A Gram-positive Polychlorinated Biphenyl-degrading Bacterium, *Rhodococcus erythropolis* Strain TA421, Isolated from a Termite Ecosystem

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Gram-positive bacteria, identified as *Rhodococcus erythropolis*, were isolated from the ecosystem of the wood-feeding termite *Reticulitermes speratus* and found to aerobically degrade polychlorinated biphenyl (PCB) compounds. *Rhodococcus erythropolis* strain TA421 and strain TA431 were isolated by enrichment culture from termites obtained from different locations and each was found to be capable of degrading polychlorinated biphenyl (PCB) compounds to chlorobenzoates. These results suggest that the termite ecosystem is one possible habitat for biphenyl- and PCB-degrading *Rhodococcus*. The spectrum of PCB-congeners degraded by strain TA421 is different from that of other, previously characterized PCB-degrading bacteria such as *Rhodococcus globulatus* strain P6 (formerly *Corynebacterium* sp. strain MB1) or *Pseudomonas* sp. strain LB400.

Rhodococci are ubiquitous in nature and have frequently been isolated from soil, fresh water and marine habitats, as well as from the gut contents of blood-sucking arthropods.1,2) Rhodococci have been implicated in the degradation of lignin-related compounds3–5) and have frequently been isolated from soil contaminated with petroleum compounds.6,7) Numerous bacterial strains representing a limited number of genera have been isolated from soil and fresh water environments and found to co-metabolize PCB congeners to chlorobenzoic acids via oxidative pathways. The majority of PCB-degrading isolates characterized to date are Gram-negative bacteria within either the *Pseudomonas*, *Alcaligenes*, or *Acinetobacter* genera. With the exception of *Rhodococcus globulatus* strain P6 (previously designated *Acinetobacter* sp. strain P6, *Arthrobacter* sp. strain M5, and *Corynebacterium* sp. strain MB1), information concerning the isolation and characterization of Gram-positive bacteria involved in the biodegradation of polychlorinated biphenyl compounds is quite sparse.8)

As lignins are a diverse class of phenolic polymers containing biphenyl moieties, we suspected that biphenyl-degrading bacteria might be present within wood and leaf litter, or possibly even within the termites that inhabit this community. Termites are terrestrial insects whose ecology is derived from the metabolism of dried wood and grasses by gut symbionts that are capable of degrading cellulose, hemicellulose, and lignin. Biphenyl moieties introduced by the breakdown of lignin within this community might represent a carbon-rich resource for biphenyl degrading bacteria, such as *Rhodococcus*.

A C-minimal medium was used for screening and isolation of strains. The C-medium consisted of (per liter of medium) (NH₄)₂SO₄, 5.0g; KH₂PO₄, 2.93 g; K₂HPO₄, 5.87 g; MgSO₄·7H₂O, 0.3 g; NaCl, 2 g; CaCl₂, 0.03 g; FeSO₄·7H₂O, 0.01 g; NiSO₄·7H₂O, 0.6 mg; yeast extract, 0.2 g; 2 ml of a trace elements solution, and adjusted to pH 7.0. The trace elements solution contained 4 mg, MoO₃; 28 mg, ZnSO₄·5H₂O; 2 mg, CuSO₄·5H₂O; 4 mg, H₃BO₃; 4 mg, MnSO₄·5H₂O; and 4 mg, CoCl₂·6H₂O. Biphenyl was added to the C-medium as insoluble crystals to a final concentration of 0.5% or 0.1% as carbon source.

Dry wood termites (*Reticulitermes speratus*) were collected from dead trees in the vicinity of Ogose, Saitama prefecture, Japan in May 1992. Termites were harvested, within 72 h of collection, from pieces of dried wood cut from termite-infested trees held at ambient temperature in small plastic containers. Worker larvae were used for all experiments. PCB/biphenyl degrading bacteria were enriched from gut homogenates by using C-medium inoculated with 1 gut equivalent per tube (i.e., approx. 1 μl gut content per tube). After 1 week of cultivation at 30°C, 0.2 ml of culture was spread onto LB plates for isolation. The ability to degrade biphenyl via an oxidative pathway was assayed spectrophotometrically by measuring an increased absorbance at 434 nm resulting from the production of the meta-cleavage product as reported by Furukawa et al.10) The single colony showing the highest 2,3-dihydroxybiphenyl dioxygenase (2,3-DHBD) activity was isolated and designated as strain TA421. Strain TA431 was isolated from a gut homogenate collected from a termite obtained in a different location in Ogose (several km from the isolation point of strain TA421).

The identification of isolates was done by standard laboratory methods as described in Bergey's Manual.9) Briefly, the cellular morphology of individual isolates was analyzed with light microscopy and the morphogenetic sequence was found to be an elementary branching-rod to coccus growth cycle. The isolates stained Gram-positive but had no spores indicating their probable inclusion within the genus *Rhodococcus*. Therefore, chemotaxonomic and biochemical analyses were done as follows: (i) cell wall peptidoglycan; (ii) mycolic acid; (iii) fatty acid pattern; (iv) menaquinone; and (v) for species identification, 35 biochemical tests. Strains TA421 and TA431 were each identified as *Rhodococcus erythropolis* by physiological and biochemical characterization. *Rhodococcus erythropolis* strain TA431 showed only weak PCB/biphenyl degrading activity and only strain TA421 was further analyzed.

To analyze the spectrum of aromatic compounds that could be metabolized as the sole carbon source for growth, *Rhodococcus erythropolis* strain TA421 was grown in liquid C-medium in the presence of various aromatic compounds at 0.1% final concentration for 7 days. Strain TA421 used the following compounds for growth: biphenyl, 4-chlorobiphenyl, benzoate, 4-hydroxybenzoic acid, protocatechuic acid, pyrocatechol, and phenol. The compounds 3-methyl catechol, 4-methyl catechol,
3-hydroxybenzoic acid, 4-chlorobenzoic acid, salicylic acid, syringic acid, vanillic acid, naphthalene, toluene and benzene, however, were not used as the sole carbon source for growth. Cells were grown in media containing biphenyl, 4-chlorobiphenyl, or phenol as the sole carbon source and 200 μl samples of media were taken after 50 h of cultivation. Addition of 2,3-DHB or 3-methylcatechol to the sample media showed yellow color, indicating the presence of enzyme activity for both 2,3-DHBD and 3-methylcatechol dioxygenase (3-MCO). These results suggest the presence of both meta- and ortho-cleavage pathways. A meta-cleavage pathway containing a 2,3-dihydroxybiphenyl dioxygenase is implicated in the degradation of biphenyl to benzoic acid (or PCB to a corresponding chlorobenzoic acid), and then an ortho cleavage pathway may be used for the further degradation of benzoic acid because addition of 2,3-DHB or 3-methylcatehol to the sample culture grown as the sole carbon source of benzoate showed no yellow color, indicating the absence of enzyme activity for both 2,3-DHBD and 3-methylcatechol dioxygenase (3-MCO). In the presence of phenol as the sole carbon source, it is possible that a phenol degradation pathway containing enzymes with either 2,3-DHBD or 3-MCO activity was induced in strain TA421.

To identify the spectrum of PCB congeners used by strain TA421, bacterial cultures were cultivated at 30°C in screw-capped test tubes containing C-medium with 0.5% biphenyl as the sole carbon source for 3 days. At that time, 0.3 ml of the 3-day culture was inoculated into 3 ml of fresh medium, and control cultures were autoclaved at 121°C for 30 min before the addition of the PCB. A 10,000 ppm solution of KC300 (polychlorinated biphenyl congener mix; GL Science Inc., Japan) in ethylacetate was added to each assay tube (33 ppm). The tubes were incubated at 30°C for 50 h with reciprocal shaking (120 rpm). Samples (3 ml cultures) were extracted by shaking thoroughly with 2 ml of ethylacetate after the addition of 100 μl of hydrogen chloride. After extraction, the ethylacetate phase was removed and dehydrated with anhydrous sodium sulfate. GC-mass spectrometry analysis was done as described previously.11 Peaks were measured by a Hewlett-Packard 5895A Chemstation and identified from standards listed in the database obtained by Quensenn.11 The PCB mixture used for PCB degradation analysis, KC300, is equivalent to Arorclor 1242 (Monsanto Co., St. Louis, MO) and mainly contains tri- or tetra-chlorine substituted biphenyls. In all, 48 congeners were identified and measured although other congeners could not be identified because of identical retention times.

From the degradation patterns (Table), the PCB congener use profile was divided into three groups: Group I (more than 90% degradation) included 33 PCB congeners; Group II (from 50-60% degradation) included 13 PCB congeners; and Group III (less than 20% degradation) included 2 PCB congeners. PCB congeners that had chlorines substituted at positions 2-, 4-, 2-, 3-, 2-, 5-, or 3, 4- were degraded well by strain TA421, but those congeners that had substituted chlorines at positions 2, 6- were less susceptible to degradation by this strain. The PCB congener degradation specificity of strain TA421 is different from that of either Pseudomonas sp. LB40012-13 or Rhodococcus globularas P6 (previously designated Corynebacterium sp. strain MB1,12-14 but now reclassified15). Strain LB400 or P6 are not particularly effective against PCB congeners substituted at positions 4, 4, 2, 4, 2, 4-4, and PCB congeners substituted at positions 2, 4, 3, 4, 2, 5-2, 2, 5-4, respectively. But strain TA421 is capable of degrading all PCB congeners substituted at these positions.

Rhodococci have been implicated in the degradation of lignin-related compounds3-5 and some Rhodococci can be isolated from the gut contents of blood-sucking arthropods1,2. Since lignin contains biphenyl moieties, we anticipated that PCB/biphenyl degrading bacteria might be present in lignin-rich ecosystems such as the digestive tract of termites and isolated a PCB/biphenyl degrading bacterium, Rhodococcus erythropolis strain TA421. A second PCB/biphenyl degrading bacterium, Rhodococcus erythropolis strain TA4431, was isolated from a gut homogenate obtained from a termite collected in a different place. Although the termite ecosystem is one possible habitat for PCB/biphenyl degrading bacteria, it is not clear if either of these strains lived within the termite hindgut or were simply present in the ingested wood at the hindgut surface. Since the termite hindgut is largely anaerobic, the oxidative PCB degradation pathway used by strains TA421 and TA431 would preclude their long-term existence within an anaerobic environment.

PCB/biphenyl-degrading bacteria have been isolated from both soil and aqueous environments throughout the world, but there is little information about Gram-positive PCB degrading bacteria other than Rhodococcus globularas P6 (previously designated Acinetobacter sp. strain P6, Arthrobacter sp. strain M5, and Corynebacterium sp. strain MB1). Rhodococcus erythropolis is suitable for further genetic analysis as in excess of sixty genetic markers have been identified in the development of an R. erythropolis genetic linkage map and temperate phages are now available for use as cloning vectors.

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References
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