termite gut should be able to mineralize resorcinol.

MATERIALS AND METHODS

Termites and gut preparation for dilution series

A small number of termite worker castes of a fungi-cultivating termite, *Macrotermes michaelseni*, were freshly collected from mounds in Jomo Kenyatta University of Agriculture and Technology, in Thika District, Kenya. The termites were used within an hour of collection to avoid any physiological changes in the intestinal tract. The termites were degutted using sterile fine-tipped forceps, and then 10 guts were homogenized in sterile glass homogenizers in 1 ml of normal saline solution (NSS).

Growth medium

Organisms were cultivated in phosphate buffered mineral salts medium KMM1. This medium was identical to basal medium MM-4 (Brune et al., 1995a) except for the following variations. The media received no additional supplements or trace elements. Basal medium KMM1 contained per liter, NaCl (1.7 g), KCl (6.5 g), $MgCl_2.6H_2O$ (0.50 g), $CaCl_2.2H_2O$ (0.10 g), NH_4Cl (5.6 g), $NaSO_4$ (1.0 g) and KH_2PO_4 (1.0 g). Resorcinol was added to the media from a 1 M sterile stock solution to a final concentration of 2 mM. All solutions, cultures and media were prepared and maintained using aerobic techniques. Cultures were incubated on a shaker (100 rpm) at 27°C and monitored for the loss of resorcinol and turbidity increase due to bacterial growth.

MPN determinations

For most-probable-number (MPN) determinations under oxic culture conditions, serial 10-fold dilutions of gut homogenates (10 guts ml^{-1}), in triplicate were prepared in basal medium KMM1 containing 2 mM resorcinol. The average number of microorganisms growing in the presence of resorcinol was determined after one week of incubation in a shaker (100 rpm) at 27°C. Numerical data was computed using MPN probability tables (Alef and Nannipieri, 1995). Growth was ascertained by checking turbidity in a spectrophotometer (Shimadzu UV240), as well as analyzing the culture supernatant for substrate utilization by High Performance Liquid Chromatography (HPLC, see analytical methods below).

Isolation and cultivation conditions

Erlenmeyers' flasks (150 ml) containing 60 ml of KMM1 medium supplemented with resorcinol (2 mM) were inoculated with a 5% (v/v) inoculum from the highest positive tube in the MPN series. The flasks were incubated with agitation (100 rpm) at 27°C until growth became constant as was evidenced by the optical density (OD_{600}). Cultures were then plated on solid KMM1 medium, which contained 1.5% agar and resorcinol (2 mM). Individual colonies, which, grew on the plates, were then re-inoculated in fresh liquid media (Yuste et al., 2000) to ascertain their ability to degrade resorcinol.

Analytical methods

The loss of the monoaromatic compound provided was monitored by high performance liquid chromatography (HPLC) (Beckman, Fullerton, Calif.) with a UV detector equipped with a Beckman ultrasphere C-18 column (250 × 4.6 mm, 5µm). A mobile phase of acetonitrile (25%) and 0.1% acetic acid (Fisher Scientific, Fairlawn, N.J.) in demineralized water was used for separation. Chromatography was carried out at ambient temperature at a flow rate of 1 ml/min at a wavelength of 260 nm. Concentrations of aromatic compounds were calculated from external standards. Compounds were identified by their retention times with reference standards.

Characterization of the isolates

Cell morphology was determined by a phase contrast Zeiss Axiophot microscope (Carl Zeiss, Oberkochem, Germany) supplemented by the classical gram staining method (Bartholomew, 1962) and the 3.0% (w/v) KOH test (Gregersen, 1978). Motility was assed by direct microscopic observation from cultures in the growth phase (in SIM agar; pH 7.3) and by testing the ability of the isolates to migrate from the point of inoculation through semisolid (0.3%) agar tube (Ball et al., 1996). Temperature and pH ranges and optima for growth were determined in nutrient broth. Growth was measured with a Shimadzu model UV240 spectrophotometer at 600 nm in cuvettes with a 1-cm light path. Biochemical characterisation of the isolate was carried out using the procedures of Cappuccino and Sherman (2002) and Atlas (1995). The ability to use various selected substrate was tested in KMM1 containing substrates at concentrations of 1% (w/v) for non-aromatic compounds and a 2 mM concentration for aromatic substrates from aerobic stock solutions. The test media was inoculated with 0.05 ml

of a 48-h old culture grown in nutrient broth and incubated at 27°C (Murray et al., 1984) in a shaker (100 rpm). Experiments were incubated for up to two weeks.

In addition, the ability to reduce nitrate under anoxic conditions was tested by inoculating the isolate into bicarbonate buffered KAM1 medium containing KNO_3 (10 mM) and resorcinol (2 mM), under an N_2/CO_2 (80%: 20%) headspace to test for ability to reduce nitrate. Basal medium KAM1 was modified from basal AM4 medium (Brune et al., 1995a) and contained (per liter) 1 g of NaCl, 0.5 g of KCl, 0.4 g of $MgCl_2.6H_2O$, 0.1 g of $CaCl_2.2H_2O$, 0.3 g of NH_4Cl, 0.2 g of KH_2PO_4 and 0.15 g of Na_2SO_4. $NaHCO_3$ (final concentration, 30 mM) was used as the buffer. The reducing agent incorporated into the medium was dithiothreitol (DTT) (1 mM). The pH was adjusted to 7.2. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 27660 were used as reference organisms in all tests.

Resorcinol tolerance

The highest concentration of resorcinol, at which the isolate(s) could initiate growth (i.e. resorcinol tolerance), was determined by monitoring the optical density of the culture growing at initial resorcinol concentrations ranging from 0.5-9 mM. Experiments were carried out in 50-ml flasks containing 30 ml of KMM1 and a 10% (v/v) inoculum. Cultures were then incubated in a shaker (100 rpm) at 27°C for one week. Isolate(s) were also inoculated into basal KMM1 without the substrates, which served as the control experiment.

Phylogenetic analysis

The genomic DNA was prepared from cells of isolate grown aerobically on nutrient agar. The bacteria were suspended in 1500 µl of phosphate-buffer solution (120 mM, Na_2HPO_4 pH 8.0) and centrifuged at 1400 rpm for 10 min (Centrifuge 5417R; Eppendorf). The supernatant was re-suspended in 300 µl of phosphate-buffer and boiled in a water bath (100°C for 10 min.). This was followed by three consecutive cycles of freeze-thaw using liquid nitrogen and a 60°C water bath for 1 minute each cycle. The DNA was pelleted by centrifugation (1400 rpm for 5 min.); the supernatant (200 µl) was then transferred into sterile 1.5 ml tubes. DNA concentration was determined photometrically (Eppendorf, Biophotometer 6131) as described by the manufacturer.A portion of the DNA extract was used in the PCR to amplify the 16S rRNA gene with primers 27F (5'- AGAGTTTGATCCTGGCTCAG-3'; *Escherichia coli* positions 8 to 27) (Edwards et al., 1989) and 1492R (5'- TAC GGY TAC CTT GTT ACG ACT T-3'; *E. coli* positions 1492 to 1512) (Weisburg et al., 1991). PCR (30 cycles) was carried out as described previously (Henckel et al., 1999), except that the annealing temperature was 48°C. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Both strands of the purified PCR products were sequenced with a model ABI 377 DNA sequencer (Applied Biosystems). Agarose gel electrophoresis with ethidium bromide staining was used to examine and check the size of the PCR products (Sambrook et al., 1989).

The sequences were compared to sequences in public databases with the BLAST search program on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to find closely related bacterial 16S rRNA gene sequences. The ARB software package (Ludwig and Strunk, 1996) was used to align the sequences. Alignments were checked and corrected manually where necessary. Highly variable regions of the 16S rRNA gene sequences and sequence positions with possible alignment errors were excluded by using only those positions of the alignment that was identical in at least 50% of all sequences. Phylogenetic trees were calculated according to the neighbor-joining method (Saitou and Nei, 1987), and maximum-likelihood (Felsenstein, 1981), and visualized using Tree View. Sequence similarity matrices were calculated, in addition.

RESULTS

Isolation and characterization of resorcinol-degrading isolates

The population of microbes capable of degrading resorcinol under oxic conditions, which were resident in the gut of *M. michaelseni*, was approximately (6.8×10^2 cells ml^{-1}). Five bacterial isolates were obtained from the highest dilution tubes after enrichment in medium KMM1 using resorcinol as the sole carbon and energy source. However, only one isolate, designated as RW was found to degrade resorcinol completely, and was thus further characterized using morphological and phenotypic properties (Table 1). Colonies of isolate RW on TSA and Nutrient Agar are cream colored, opaque and convex with circular margins. Isolate RW cells typically stains gram-positive revealing long, rod-shaped cells approximately 1 by 10 µm, which may be in chains of 2-3 cells showing some branching during exponential phase of growth (Figure 1A). In the stationary phase of growth, most cells are spherical (Figure 1B). RW grew relatively slowly in rich media (KMM1 with resorcinol and agar [1.5%] or nutrient agar or nutrient broth only). Growth on TSA was slow, resulting in pinpoint colonies after 48 h. The optimum temperature of growth was 27°C but the isolate could grow between 4 and 30°C but not above 37°C (Figure 2). The pH range for growth at ambient room temperature was ascertained to be 5-8.5, with optimum growth at pH 7.0. Isolate RW also flocculates upon depletion of its carbon source. The cells are faintly acid-fast and no spores were demonstrated by malachite green staining (Cappucino and Sherman, 2002) of a 1-week old culture. In addition, cells did not remain viable, after treatment of the culture with 50% ethanol for 1 h (Koransky et al., 1978) or pasteurization of 1-week old cultures at 80°C for 10 min followed by inoculation in nutrient agar. Isolate RW could not initiate growth on resorcinol at concentrations above 6 mM.

Metabolic versatility

Isolate RW was tested for its ability to mineralize or transform a variety of simple aromatic compounds under oxic and denitrifying conditions, and non-aromatic compounds under oxic conditions. The isolate grew on phenol and benzoic acid, both aerobically and anaerobically. The compounds tested but not mineralised by isolate RW include 4-nitrophenol, ninhydrin, alpha-naphthol, 2,6-dimethylphenol and 4-dimethylamino

Table 1. Morphological and biochemical characteristics of isolate RW.

Characteristics	Isolate RW
Gram staining	Positive
Acid fastness	Positive
Cell shape	Rod
Cell size	0.8-1 × 9-10 µm
Colony size	10-15 mm
Motility	Negative
Spore formation	Negative
Growth behaviour to O_2	Aerobic
Assimilation of C_1 compounds	Negative
Growth on or in peptone	Yes
Tolerance to NaCl (6%)	Negative
Oxidase	Negative
Catalase	Positive
Gelatin Liquefaction	Positive
Nitrate reduction	Positive

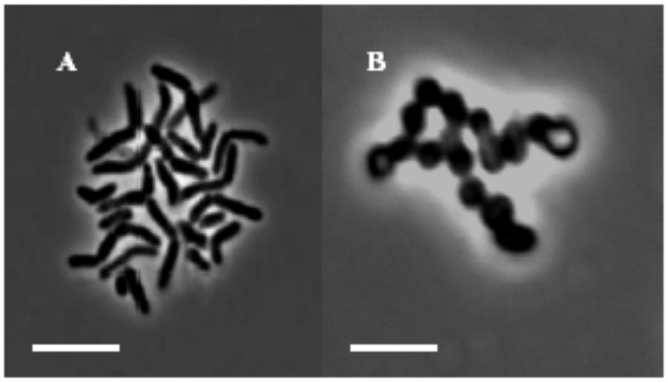

Figure 1. Phase contrast photomicrographs of isolate RW in cells from the logarithmic phase (A) and cells from the lag-phase (B). Bar = 10 µm.

benzaldehyde. The isolate could not grow on glucose, fructose, xylose, sucrose, starch, mannose, maltose, mannitose, mannitol, inositol, sorbital, rhamnose, saccharose, melibiose, amygdaline, arabinose, but could grow on succinate and lactose.

Resorcinol degradation

Isolate RW grew in resorcinol after a lag phase of approximately 24 h (Figure 1). Under these conditions, complete disappearance of the resorcinol peak took approximately 150 h with a corresponding increase in cell biomass (turbidity). The generation time for the isolate

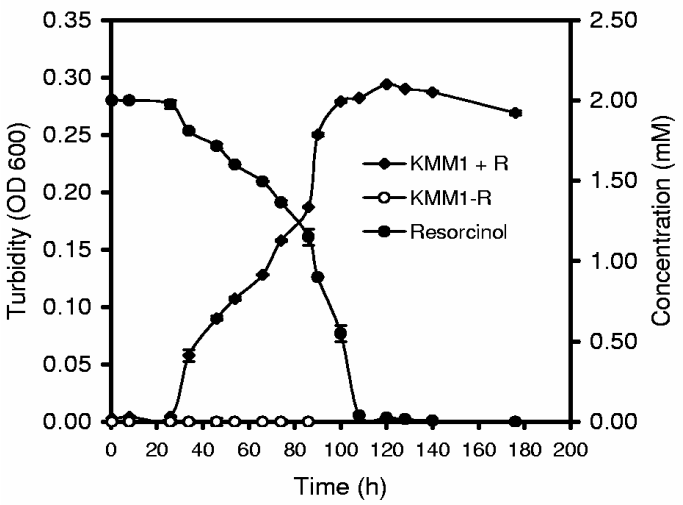

Figure 2. Growth of strain RW in medium containing resorcinol (KMM1 + R), and the corresponding depletion of resorcinol in the medium (Resorcinol). In the control without Resorcinol (KMM1 –R) the isolate did not show any increase in turbidity (no growth) (n=2).

Table 2. 16S rRNA sequence similarity of species of the genusRhodococcus and other relatives of the mycolic-containingbacteria related to the isolate RW.

Strain	Accession number	% Relatedness to RW
R. opacus	AF095715	99.6
Tsukamurella wratislaviensis	Z37138	99.6
R. opacus	X89710	99.0
R. rhodnii	X80622	94.8
Nocardia asteroides	X80606	94.8
R. rhodochrous	X79288	94.4

was determined using the data generated from the direct growth curve method, as approximately 33 h. Nitrate reduction to nitrite and/or nitrogen was detected in cultures of isolate RW following the addition of sulfanilic acid and alpha-naphthylamine (Cappuccino and Sherman, 2002).

Phylogenetic analysis

The 16S rRNA gene sequences of the isolate RW comprising 1500 nucleotides (of *E. coli* sequence) were determined. Figure 3 shows the phylogenetic position of isolate RW, clustering on the branch of mycolic-acid-containing bacteria that comprises the genera *Corynebacterium*, *Mycobacterium* and *Norcadia*, and was more closely related to the genus *Rhodococcus*. The 16S

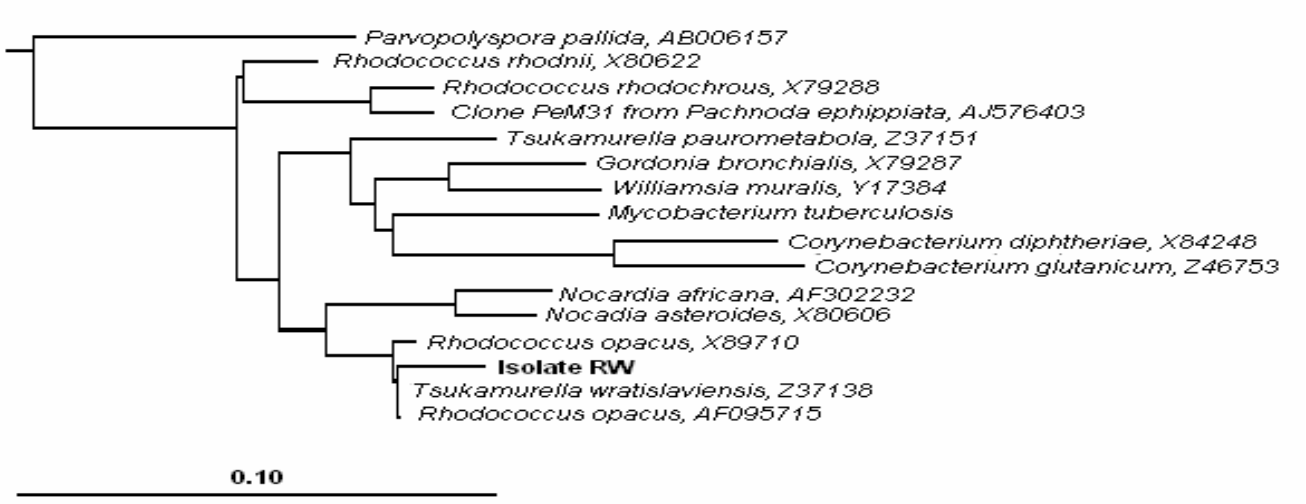

Figure 3. Phylogenetic tree showing the position of isolate RW with reference organisms from the genus *Rhodococcus*. The GenBank accession number for the 16S rRNA sequence of each reference species is shown at the end of the species name. The bar indicates the estimated substitution per nucleotide position.

RNA gene sequence similarity values of isolate RW to other strains of the genus *Rhodococcus* are in the range of 94.8-99.6% (Table 2). These values and the phylogenetic position shown in the phylogenetic tree (Figure 3) indicate that isolate RW belongs to the genus *Rhodococcus,* and is closely related to *R. opacus*, with 99.6% sequence similarity.

DISCUSSION

Rhodococci are described as aerobic, gram positive, non-motile, non-sporulating, and catalase positive microorganisms. They have a variety of growth patterns in which they form filaments with short projections, elementary branching, or, in some species, extremely branched hyphae. These filaments can undergo fragmentation into rod shaped or cocci elements (Finnerty, 1992). Isolate RW adheres to all the signature phenotypic traits of the genus *Rhodococcus* including the fact that the cells in the exponential phase are gram-positive, partially acid-alcohol fast, aerobes, catalase positive, growing on standard laboratory media at 15 - 40°C (Goodfellow, 1984). These characteristics together with other biochemical properties distinguish the isolate as a member of the genus *Rhodococcus*. Phylogenetically, the isolate RW is closely related (with the 16S rRNA sequence) with more than 99% similarity to *R. opacus* strains including *Tsukamurella wratislaviensis*, which was originally misclassified and has recently been officially classified as *R. wratislaviensis* (Goodfellow et al., 2002).

The isolation of a resorcinol-degrading bacterium and the low abundance of resorcinol degrading micro-organisms from the fungi-cultivating termites maybe directly related to the host diet and the relatively low occurrence of resorcinol (Hans, 1994) in plant biomass and its toxicity. The diversity of geographical locations and environments in which *Rhodococcus* species can be found is reflected in the first publication of the genus (Goodfellow, 1986). The genus *Rhodococcus* encompasses a multi-faceted group of bacteria of clinical, industrial and environmental significance (Fernandes et al., 2001). Members of this genus have been found in soil (Zhang et al., 2002; Matsuyama et al., 2003), rocks, boreholes, groundwater, marine sediments, animal dung, healthy and diseased animals and plants (Bell et al., 1998), and from the gut of blood-sucking insect *Rhodnius prolixus* (Ben-Yakir, 1987), with which they may form a mutualistic association.

The genus *Rhodococcus* is an industrially important taxon that includes many strains that degrade a variety of toxic organic and aromatic compounds. They exhibit diverse metabolic activities including the degradation of various aromatic hydrocarbons (Warhust and Fewson, 1994), chlorinated polycyclic aromatics such as polychlorinated biphenyls (PCBs), nitroaromatics and other recalcitrant toxic pollutants (Bell et al., 1998). In the resent study it was demonstrated that isolate RW was capable of growing not only with resorcinol but also with benzoic acid and phenol. This is not surprising given that members of the genus *Rhodococcus* have been shown to possess the ability to degrade a wide range of organic compounds. Indeed, many of the xenobiotic transformations traditionally associated with nocardiae are due to rhodococci (Goodfellow, 1984).

However, no species of this genus has been isolated from the guts of termites and characterized. Ben-Yakir (1987) isolated a rhodococcus bacterium, *R. rhodnii* from

the gut of the insect *Rhodnius prolixus* that is an important vector of American trypanosomiasis (Chagas' disease). It has been suggested that biological control of the disease could be accomplished by targeting the insect via *R. rhodnii* as the growth and development of the insect is apparently dependent on the bacterium. Indeed, the isolation of RW suggests that components of decomposed plant materials as well as those resulting from the condensation of smaller molecules through biological and physical processes, containing chemically complex organic acids such as resorcinol and other phenolic compounds may support bacterial growth in the termite gut. Given the ability of *Rhodococcus* species to degrade many pollutants and other compounds, isolate RW may therefore offer a rich source of enzymes that may be of use in biotechnology. Whether this ability has any physiological benefit to the host would be speculative and therefore necessitates further comprehensive investigations to be undertaken.

The role of bacteria in the degradation of lignin is not clear. However, *Rhodococcus* spp. has been implicated in the degradation of lignin-related compounds and humic acids (Goodfellow, 1986). They have been shown to cleave intermonomeric linkages that are characteristic of lignin (Rüttimann et al., 1991). Therefore, the degradation of monoaromatic compounds by the isolate RW also suggests that similar schemes of metabolic processes might be present in the gut of *M. michaelseni*. However, whether the bacteria mineralize these aromatic acids *in situ* is still unknown and needs to be investigated employing microinjection of radiolabeled compounds (Kappler et al. 1999).

In conclusion, based on the above morphologic and phenotypic characterization, the taxonomic placement of isolate RW as *R. opacus* strain RW is proposed. To our knowledge this is the first resorcinol-utilizing bacterium isolated from fungi cultivating termites, *Macrotermes* sp. The genus *Rhodococcus* has proven to be an important and metabolically versatile group, transforming various environmental contaminants, which include recalcitrant and ubiquitous xenobiotics. Hence, the possibility of using termite gut microorganisms for biotechnological research should be explored. The wide range of chemicals transformed or degraded by these genera makes them potentially useful in environmental and industrial biotechnology such as their ability to synthesize surfactants, flocculants, amides and polymers.

ACKNOWLEDGEMENTS

This work was supported in part by Jomo Kenyatta University of Agriculture and Technology (JKUAT). We thank Dr. Andreas Brune of Max Planck Institute of Terrestrial Microbiology for providing research fellowship for David; Dr. Dirk Schmitt-Wagner, Katja Meuser and Dr. Ai Fujita for technical support with 16S rRNA sequencing; and Paul Karanja of Food Science and Post Harvest Technology Department, JKUAT for technical assistance on HPLC.

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