The Journal of Microbiology, June 2003, p.165-168
Vol. 41, No. 2

NOTE

Isolation and Identification of a Pentachloronitrobenzene (PCNB) Degrading Bacterium *Alcaligenes xylosoxidans* PCNB-2 from Agricultural Soil

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(Received March 10, 2003 / Accepted May 6, 2003)

We report a new PCNB-degrading strain (PCNB-2) that is able to utilize and grow on PCNB (100 ppm) as a sole carbon source. This strain was identified as *Alcaligenes xylosoxidans* based upon 16S rDNA sequence analysis, API 20 NE tests and cell membrane lipid analysis. The new PCNB degrader *Alcaligenes xylosoxidans* PCNB-2 could find use in bioremediation of PCNB, which is environmentally persistent.

Key words: Pentachloronitrobenzene (PCNB), *Alcaligenes xylosoxidans*, pesticides, bioremediation

Pentachloronitrobenzene (PCNB) was first developed by Bayer AG (Germany) in 1930 and was primarily used as a fungicide for seed and soil treatments to control the damping-off diseases of seedlings. PCNB was first used in Korea in 1969 but its usage was terminated due to its environmental persistency. The compound is relatively mildly toxic to mammals and fish but its half-life in the soil is relatively long (5-10 months) (Yoon, 1996). Although its domestic usage was banned in 1989, residual amounts of PCNB and of its metabolites are still detected in crops and in agricultural soils. The persistence of the pesticide in edible crops (Cairns *et al.*, 1983) is a particularly problem since PCNB poses a health hazard to humans. Environmentally, PCNB showed relatively high initial concentrations in biota and a slow buildup followed by a subsequent decline in sediment (Schauerte *et al.*, 1982). The degradation products pentachloroaniline (PCA) and pentachlorothioanisole (PCTA) have been found in farming soils and in river sediments (Fushiwaki *et al.*, 1990). These metabolites appear to be formed by anaerobic biodegradation process (Ko and Farley, 1969; Murthy and Kaufman, 1978; Kamal *et al.*, 1983). No study has been conducted on either the aerobic biodegradation or mineralization of PCNB. Here we report on the isolation and identification of an aerobic utilizer of PCNB and a good candidate for soil bioremediation.

PCNB degraders in a soil from a ginseng growing area (Jeungpyong, Korea) with a PCNB application history were cultivated. Briefly, 2.5 grams of the soil were added to 20 ml of a mineral salt medium (McCullar *et al.*, 1994) containing 100 or 300 ppm of PCNB as a final concentration. Soil suspensions were incubated in a shaking incubator (200 rpm, 28°C) for the first week of this enrichment process. Three additional sequential enrichments were then performed before spreading out the enriched microbial populations onto agar plates containing PCNB (100 ppm) as a sole carbon source. Potential degraders were screened and grown again on a MSM broth containing PCNB (100 ppm) as a sole carbon source. Growth was monitored by measuring absorbance at 525 nm and the concomitant disappearance of PCNB. Quantitative analyses of PCNB from the aqueous and biomass phases were performed by hexane extraction and gae chromatography (GC). Briefly, the blank (control; no-inoculation) and inoculated liquid media were filtered using a membrane filter (MF-Millipore, 0.22 μm pore size). Filtrates and aqueous suspensions of biomasses were extracted twice with 50 ml and 20 ml of *n*-hexane, respectively. All extracts were evaporated under vacuum at 45°C. Residual PCNB was dissolved in *n*-hexane and analyzed using a GC (Hewlett-Packard 6890N) equipped

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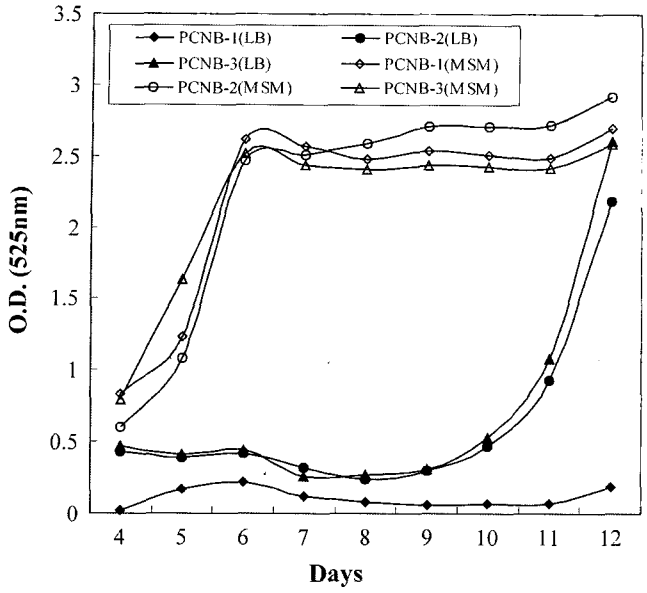

Fig. 1. Growth curves of the three enriched PCNB degraders, which were grown on MSM containing PCNB (100 ppm) as a sole carbon source. "LB" and "MSM" in the parentheses indicate LB and MSM agar plates in which each inoculum was grown, respectively.

with a μ-ECD detector and a DB-1MS, 60 m×0.25 mm, 0.25 μm (i.d.) column (J&W Scientific, USA), using splitless injection, at injector and detector temperatures of 250°C and 290°C, respectively. Run temperature conditions were as follows: 180°C for 1 min, 180-290°C at 5°C/min, and 290°C for 30 min. The flow rate of the carrier gas (N_2) was 28 (cm/sec). Characterization of morphological, cultural, physiological and biochemical features was performed. We also performed 16S rDNA sequence analysis (Geiss *et al.*, 1985), and cell membrane lipid analysis by using the Sherlock Microbial Identification System (MIDI Inc., USA). The 16S rDNA sequence similarity matrix was corrected for multiple base changes at single positions using the method of Jukes and Cantor (1969) and a phylogenetic tree was constructed using the neighbor-joining method of Saitou and Nei (1987).

The three PCNB-degrading strains (PCNB-1, PCNB-2 and PCNB-3) were enriched and isolated in the presence of PCNB as the sole carbon source. Growth curves of these strains utilizing PCNB as a sole carbon source are shown in Fig. 1. As shown in Fig. 1, the strain PCNB-2 whose inoculum was grown on MSM plates (containing 100 ppm of PCNB) for one week gave the best growth and this strain was selected and further identified. Inoculum grown on LB plates, however, showed a longer lag phase than that grown on MSM containing PCNB (at least 6 days). Residual PCNB was hardly detectable in filtrates from all the inoculated media, but was detected in the blank (no-inoculation). The rate of disappearance of PCNB from the bacterial biomass is shown in Fig. 2A, and its half-life of PCNB was calculated using first order

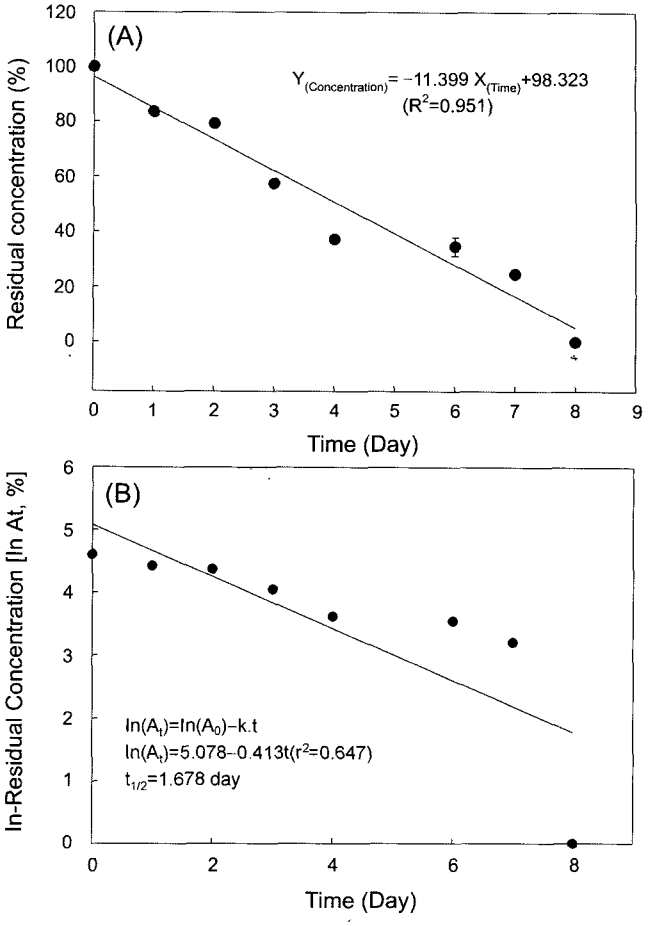

Fig. 2. Rate and kinetics of PCNB disappearance by the degrader PCNB-2: % disappearance of PCNB (panel A) and kinetics of this disappearance (panel B).

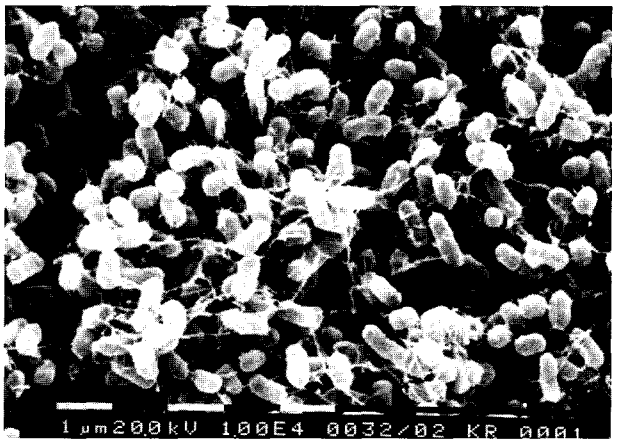

Fig. 3. Scanning electron micrograph of the PCNB degrader PCNB-2.

kinetics (Fig. 2B). The estimated half-life of PCNB in the bacterial biomass was 1.68 days and 90% disappearance was observed in 5.58 days. Lièvremont *et al.* (1998) reported the removal of PCNB from aqueous solution by a fungal mycelia using *Mucor racemousus*, *Rhizopus arrhizus* and *Sporothrix cyanescens*. They reported that

Table 1. 16S rDNA sequence (1552 bp) similarity of the strain PCNB-2 to those of various type cultures

Strain	Accession No	% Similarity
Alcaligenes xylosoxidans subsp. *denitrificans* DSM30026^T	AJ278451	99
Alcaligenes faecalis	AJ509012	99
Alcaligenes xylosoxidans	AJ491845	99
Bordetella hinzii	AF177667	98
Bordetella avium	AF177666	97
Bordetella parapertussis DSM4922^T	AJ278450	97
Bordetella holmesii CDC F5101^T	U04820	97
Alcaligenes faecalis ATCC^T	M22508	92

^{T}type culture

the removal process was achieved via adsorption, which was presented using the Freundlich adsorption isotherm. Moreover, the most efficient adsorbent was not the cell wall but the dead biomass of *R. arrhizus* and *M. racemousus*. In our study, however, we speculate that the PCNB was first adsorbed onto the PCNB degrader and then biodegraded because the strain actively grew on PCNB as the sole carbon source.

The cells of PCNB-2 were identified as those of a rod, non-motile Gram-negative bacterium (Fig. 3). The colonies of the strain on MSM agar containing PCNB (100 ppm) were 0.5~1.5 mm in diameter, white, non-pigmented, circular, smooth, raised and opaque. To isolate DNA from the strain PCNB-2, the bacterial cells were cultured in 5 ml of TSB (DIFCO, MD, USA) for 8 h at 30°C. The bacterial cells were harvested by centrifugation and then the chromosomal DNA was isolated as described by Oh *et al.*, (2002). To amplify 16S rDNA, universal primers and polymerase chain reaction (PCR) method were used: fD1 (5'-AGAGTTTGATCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3'), which supposedly yield a 1.5 kb fragment (Weisburg *et al.*, 1991). The amplification and the visualization of PCR product were carried out as previously described by Sambrook and Russel (2001). The PCR products were extracted and purified from the agarose gel using High Pure PCR Product Purification Kit (Roche, Germany). The purified PCR products were cloned into pGEM-T_{easy} vector (Promega, USA) and cloned DNA was used to transform *Escherichia coli* JM109 by electroporation (1,200 V, 25 mA and 15 ms). The transformants were selected on LB containing 50 μg/ml of ampicillin, 20 mg/ml of X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside), and 100 mM of IPTG (isopropyl-1-thio-β-D-galactoside). The appropriate cloned plasmid was prepared by QIAprep (Qiagen, USA) and the sequences of 16S rDNA were determined by ABI 377 automated DNA sequencer (Perkin Elmer, USA) at Bionex, Inc. (Seoul, Korea). The 16S rDNA sequences determined in this study have been deposited in the GenBank database (accession number: AY283260). The molecular identification based upon 16S rDNA sequence analysis (1552 bases) showed that it has 99% similarity to *Alcaligenes xylosoxidans* subsp. *denitrificans* DSM30026^T while this strain has less homology with the species of the genus *Bordetella* (Ruger and Tan, 1983) (Table 1). The strain PCNB-2 was, therefore, confirmed as *Alcaligenes xylosoxidans*. However, the data obtained from the API 20 NE test showed that the strain PCNB-2 possessed 47% homology with *Alcaligenes faecalis* ATCC 8750^T (Table 2). The strain was also identified based upon its cellular fatty acid profile (MIDI method), which showed that it had 71.4% homology with *Alcaligenes xylosoxidans* (Table 3). Taken together, it would be reasonable to classify the strain as

Table 2. Physiological characteristics of the PCNB-2 strain based upon the API NE 20 test

Characteristics	Reactions of PCNB-2
Reduction of nitrates to nitrites	−
Tryptophan assimilation	−
Arginine dihydrolase	−
Urease	−
Gelatine Hydrolysis	−
Glucose assimilation	−
Acidification from	
Arabinose	−
Glucose	−
Mannitol	−
N-acetyl-glucosamine	−
Maltose	+
Gluconate	+
Caprate	+
Adipate	+
Malate	−
Citrate	+
Phenyl-acetate	+
Cytochrome oxidase	+

Table 3. Profile of cellular fatty acids of the strain PCNB-2 as determined by the MIDI method[a]

Fatty acid profile	Strain PCNB-2
C12:0 2OH	2.00
C14:0	4.89
C16:1 Iso I/14:0 3OH	7.48
C16:1 ω 7c/15 Iso 2OH	25.55
C16:0	34.12
C17:0 CYCLO	11.82
C18:1 ω 7c	8.01
C18:0	1.79
Identification	*Alcaligenes xylosoxydans* (similarity 0.714)

[a]Analyzed using a Hewlet-Packard model 6890 A gas chromatograph and by the MIDI Aerobe Method, Chem Station V. 4.02.

Alcaligenes species, and particularly with the species *xylosoxidans* based on the high homology of 16S rDNA sequences. The isolation and characterization of new PCNB degraders, such as the strain *Alcaligenes xylosoxidans* PCNB-2 should be useful in facilitating the accelerated bioremediation of PCNB and potentially other relevant persistent chemicals in the environment and for the monitoring of bioremediation processes.

This work was supported by a grant from the Korea Science and Engineering Foundation (No. R01-2000-000-00196-0). We express our gratitude for this financial support.

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