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Full Length Research Paper

Isolation of biphenyl-degrading microorganism and its cometabolic transformation of polychlorinated biphenyls (PCBs)

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Polychlorinated biphenyls (PCBs) are ubiquitous pollutions that cause public concern due to their long persistence, low degradability, strong bioaccumulation and high toxicity in the environment. A bacterium strain G-2 was isolated from an activated sludge of a chemical factory wastewater treatment plant in China. Based on its phenotypic typing coupled with 16S rRNA gene analysis, the strain was classified as *Paracoccus* sp. The strain G-2 can use biphenyl as sole carbon and energy sources, and almost complete degraded it within 7 days. Although this strain can only utilize 24% of PCB 77, more substrate may degrade when added biphenyl as cometabolic compound. The result may provide evidence for the potential application of Paracoccus in bioremediation of PCB-contaminated soil.

Key words: Polychlorinated biphenyls (PCBs), biphenyl, *Paracoccus*, cometablism.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are a large group of chlorinated biphenyls with 209 possible congeners. The aromatic biphenyl rings may carry on to ten chlorine atoms. Due to their recalcitrant, PCBs were widely used as heat transfer agents in transformer, as stable fluid insulators in high-voltage electric transformer, inertness, dielectric properties, capacitors and hydraculic fluids and other industrial products (Field et al., 2008). Although PCBs production was banned several years ago, they can stay in the environment for long periods of time. Once in the environment, they can easily cycle between air, water, and soil.

As a consequence, PCBs are nowadays distributed ubiquitously in the global ecosystem, such as estuary (Evy et al., 2012), soil (Dömötörová et al., 2012), water (Hong et al., 2012) and air (Aliyeva et al., 2012). Even today, large proportion of the original amounts of PCBs, still remain in old transformers and capacitors in China (Jiang et al., 1997). Moreover, PCBs are accumulating in the food web and may cause a number of toxic effects from aquatic organisms to fish and to humans. Many researches investigate the concentrations of PCBs contaminants in benthos (Dinn et al., 2012), fish (Xia et al., 2012), cow's milk (Durand et al., 2008), and city inhabitants (Amodio et al., 2012). The toxic effects of PCBs vary according to the chlorine substitution from mammals to human beings. The U.S. Environmental Protection Agency (EPA) reported that PCBs have been shown to cause cancer in animals, and they may cause cancer such as breast cancer (Fredslund and Bonefeld-Jørgensen, 2012) in humans. They have become some of the most serious environmental pollutants worldwide.

Concerned over the impact of PCBs on the environment and their persistence, they are strictly regulated in the environment. And to remediate the contamination, efficient and economically feasible technologies are needed. Many publications have reviewed PCB chemical, physical and biological degradation (Centeno et al., 2012). Among these treatments, microorganisms can eliminate those compounds and can be cost-effective, that biodegradation is playing a very important role in remediation. Microbe degradation of PCBs has been extensively studied in recent years. Microbial modify the organic pollutant into simpler and low toxic compounds by producing degradation enzymes. Isolation micro-

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organisms with biodegradation ability are first and crucial step. Several microorganisms that able to biodegrade PCB congeners were isolated. Some of these strains are aerobic bacteria (Jia et al., 2008; Dercova et al., 2008) that degraded lower chlorinated congeners of PCB; some are anaerobic bacteria which may degrade highly chlorinated PCB congeners (Payne et al., 2011; Bedard, 2008). It is found that degree of chlorination of the congener is a major factor, and degradation rate of PCBs decreased as chlorine substitution increases (Rodenburg et al., 2010).

Although PCB complete mineralization is the ultimate goal, due to the complex metabolic network responsible for PCBs degradation, optimizing degradation by single bacterial species is necessarily limited (Pieper 2005; Field et al., 2008).

In this paper, one aerobic bacteria that degraded biphenyl and 3, 3', 4, 4'- tetrachlorobiphenyl (PCB77) was isolated from wastewater treatment plant. The metabolic ability of biphenyl and cometablic PCBs were investigated.

MATERIALS AND METHODS

Samples

Activated sludge was used as starting material for the isolation of pure cultures. The activated sludge was obtained from Tianjin Dagu Chemical Co., Ltd, Tianjin, China.

Media and enumeration of cultivable bacteria

The activated sludge was serially diluted. Indigenous biphenyl and 3, 3', 4, 4'-tetrachlorobiphenyl (PCB77) degraders were selectively enumerated on minimal salts medium (MM) plates. The final pH of the medium was 7.0, using 0.5 g/L biphenyl and 5 mg/L PCB77 as carbon and energy sources. All plate counts were conducted after incubation of plates at 30℃ for 7 days.

Enrichment procedures for isolation

Enrichments were performed in 250 ml Erlenmeyer flasks containing a liquid volume of 50 ml. Transfers (10% volume) were done every 7 days into fresh MM containing biphenyl. After growth the culture suspension was serially diluted and seeded on mineral medium plates containing biphenyl. After incubation at 30℃, colonies that appeared on the plates were isolated on the basis of their morphology. The isolates were grown on mineral medium supplemented with biphenyl for 5 cycles of growth.

Sequence analysis of 16S rDNA

1.5 mL of bacterial suspension was obtained from the medium and put in Luria Bertani (LB) medium, then the mixture was cultured under optimal bacterial growth conditions (25℃, pH 7.5) which had been explored at 150 rpm for 24 h. Bacterial genomic DNA was extracted by Biospin Bacteria Genomic DNA Extraction Kit (Bioer Technology CO., LTD, Hangzhou, China) according to the manufacturer's instructions and was detected by 1% (w/v) agarose gel electrophoresis with 1×TAE buffer. Genomic DNA purified was used as templates for PCR amplification with gradient PCR instrument (Eppendorf 5331, Germany).

Primers *E. coli* 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 492R (5'-TACCTTGTTACGACT-3') were employed. Amplification was performed in 50 μL reactions containing 10-100 ng of template, 5 μL of 10×PCR buffer (Shanghai Sangon Biotech Co. Ltd.), 5 μL of 25 mmol/L $MgCl_2$, 200 μmol/L of dNTPs (Shanghai Sangon Biotech Co. Ltd.), 0.5 μmol/L of each primer and 2.5 U of Taq DNA polymerase (Promega, USA), and reactions was made up to 50 μL with sterile ultra-pure water. To improve the specificity of the reaction, a "Touch-down" PCR was performed. An initial denaturation of 5 min at 94℃, 30 cycles of 1 min at 95℃, 1 min at 55℃, 2 min at 72℃, the following 10 cycles of 1 min at 94℃, 1 min at 55℃, 1 min at 72℃, finally, extension of 2 min at 72℃. Amplified products were detected by 2% (w/v) agarose gel electrophoresis with 1×TAE buffer.

The PCR products were sent to Shanghai Sangon Biotech Co. Ltd. The sequence of about 1500 bp of 16S rDNA was compared with the National Center for Biotechnology Information (NCBI) public database using the blast tool (http://www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic tree was done by the soft of MEGA v.4. Other phenotype and chemotaxonomic characters were studied according to the Bergey's Manual of Systematic Bacteriology (Holt et al., 1994).

Biodegradation ability of biphenyl and PCB77 by isolated strain

Isolated strain was cultured in nutrient broth at 30℃ for 24 h. The cells were collected by centrifugation at 12000×g for 10 min (4℃) and washed with 0.9% NaCl. The cell suspension was added to 250-ml Erlenmeyer flasks containing 50 ml mineral medium amended with biphenyl (0.5g/L), PCB77 (5mg/L), biphenyl plus PCB77 mix, and adjusted optical density at 550 nm (OD550) to about 0.16. Then cultivated at 30℃ at 130 r/min.

Growth, residual biphenyl and PCB77 concentrations were monitored at regular intervals.

Analytical methods

Growth was measured by the optical density at 550 nm using Cary 100 UV-Visible Spectrophotometer (Varian, USA).

Analysis residual PCB77: These extracts were analyzed using a Hewlett Packard gas chromatograph (HP, USA) with an electron capture detector (ECD) and fused silica capillary column (30 m × 0.32 mm × 0.25 μm) with nitrogen as carrier gas at a constant flow rate of 1.5 ml/min. The temperature program was 150℃ for 1 min, followed by an increase at a rate of 10℃ min^{-1} until the temperature was 200℃, then increase at a rate of 5℃ min^{-1} until the temperature was 280℃, and then kept at this temperature. Results were calculated from the residual amounts of congener peaks present in each sample.

RESULTS

Biphenyl and polychlorinated biphenyls biodegradation

One bacterial strain (G-2) was isolated from activated sludge of Tianjin Dagu Chemical Co., Ltd, using biphenyl as sole carbon and energy sources. This strain was used for further analysis. Polychlorinated biphenyls are among the most persistent substances in the environment, and

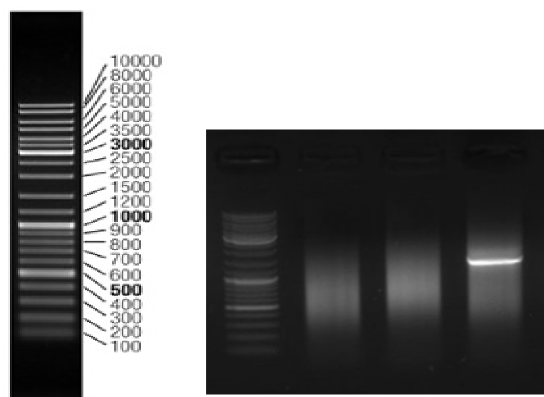

Figure 1. 16S rRNA gene PCR products. M refer to the marker, which is 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp.

GCTACACATGCAAGTCGAGCGAACCCTTCGGGGTTAGCGGCGGACGGGTGAGTAACGCGTGGGAACGTGCCCTTTGCTACGGA
ATAGCCCCGGGAAACTGGGAGTAATACCGTATACGCCCTTAGGGGGAAAGATTTATCGGCAAGGGATCGGCCCGCGTTGGATTA
GGTAGTTGGTGGGGTAATGGCCTACCAAGCCGACGATCCATAGCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATCTTAGACAATGGGGGCAACCCTGATCTAGCCATGCCGCGTGAGTGATG
AAGGCCCTAGGGTTGTAAAGCTCTTTCAGCTGGGAAGATAATGACGGTACCAGCAGAAGAAGCCCCGGCTAACTCCGTGCCAGC
AGCCGCGGTAATACGGAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGACCAGAAAGTTGGGGGT
GAAATCCCGGGGCTCAACCTCGGAACTGCCTTCAAAACTATTGGTCTGGAGTTCGAGAGAGGTGAGTGGAATTCCGAGTGTAGA
GGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGCTCGATACTGACGCTGAGGTGCGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCCAGTCGTCGGGCAGCATGCTGTTCGGTGA
CACACCTAACGGATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAACCCTTGACATCCCCGGACCGGCCTGGAGACAGGTCTT
CCACTTCGGTGGCCGGGTGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTCGGTTAAGTCCGGCAACGAGC
GCAACCCACACTCTTAGTTGCCAGCATTTGGTTGGGCACTCTAAGAGAACTGCCGATGATAAGTCGGAGGAAGGTGTGGATGAC
GTCAAGTCCTCATGGCCCTTACGGGTTGGGCTACACACGTGCTACAATGGTGGTGACAGTGGGTTAATCCCCAAAAGCCATCTC
AGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTTGGAATCGCTAGTAATCGCGGAACAGCATGCCGCGGTGAATACGTTC
CCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGGTCTACCCGACGGCCGTGCGCTAACCAGCAATGGGGGCAGC
GGACCACGGTAGGCTCAGCGACTGGGGTGAAGTCGAACAAGATGCC

Figure 2. 16S rDNA sequence of G-2.

are difficult to degrade by microgram. It was generally believed that bacteria able to grow on biphenyl usually have the ability to cometabolize PCBs. And the biphenyl, use as carbon source, can induce the necessary enzymes for biodegradation PCBs.

Characterization and identification of biodegradation strains

The phenotype and chemotaxonomic characters of the biodegradation strain was studied. G-2, two-day-old colonies were round, uniform, and white colonies which were 2-4mm in diameter. The G-2 strains were Gram-negative, aerobic, non-motile. Strains tested positive for catalase and oxidase. Strains were negative for the degradation of starch, gelatin. They can reduce nitrate to nitrite. Genomic DNA was extracted, and amplified by PCR. The PCR products were detected by agarose gel electrophoresis, as shown in Figure 1.The 16S rDNA sequence of G-2 is shown in Figure 2. The 16s rDNA sequence of strain was determined and deposited in the NCBI sequence databank. The nucleotide sequence was compared with previously described 16s rDNA sequences and a similarity matrix was constructed (data not shown). Phylogenetic tree was constructed by using a least-squares algorithm and the neighbor-joining method. Neighbour-joining tree based on almost complete 16S rDNA sequences is shown in Figure 3, showing relationships between strain G-2 and *Paracoccus* and *Rhodobacter* related taxa. The chemical and phenotypic properties of strain G-2 are consistent with its assignment to *Paracoccus*. The close relationship found between strain G-2 and the type strain of *Paracoccus* is supported both by treeing algorithms and by a high bootstrap value (Figure 3). The chemotaxonomic, molecular systematic and phenotypic data show that strain G-2 should be given species status within the genus *Paracoccus*.

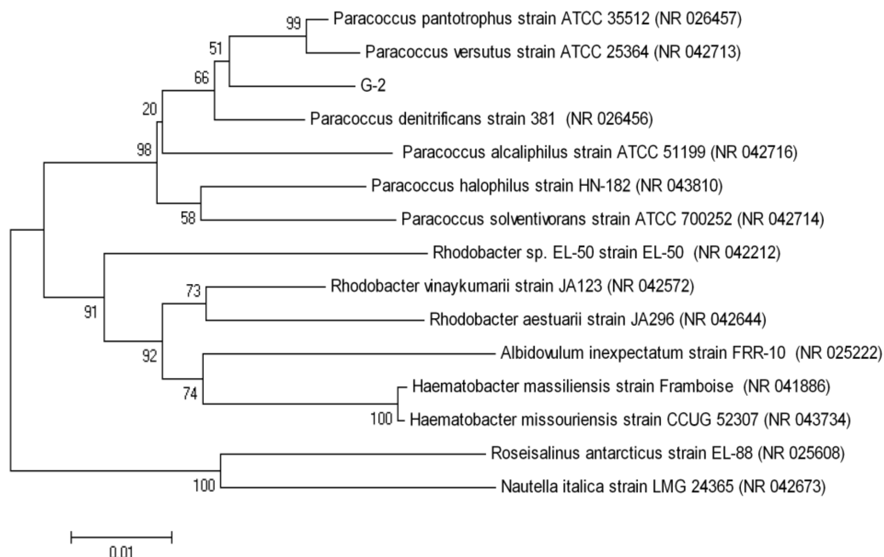

Figure 3. Phylogenetic tree of G-2 based on 16S rDNA sequence. Figures in parentheses indicate the accession numbers of 16S rDNA sequence data from NCBI.

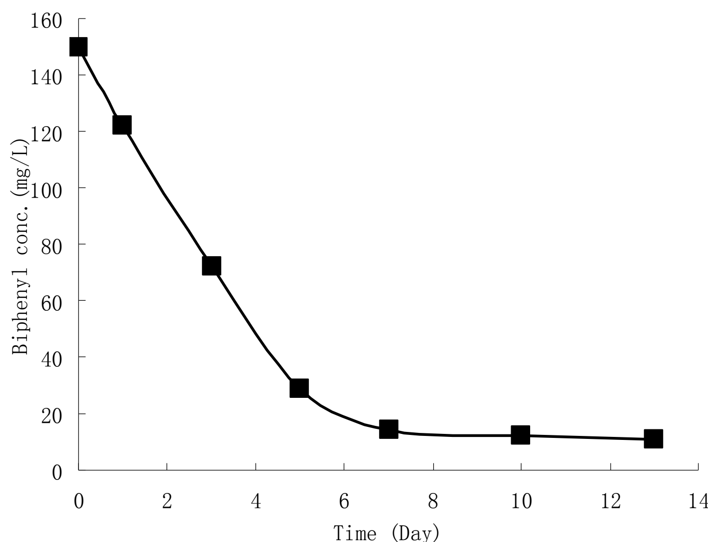

Figure 4. Degradation of biphenyl by strain G-2.

Biodegradation ability of isolated strain on biphenyl and PCB77

Degradation ability of biphenyl (BP) by G-2 at 30℃ was tested (Figure 4) .The organism grew exponentially with biphenyl as sole source of carbon and energy. The biphenyl degradation rate and the chloride release rate were found to be somehow dependent on the biomass production rate. The G-2 started degradation after the lag period. Before 3 days, the biphenyl was degraded only 17%. After that the degradation rate grew rapidly. On the 5 day, the strain can utilize 75% of biphenyl. More than 95% of biphenyl was degraded in the 7 day. The degradation ability on the medium contained PCB77 only and PCB77+biphenyl are shown in Figure 5.Strain G-2 can utilize the PCB77 as sole carbon resource, but only 24% of PCB77 was degraded. However, when biphenyl was added to the medium, it resulted in more efficient PCB77 degradation, with about 32% of PCB77 degraded. This phenomenon was the same with the Kohler's

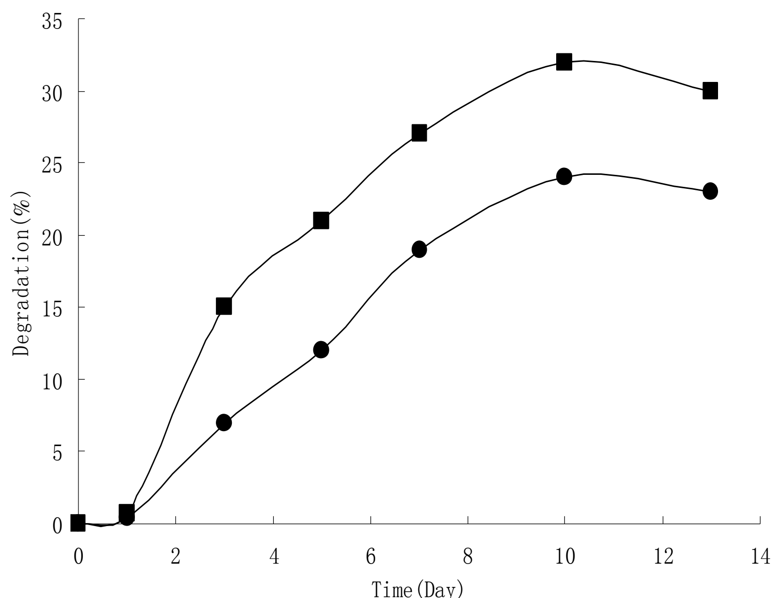

Figure 5. Degradation ability on the medium contained PCB77(-●-) only and PCB77+biphenyl (-■-).

(Kohler et al., 1988) study, which showed that PCB degradation growing on biphenyl provided more physiological stability leading to more active cells. Billingsley et al. (1997) also reported that LB400 can cometabolize PCBs when the medium conditioned to biphenyl.

DISCUSSION

In this study, a PCB-degrading bacterium was isolated and characterized. The G-2 strain was identified as *Paracoccus*.

As PCBs are produced as complex congeners, the degradation is very complicated. Some degraded microbial, such as *Burkholderia xenovorans* LB400, *Alcaligenes eutrophus* H850, *Rhodococcus* sp. RHA1 and *Alcaligenes* sp. JB1 are well characterized and widely studied (Bedard et al., 1987; Commandeur et al., 1996-1997; Seto et al., 2005; Rodrigues et al., 2006), as those strains have exceptional abilities to degrade wide range of PCB mixtures.

To our knowledge, no information concerning PCB77 degradation by *Paracoccus* was detected; however, this genus has been associated with the degradation of varies compounds, such as polycyclic aromatic hydrocarbons (Zhang et al., 2004).

Thus, this is the first report on the degradation of a fraction of PCB congeners by *Paracoccus*. Our result may provide evidence for the potential application of *Paracoccus* in bioremediation of PCB-contaminated environment. Our future work will include screening bacterial strains for efficient PCBs degradation, investigating the metabolic pathway of PCB degradation by *Paracoccus* G-2 and the genes that encoding the key enzymes in the pathway.

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