World Journal of Microbiology & Biotechnology (2005) **21**: 265–269
DOI 10.1007/s11274-004-3628-8

Isolation and characterization of a new carbendazim-degrading *Ralstonia* sp. strain

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Received 24 February 2004; accepted 13 July 2004

Keywords: 16S rDNA, carbendazim, phenotypic features, phylogenetic analysis, *Ralstonia*

Summary

A bacterial strain 1-1 capable of utilizing carbendazim was isolated from carbendazim-treated Qiyang red soils Hunan Province, China. It is gram-negative, rod-shaped, motile with peritrichous flagella, which formed round, smooth, convex and transparent colonies of about 1.1 mm diameter after 3 days of incubation on the isolation and purification medium using carbendazim as the sole carbon and energy sources. The degradation ratios of carbendazim by strain 1-1 were 19.16 and 95.96% in the carbendazim (500 mg/l)-degrading medium and the carbendazim (500 mg/l)-degrading medium supplemented with yeast extract (150 mg/l) within 24 days, respectively. Strain 1-1 was identified as *Ralstonia* sp. (β-Proteobacteria) based on the results of phenotypic features, G + C mol% and phylogenetic analysis of 16S rDNA. Strain 1-1 could become a new bacterial resource for biodegrading carbendazim and might play a bioremediation role for soils contaminated by carbendazim.

Introduction

Carbendazim (methyl benzimidazol-2-ylcarbamate) is the most widely used benzamidazole fungicide and is also the major degradation product of other systemic fungicides, benomyl and thiophanate-methyl (Fleeker *et al*. 1974; Mongomery *et al*. 1997). (Figure 1). It is a stable compound with a long half-life (Carbendazim is decomposed in the environment with half-lives of 6–12 months on bare soil, 3–6 months on turf, and half-lives in water of 2 and 25 months under aerobic and anaerobic conditions, respectively.) in the environment (WHO 1993). Hence, it can persist at application sites and easily induce cumulative effects; its residue in fruits, plants and soils could be harmful to human health through food chains. Some studies indicated that carbendazim could do harm to the liver to some extent (WHO 1993) and it has been documented as mutagenic and has teratogenic effects on mammals at single, low-level doses (Sarrif *et al*. 1994;Nakai *et al*. 1998).

The degradation of carbendazim in the environment has received extensive attention. Carbendazim may be degraded by way of both photolysis and biodegradation (Fleeker & Lacy 1977; Yarden *et al*. 1990). Helweg (1977) reported that carbendazim in soil was decomposed mainly by microorganisms. The isolation and screening of efficient carbendazim-degrading microorganisms is a useful approach in bioremediation of carbendazim contamination. However, the isolation and identification of bacterial pure cultures capable of degrading carbendazim have been seldom reported so far. Fuchs & de Vries. (1978) reported that carbendazim could be degraded to 2-aminobenzimidazole by *Pseudomonas* spp., whereas Holtman & Kobayashi (1997) found carbendazim could be degraded by *Rhodococcus erythropolis*. This paper reports the isolation and characterization of a strain of carbendazim-degrading bacterium from carbendazim-contaminating Qiyang red soil from Hunan Province, China.

Materials and methods

Enrichment, isolation and purification of carbendazim-degrading bacteria

The soil which had been treated with carbendazim for 3 months at the time of sampling was collected from Qiyang District, Hunan Province, China. For the isolation of potential carbendazim-degrading bacteria, a continuous enrichment culture method was used. 7.5 g sample of soil was added to 75 ml sterilized enrichment medium (g/l): NaCl 1.0, K_2HPO_4 1.0, $MgSO_4{\cdot}7H_2O$ 1.0, $CaCO_3$ 1.0, carbendazim (94.6% pure) was firstly dissolved in 0.01 M hydrochloric acid, at five concentrations (g/l): 0.2, 0.4, 0.5, 0.6, 0.8, distilled water, pH 7.0 and incubated with shaking at 30 °C in the dark. After 3 days, 1 ml aliquot from each conical flask was transferred to a new flask containing fresh medium, then was still incubated at 30 °C with shaking in the dark. This process was repeated five times before each culture was dilution-plated (five repeats) onto sterilized isolation and purification medium (g/l): NaCl 1.0, K_2HPO_4 1.0, $MgSO_4{\cdot}7H_2O$ 1.0, $CaCO_3$ 1.0, $(NH_4)_2SO_4$ 2.0,

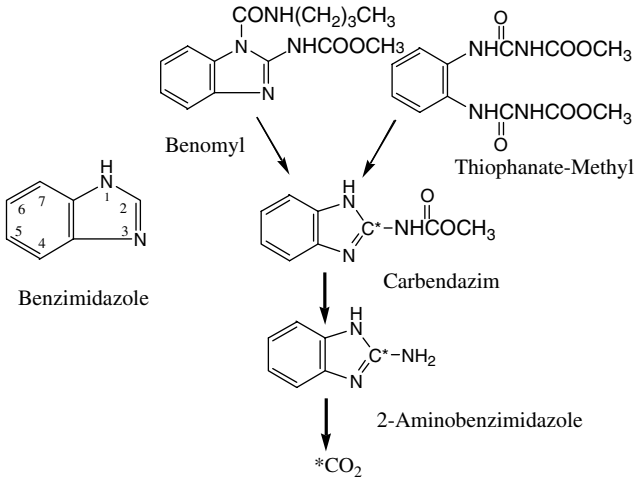

Figure 1. Structural formulae of benzimidazoles and a suggested biotransformation pathway. Benomyl, thiophanate-methyl and carbendazim (= MBC, = methyl benzimidazol-2-ylcarbamate) are systemic fungicides; 2-AB (= 2-aminobenzimidazole) and benzimidazole are non-fungicides. *C = labelled carbon.

carbendazim (five concentrations are the same as those of enrichment medium), agar 20, distilled water, pH 7.0. All plates were incubated at 28 ± 1 °C in the dark for bacterial isolation. After five subcultures, the pure bacterial cultures that could grow on the isolation and purification medium were selected. One of these pure cultures, strain 1-1 which was from the highest carbendazim concentration (0.5 g/l) of isolation and purification medium , was maintained on LB agar medium and used to carry out further studies.

Carbendazim degradability of strain 1-1

For the study of bacterial carbendazim degradability, five 500 ml conical flasks containing 200 ml carbendazim-degrading medium (g/l): NaCl 1.0, K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 1.0, $CaCO_3$ 1.0, $(NH_4)_2SO_4$ 2.0, carbendazim 0.5, distilled water, pH 7.0, five 500 ml flasks containing 200 ml carbendazim-degrading medium supplemented with yeast extract (150 mg/l) and five control flasks containing carbendazim-degrading medium were designed. The strain 1-1 cells were inoculated into each sample flask. Five control flasks were inoculated with aseptic distilled water instead of strain 1-1. All flasks were incubated at 28 ± 1 °C with shaking in the dark.

The concentration of carbendazim was determined as follows: 5 ml samples (1 ml each flask, three repeats) were drawn periodically (every 3 days) under aseptic conditions. The 5 ml solution was placed in a 50 ml conical flask, to which was then added 20 ml 1,4-dioxane, heated for 5 min in boiling water, then 1 ml acetic acid was added to and cooled to room temperature, then filtered into a 50 ml volumetric flask by G4 sand core funnel. About 10 μl of filtered solution was taken and samples spotted on GF254 glass-backed plates (10cm × 20 cm) and dried with the aid of a hair drier. The plate was then developed in the solvent system benzene-acetone-acetic acid (70:30:5, v/v) and then dried. Carbendazim could be easily identified as violet spots under u.v. light and the R_f of the carbendazim spots were about 0.65. The content and the standard curve of carbendazim were determined with the same process mentioned above. Concentrations of carbendazim were calculated according to the standard curve of carbendazim. The recovery rate of this method was 94.56%, the lowest limit of determination was 3.5 mg/l. The carbendazim degradation of strain 1-1 was analysed by using a Shimadzu CS930 dual-wavelength thin-layer chromatogram (TLC) scanner at 280 nm (Braithwaite & Smith 1985).

Identification of strain 1-1

The morphology and motility of strain 1-1 were determined by conventional methods. Flagella were observed using JEM-1200EX electron microscope (JEOL Inc., USA).

Physiological and biochemical characterization was performed by biochemical test tubes (Hangzhou Tianhe Microorganism Reagent Co., Ltd., China) according to the protocol supplied by the manufacturer.

DNA G + C content (T_m method) was analysed on Shimadzu UV-2550 spectrophotometer (Shimadzu Corporation, Japan) equipped with water cycle heating-up system.

PCR amplification and sequencing of 16S rDNA

Strain 1-1 cells that had been cultured on LB medium for 24 h were transferred to an Eppendorf tube containing 200 μl aseptic double-distilled water. The mixture was centrifuged for 5 min after it was kept in boiling water for 3 min, the supernatant of which was directly used amplification as template DNA.

Amplification was done by PCR with primers (Devereux & Willis 1995) named BSF8/20 (5′-AGAGT TTGAT CCTGG CTCAG-3′) and BSRl541/20 (5′-AAGGA GGTGA TCCAG CCGCA-3′). The reactions were performed in a final reaction mixture of 50 μl containing 5 μl 10 × PCR buffer (containing Mg^{2+}), 1 μl dNTP (5 mM),1 μl (0.2 μM) each primer, l.5 μl template DNA,0.5 μl Taq DNA polymerase (5 U/μl),40 μl ddH_2O and 30 μl liquid paraffin. The amplification reactions were performed with the following cycles of parameters: 94 °C for 5 min, followed by three cycles of 45 s at 94 °C, 2 min at 50 °C and 1 min at 72 °C and 29 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, with a final extension at 72 °C for 7 min. The amplification products were checked by 2% agarose gel electrophoresis and staining with ethidium bromide. PCR products were purified and sequenced by Shanghai Bioasia Biologic Technology Co., Ltd., China.

Phylogenetic analysis

The 16S rDNA gene sequence (605 bp, GenBank accession number AY447043) of strain 1-1 aligned with all the sequences available from the GenBank database by BLAST and all sequences were retrieved from Genbank database individually and aligned using ClustalX 1.8.1 with default settings (Thompson et al. 1997). Phylogenetic analysis was performed by means of MEGA version 2.1 (Kumar et al. 2001) software using UPGMA method and selecting Kimura 2-parameter distance model, which was tested by Bootstrap method (1000 repetitions). The 16S rDNA sequences included in the phylogenetic analysis can be seen in Figure 4.

Results and discussion

Isolation and taxonomic characteristics of bacteria

Bacterial colonies could be observed on the isolation and purification medium using carbendazim as the sole carbon and energy sources after 3–5 days except at 0.6 and 0.8 g/l (carbendazim concentration). All the colonies were identical morphologically, from which strain 1-1 was isolated.

The cells of strain 1-1 were 0.5–0.6 μm × 0.6–2.7 μm, gram-negative, short rod-shaped, motile with peritrichous flagella (Figure 2), non-spore-forming. It could form round, smooth, convex and transparent colonies of about 1.1 mm diameter on the isolation and purification medium after 3 days. However, ivory-white, opaque colonies were formed on LB medium after 1–2 days. It was observed that yeast extract could accelerate growth of strain 1-1.

Strain 1-1 could grow at 20–41 °C, but not at 42 °C, and could also grow on 3% NaCl LB medium, but not on 5% NaCl LB medium. The following enzyme reactions were positive: oxidase, catalase, urease, arginine dihydrolase, lysine decarboxylase, alkaline (acid) phosphatase and ornithine decarboxylase. Nitrate was reduced, nitrite was not. No indole was produced from tryptophan. Glucose was not fermented. D-ribose, D-gluconate, L-malate and citrate were assimilated. No assimilation of galactose, D- & L-arabinose, lactose, maltose, mannose, mannitol, melezitose and raffinose was detected. The DNA G+C content was 64.9 mol% (T_m). These phenotypic characteristics are very close to those of four species of *Ralstonia* spp. (Yabuuchi *et al.* 1995; Goris *et al.* 2001).

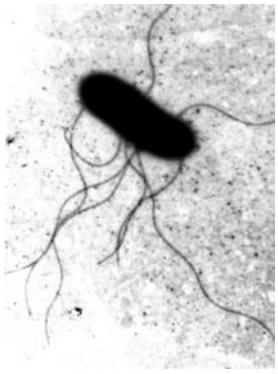

Figure 2. Electron micrograph of negative-stained 1-1 cell with peripheral flagella (12000×).

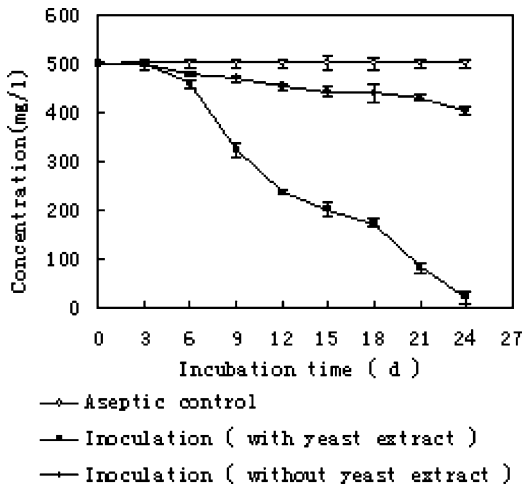

Figure 3. Degradation kinetic curves of carbendazim by cells of strain 1-1 within 24 days expressing that carbendazim (500 mg/l)-degrading medium supplemented with yeast extract (150 mg/l) can distinctly accelerate the degradation of carbendazim and carbendazim cannot be degraded without cells of strain 1-1 in the dark.

Carbendazim degradation of strain 1-1

The results of carbendazim degradation by strain 1-1 (Figure 3) showed that the concentration of carbendazim fell from 500 to 404.2 mg/l in carbendazim-degrading medium and to 20.2 mg/l in carbendazim-degrading medium supplemented with yeast extract within 24 days, respectively. The degradation ratios and the average degradation rates were 19.16%, 3.99 mg/(l·d) and 95.96%, 19.99 mg/(l·d) , respectively. The addition of yeast extract accelerated the degradation of carbendazim by strain 1-1. It is most probable that the compound is a poor microbial energy source and not the most suitable substrate for strain 1-1, which indicated that the degradation may be a co-metabolic process similar to that suggested in the earlier studies of Helweg (1977).

Holtman & Kobayashi (1997) have reported that five strains of *Rhodococcus erythropolis* could completely degrade 16 mg carbendazim/l in M9 salts medium within 15 days, the average degradation rate of which was 1.07 mg/(l·d). However, the average degradation rate by strain 1-1 was 3.99 mg/(l·d) in carbendazim-degrading medium. Hence, the carbendazim degradability of strain 1-1 exceeded that of *Rhodococcus erythropolis*.

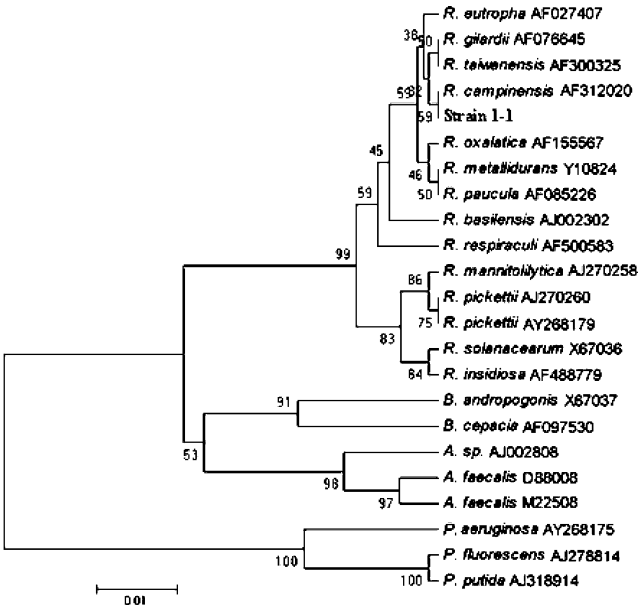

Figure 4. UPGMA phylogenetic tree of strain 1-1, validly described *Ralstonia* species, carbendazim degradation bacteria *Pseudomonas* spp. based on the 16S rDNA sequences comparisons. Bootstrap values obtained with 1000 repetitions are indicated as percentages at all branches. The 16S rDNA sequences of *Alcaligenes faecalis*, *Burkholderia cepacia* and *Burkholderia andropogonis* were included as closely related species. Scale bar indates evolutionary distance. Abbreviation: A., *Alcaligenes*; B., *Burkholderia*; P., *Pseudomonas*; R., *Ralstonia*.

Phylogenetic analysis of 16S rDNA sequence

A phylogenetic tree including all known representatives of validly described *Ralstonia* species and other correlative species is given in Figure 4. Strain 1-1 and *Ralstonia* species constituted one big cluster on the phylogenetic tree. The 16S rDNA gene of strain 1-1 showed high sequence similarity (more than 97%) to the 16S rDNA genes of all *Ralstonia* species including the type species: *R. pickettii*. The highest sequence homology between strain 1-1 and *R. campinensis* with heavy-metal-resistance (Goris *et al*. 2001) was 99.8% though they have slightly difference in phenotypic features. The 16S rDNA gene sequences of *Burkholderia cepacia*, *Burkholderia andropogonis* and *Alcaligenes faecalis* apparently formed a big cluster, which was close to that of *Ralstonia* spp. and the three species were suggested to be emended to *Ralstonia* spp. by Yabuuchi *et al*. (1995).

According to some phenotypic characteristics mentioned above, G + C content, phylogenetic analysis, strain 1-1 should be assigned to *Ralstonia* sp. (β-Proteobacteria). DNA–DNA hybridizations with related species should be performed for species taxa.

The genus *Ralstonia* (Yabuuchi et al. 1995) has been created for a group of organisms from ecologically diverse niches to accommodate bacteria that were formerly classified as members of *Burkholderia* (Yabuuchi et al. 1992) and *Alcaligenes*, which so far has 13 species (Euzéby 1997) that have been validly published including *R. eutropha* JS705 (Van der Meer *et al*. 1998) and *R. basilensis* RK1 (Steinle *et al*. 1998) that could degrade chlorobenzene and 2,6-dichlorophenol, respectively. Furthermore, the isolated strain 1-1 in this study is a carbendazim-degrading bacterium.

To date, pure cultures of bacteria capable of degrading carbendazim that have been documented have been only *Pseudomonas* spp. (Fuchs & de Vries 1978) and *Rhodococcus erythropolis* (Holtman & kobayashi1997). The former and strain 1-1 belong to the Proteobacteria, so they were comparatively close in taxonomic status, which could be seen on the phylogenetic tree, however, the *Rhodococcus* sp. belongs to an unrelated group: Nocardiaceae, Actinomycetales, Actinobacteria.

In this study, strain 1-1 that we have isolated is a new bacterial resouce for biodegrading carbendazim and might have a possible bioremediation role for soils contaminated by carbendazim.

Acknowledgements

We are grateful to Professor Ren-lin Cao at Institute of Agro-Environment Protection, Ministry of Agriculture, PR China, for his providing us with carbendazim (94.6% pure) samples. This project was supported by the Research Fund for Public Welfare of Ministry of Science and Technology, PR China (Grant number 2001-177).

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