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Biodegradation of benzene homologues in contaminated sediment of the East China Sea

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HIGHLIGHTS

- To enrich moderately halophilic to degrade BTEX in a wide range of salinity.
- The optimal degradation was 120 mg/L toluene within 5 d in the presence of 2 M NaCl.
- Marinobacter, *Prolixibacter*, *Balneola*, *Zunongwangia*, *Halobacillus* were the dominant genus.
- Ring fission was catalysed by catechol 1,2-dioxygenase and catechol 2,3-dioxygenase.
- Bacteria adjusted osmotic pressure by ectoine and hydroxyectoine as compatible solutes.

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ABSTRACT

This study focused on acclimating a microbial enrichment to biodegrade benzene, toluene, ethylbenzene and xylenes (BTEX) in a wide range of salinity. The enrichment degraded 120 mg/L toluene within 5 d in the presence of 2 M NaCl or 150 mg/L toluene within 7 d in the presence of 1–1.5 M NaCl. PCR–DGGE (polymerase chain reaction–denatured gradient gel electrophoresis) profiles demonstrated the dominant species in the enrichments distributed between five main phyla: Gammaproteobacteria, Sphingobacteriia, Prolixibacter, Flavobacteriia and Firmicutes. The *Marinobacter*, *Prolixibacter*, *Balneola*, *Zunongwangia*, *Halobacillus* were the dominant genus. PCR detection of genotypes involved in bacterial BETX degradation revealed that the degradation pathways contained all the known initial oxidative attack of BTEX by monooxygenase and dioxygenase. And the subsequent ring fission was catalysed by catechol 1,2-dioxygenase and catechol 2,3-dioxygenase. Nuclear magnetic resonance (NMR) spectroscopy profiles showed that the bacterial consortium adjusted the osmotic pressure by ectoine and hydroxyectoine as compatible solutes to acclimate the different salinity conditions.

1. Introduction

BTEX contaminants have been widely detected at numerous chemical industrial facilities and oil basin, especially nearby the petroleum and related products manufacture area. As neurotoxicants BTEX was able to cause abnormality, sudden change and cancer, like leukemia (Smith et al., 2007). Recently, microbial bioremediation has been the most popular removal method which is environment friendly (Eldon et al., 2012). Lots of BTEX-degrading bacteria were isolated in the past few years, such as *Moraxella* sp. (Hogx and Jaenicee, 1972), *Nocarida* sp., *Alcaligenes denitrificans*, *Micrococcs* sp., *Arthrobacter* sp. (Weber and Corseuil, 1994), *Thermus* sp. (Chen and Taylor, 1997), *Rhodococcus rhodochrous* (Deeb and Alvarez-Cohen, 1999) and *Pseudomonas* spp. which were the most commonly reported bacteria to degrade BTEX compounds (Brusa et al., 2001). However, the bioremediation of the BETX contamination often met a bottleneck when the pollution occurred in the high salinity environment, because the degrading bacteria normally were not suit to the high salinity condition. Numerous oil seeps and abandoned drilling fluids from petroleum exploring procedure make serious BTEX contamination in saline-alkali soil and sea water. Industrial water also contains high salinity and BTEX,

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especially in many chemical and pharmacy plat effluent where BTEX was popularly used as solvent or raw materials (Boonsaner et al., 2011).

Some halophilic bacteria have been found to remove organic pollutant including BETX in high salinity soil and water, such as *Halobacter halobium*, *Staphylococcus* sp. and *Bacillus cereus* (Olivier and René, 2006). In a previous work, acclimated an aerobic bacterial enrichment which completely degraded 625 μM benzene in the presence of 14% NaCl in 1 week (Sei and Fathepure, 2009). Interestingly, moderately halophilic bacterial widely existed in the presence of 0.5–20% (w/v) NaCl natural habitats (Ventosa et al., 1998), which could degrade hexadecane, benzoate, aromatic hydrocarbon and other organics effectively. *Methylomicrobium* spp. was able to oxidize trichlorethylene in the presence of 2–6% NaCl (Margesin and Schinner, 2001). However, a limited number of studies have investigated the BTEX biodegradation in the wide salinity condition (1–3.5 M) or the activated sludge contained halophilic and salt-tolerant bacteria to degrade BTEX. Recently, molecular techniques have been used to evaluate the substantial shift of microbial diversity when the biomass was exposed to the toxic substance including BTEX, such as PCR–DGGE profile was effective in reporting the variation of microbial diversity and community structure during the exposure to contaminants (Liu et al., 2010). However, there were limited number of studies describing the diversity when the community grew in different levels of salinity with high concentration of BTEX.

Many researchers proved the PCR detection of the genotypes expressed during bacterial aerobic BTEX-degrading process was a rapid and accurate technique to determine the BTEX-biodegrading pathway (Baldwin et al., 2003). The initial oxidative step of BTEX possesses three routes including a direct oxidation of the aromatic ring via mono-oxygenase, dioxygenase attack or oxidation of the alkyl side chain catalyzed by mono-oxygenase. In all of these routes, BTEX was oxidized to three intermediate products as catechol, catechol-like intermediates and protocatechuic acid (Andreoni and Gianfreda, 2007). Then they undergo ring fission which takes place between the two hydroxyl groups or proximal to one of the two hydroxyl groups, involving action of catechol 1,2-dioxygenase or catechol 2,3-dioxygenase, respectively. In order to assess the genotypes expressed during aerobic BTEX-degrading process, some primers were designed to amplify the conserved region of the mono-oxygenase, dioxygenase or catechol 2,3-dioxygenase gene sequences (García et al., 2005; Hendrickx et al., 2006). In addition, the compatible solutes play an important role in moderately halophilic bacteria to maintain osmotic balance in high salt medium (Shivanand and Mugeraya, 2011). Brown and Simpson (1972) firstly stated the concept of compatible solutes as the low-molecular weight and highly water-soluble substance, including sugars, alcohols, amino acids, betaines, ectoines and their derivatives (Brown and Simpson, 1972). Halotolerant and halophilic bacteria could synthesize and accumulate compatible solutes to maintain osmotic balance between their cytoplasm and the saline environment (Ventosa et al., 1998). The moderately halophilic bacteria regulate the osmotic pressure by these compatible solutes under osmotically unfavorable conditions (Bursy et al., 2008). Nuclear magnetic resonance (NMR) spectroscopy is an effective technique to detect the compatible solutes (Jakeman et al., 1998) and both ^{1}H and ^{13}C NMR have been developed to rapidly and sensitively determine the bacteria metabolized compatible solutes (Roberts, 2005).

The aim of this study was to investigate a halophilic bacterial community able to degrade BTEX in a wide range of salinity conditions. In order to obtain a moderately halophilic bacterial community able to efficiently degrade BTEX, a serious of microcosm experiments were acclimated from the beach sediments of East China Sea at various concentration levels of NaCl. Also, this study determined the bacterial diversity, detected the BTEX-degrading pathway and revealed the salt tolerance mechanism of the bacterial enrichments through an integrated approach.

2. Methods

2.1. Acclimating and enrichment of the microbial community

For the aerobic culture, upper sediment samples (5–10 cm) in the East China Sea were collected near the oil chemical production contaminated site (Latitude °N, 30.82; Longitude °E, 121.53) by using sterilized spatulas. The sampling points were chosen because of its salinity 2.63%, and the presence of high levels of BTEX contaminants (91.57 mg/kg), where was near a sewage draining exit of a proleum chemical industrial factory. The samples (approximate 200 g) were placed in sterile mason jars with ample headspace. The samples were carried to the laboratory on ice and put in 4 °C. The samples were used to prepare microcosms in this study. The microcosms prepared in 125-mL capacity serum bottles using 10 g of sediment sample and 40 ml of mineral salts medium (MSM) with pH 6.9, amended with 1, 1.5, 2, 2.5, 3, 3.5 M NaCl, respectively. The composition of MSM is described by Nicholson and Fathepure (2004). Each bottle of microcosm experiments containing 1, 1.5 M NaCl were amended with 9.0 μl toluene, and the bottles containing 2, 2.5, 3, 3.5 M NaCl were amended 6.0 μl toluene as the only source of carbon. Autoclaved bottles prepared as mentioned were used as controls. Bottles were closed with Butyl Rubber Stopper and aluminum caps and incubated in an inverted position at 30 °C. Air in the headspace served as the source of oxygen. Enrichment cultures were developed from the bottles that showed significant degradation of added toluene. The bottles were repeatedly spiked with toluene 6–7 times to acclimatize the BTEX degrading bacteria. And the enrichments were transferred 5–6 times to obtain the sediment-free cultures.

2.2. BTEX degradation assay

Biodegradation of added toluene was monitored using a gas chromatograph (GC). Aerobic media samples (10 μL aerobic medium in 10 mL double distilled water) were obtained from the culture vessels every day. Biodegradation of BTEX compounds was analyzed by GC (Agilent 6890) equipped with a flame ionization detector (FID) and a DB-VRX capillary column, 60 m × 0.25 mm × 1.4 μm. N_2 (ultra pure) served as both carrier and make up gas at flow rates of 20 and 25 ml/min, respectively. The flow rates of H_2 and air were at 40 and 400 mL/min, respectively. The GC was operated using the following conditions: set at 45 °C for 0 min, then to 190 °C at a rate of 12 °C/min. Nitrogen was used as the carrier gas. Inlet temperature: 240 °C, samples were injected using a split rate, 10:1 and detector temperature, 300 °C. Samples were collected from the solutions, and 40 mL of the water samples were injected into the purge-and-trap system. Additional pre-treatment procedures were carried out prior to the analysis of toluene in plant materials. Hybrid poplar roots, stems, and leaves were separated at the end of the incubation, dipped into liquid nitrogen, and sealed in bottles for the analysis of toluene in each component of the biomass. All samples were stored at −20 °C and isolated from light until the concentration of toluene was determined. The toluene in the biomass was analyzed using the same techniques as in the aqueous solution samples.

2.3. Total DNA extraction, 16S rRNA gene V3 amplification and DGGE analysis

Aerobic medium was obtained from the enrichment culture maintained on toluene and MSM containing 1–3.5 M NaCl. Five mil-