Protection of *Polaromonas naphthalenivorans* CJ2 from Naphthalene Toxicity by Extracellular Polysaccharide Capsules

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***Polaromonas naphthalenivorans* CJ2, responsible for naphthalene degradation at a coal tar contaminated site, was isolated on MSB agar media supplied with naphthalene vapor as the sole carbon source at 10°C. The strain is not isolated under the same isolation condition using the same soil sediment at 20°C although its optimum temperature is about 20°C. In this work we explored the reason why strain CJ2 could not have been isolated on MSB agar with naphthalene vapor at 20°C. Dispersed CJ2 cells in PBS buffer formed colonies on MSB agar with naphthalene vapor at 10°C with low naphthalene vapor pressure, but not at 20°C with high naphthalene vapor pressure. However, streaked cells without resuspension grew on MSB agar with naphthalene vapor at 10°C, 20°C, and even 25°C. Investigation of scanning electron microscopy showed that CJ2 cells formed extracellular polysaccharide (EPS) capsules, which were released easily from CJ2 cells by just dispersion. Therefore, it is concluded that strain CJ2 is able to overcome the naphthalene toxicity by forming a capsule-type barrier around the cells although it is susceptible to naphthalene toxicity at high temperature.**

Key words: *extracellular polysaccharide, naphthalene toxicity,* P. naphthalenivorans *CJ2*

Among the many goals of microbial ecology, identification and understanding of the genes as well as the microorganisms that are responsible for catalyzing biogeochemical reactions in soils, water, and sediment are very important to analyze the environmental processes [Jeon *et al.*, 2006; Madsen, 2000; Radajewski *et al.*, 2000]. These days techniques that focus on markers of *in situ* metabolism that are specific and transient, such as unstable metabolites and mRNA, are very popular and can offer the possibility of measuring activity that is taking place at the time of sampling [Wilson *et al.* 1999; Wilson and Madsen, 1996]. However, isolation of microorganisms is still a prerequisite for understanding of environmental processes and biotechnology in detail, but it is not always successful due to many obstacles.

Polycyclic aromatic hydrocarbon (PAH) contamination of the environment is of concern and numerous studies have been conducted on PAHs degrading bacteria because they are toxic and carcinogenic [Zhang *et al.*, 2004]. There has been extensive information on PAH biodegradation by both gram-positive and gram-negative bacteria, especially *Pseudomonads* because *Pseudomonads* could be isolated and cultivated easily [Park *et al.*, 2002; Serdar *et al.*, 1989; Shuttleworth and Cerniglia, 1995; Simon *et al.*, 1993]. Coal-tar waste containing high concentrations of PAH was buried at our study site (site 24, NY, USA) approximately 40 years ago, which has provided an excellent opportunity to examine the naturally occurring microorganisms to PAH [Madsen *et al.*, 1991]. Therefore, previous researchers have studied on active bacteria capable of metabolizing aromatic hydrocarbons at a coal tar waste-contaminated site [Bakermans and Madsen, 2002; Jeon *et al.*, 2003; Park *et al.*, 2003] and isolated a variety of Gram-negative and Gram-positive naphthalene degrading bacteria from the site [Herrick *et al.*, 1997; Stuart-Keil *et al.*, 1998].

Recently we isolated a novel Gram-negative bacterium, *Polaromonas naphthalenivorans* CJ2, on MSB agar supplied with naphthalene vapor as the sole carbon and energy source at 10°C [Jeon *et al.*, 2004]. However, previous researchers could not isolate strain CJ2 on MSB agar with naphthalene vapor from the same soil sediment

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Abbreviations: EPS, extracellular polysaccharide; SEM, scanning electron microscopy

at 20°C. The only difference between the previous isolation conditions and our conditions was temperature. By lowering the incubation temperature from 20°C to 10°C, we succeeded in isolating the naphthalene-degrading bacterium. In this paper we describe in the light of naphthalene toxicity the reasons why strain CJ2 is not isolated on MSB media supplied with naphthalene vapor at 20°C. This was one important achievement since numerous previous efforts to isolate such a bacterial strain had turned out to be failed at the similar isolation conditions.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. *Polaromonas naphthalenivorans* strainCJ2 and *Pseudomonas putida* NCIB 9816-4 have been described previously [Jeon *et al.*, 2003; 2004; 2006; Simon *et al.*, 1993; Stuart-Keil *et al.*, 1998; Park *et al.*, 2002; 2004]. *P. naphthalenovorans* CJ2 was grown and maintained on mineral salts basal medium (MSB) [Stanier *et al.*, 1966] with either naphthalene vapor as the sole carbon source or a complex medium R2A agar. Naphthalene was supplied as vapor in a closed chamber. Other culture media were prepared according to the procedures described previously [Stuart-Keil *et al.*, 1998].

Toxicity tests of *P. naphthalenivorans* CJ2 to naphthalene. Toxicity tests of *P. naphthalenivorans* CJ2 were performed following the procedure that was used for strain CJ2 isolation [Jeon *et al.*, 2004]. Strain CJ2 colonies grown on R2A agar (Difco) were resuspended in PBS (phosphate buffered saline; 0.01 M sodium phosphate, 0.9% (w/v) NaCl, pH 7.2) buffer. The resuspended cells were spread on MSB agar supplied with naphthalene vapor or R2A agar, respectively. The agar plates were incubated at 10, 20, and 25 °C and colony formation was checked after 10 days. Toxicity tests of unsuspended *P. naphthalenivorans* CJ2 were also carried out. Strain CJ2 colonies grown on R2A agar were transferred on MSB agar supplied with naphthalene vapor or R2A agar by just streaking and incubated at 10°C and 20°C and 25°C, respectively. Growth was checked after 10 days. Our experimental design for toxicity tests included an archetype naphthalene degrader, *P. putida* NCIB 9816-4 as a reference test.

Measurement of naphthalene vapor pressure. To measure naphthalene vapor pressure at different temperatures, crystals of naphthalene were added into empty serum bottles and stored at 10, 20, and 25°C. Headspace sampling (250 μl gas-tight syringe) occurred 3 times after two days, naphthalene vapor contents in the serum bottles were measured using a Hewlett Packard 6890 GC equipped with an HP-5MS column (crosslinked 5% PH ME Siloxane) coupled to an HP 5973 MSD (mass selective detector) according to the method described previously [Jeon *et al.*, 2004].

Observation of extracellular polysaccharide from strain CJ2. For SEM, CJ2 cells were fixed with 3% glutaraldehyde and 1% osmium tetroxide and dehydrated in an ethanol series (20, 40, 60, 80%) for 10 min at each gradation followed by three times in 100% ethanol for 10 min. The dehydrated samples were dried with a critical point dryer using liquid carbon dioxide as the transition fluid. A piece of the dried membrane was attached to an SEM stub with double-sided tape, then coated with ~40 nm of gold-palladium in a sputter coater under vacuum. The coated samples were viewed in a Hitachi 4500 field emission scanning electron microscope operated at 5.0 kV at the Cornell Integrated Microscopy Center.

Results

Isolation background of *Polaromonas naphthalenivorans* CJ2. *P. naphthalenivorans* strain CJ2, responsible for naphthalene degradation at a coal-tar contaminated site (site 24, NY, USA), was isolated on MSB media supplied with naphthalene vapor at 10°C [Jeon *et al.*, 2003, 2004]. However, strain CJ2 is not isolated from the same soil sediment that was used for CJ2 isolation on MSB media supplied with naphthalene vapor at 20°C although its optimum temperature is approximately 20°C [Jeon *et al.*, 2004]. Previous researchers also could not isolate strain CJ2 or its relatives at 20°C. Most of the previous isolates were just traditional naphthalene degraders such as *Pseudomonas*, *Burkholderia*, *Paenibacillus*, and *Rhodococcus* from the site at 20°C [Wilson *et al.*, 2003]. The sole difference between success and failure of strain CJ2 isolation was just incubation temperature.

Toxicity tests of *P. naphthalenivorans* CJ2 to naphthalene. To investigate the reasons that strain CJ2 had not been isolated from the coal tar contaminated site at 20°C although its optimum temperature is 20°C, growth tests were performed using already isolated strain CJ2. The resuspended cells of strain CJ2 in PBS buffer were spread on MSB agar and incubated under naphthalene vapor at 10, 20, and 25°C, respectively. As the results, they formed colonies at 10°C, but not at 20°C or 25°C, whereas they formed colonies at all temperatures on R2A agar (Table 1). On the other hand when the strain CJ2 cells were transferred on fresh MSB agar by streaking method without resuspension, which can keep the EPS capsules on the cells, and incubated under naphthalene vapor at 10, 20, and 25°C. As the result, the streaked CJ2 cells grew well on MSB agar with naphthalene vapor at all temperatures (Table 1). For the reference experiment, the

Table 1. Growths of dispersed and streaked cells of strain CJ2 on MSB agar supplied with naphthalene vapor or MSB agar with pyruvate

		Media					
		MSB with naphthalene vapor			MSB with pyruvate		
		Temperature, °C			Temperature, °C		
		10	20	25	10	20	25
CJ2	Dispersed	+	-	-	+	+++	+++
CJ2	Streaked	+	++	++	+	+++	+++

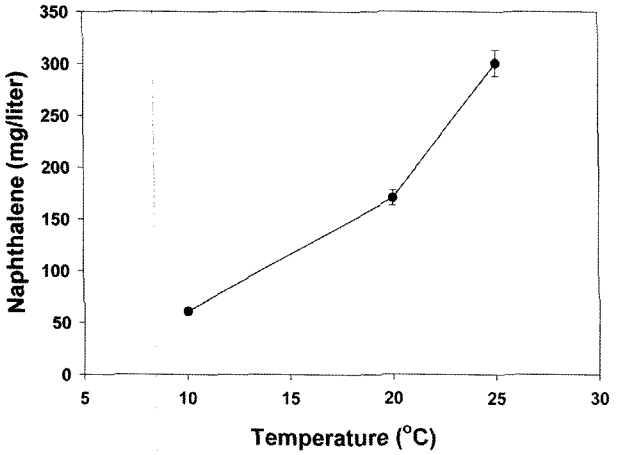

Fig. 1. Naphthalene vapor pressure in serum bottles at different temperatures.

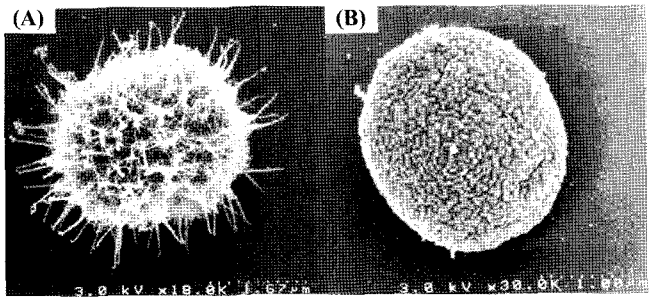

Fig. 2. Photomicrographs of strain CJ2. (a) CJ2 cell with rough surface texture from extracellular polysaccharide visible in SEM image. (b) CJ2 cell with wrinkled surface by releasing EPS material in SEM image.

growth tests of *P. putida* NCIB 9816-4, a well-known naphthalene degrader, showed that the resuspended cells of strain NCIB 9816-4 formed colonies on MSB agar with naphthalene vapor at all temperatures (Data not shown).

Naphthalene vapor pressure at different temperatures. Because naphthalene as a sole carbon source was supplied with vapor form by sublimation from naphthalene pellets, its vapor pressure is closely related to temperature of incubation chambers. As shown in Fig. 1, naphthalene vapor pressure increased sharply according the temperature increase. The amount of vaporized naphthalene at 20°C was three times higher than that at 10°C, suggesting that the vulnerability of strain CJ2 on naphthalene vapor at the elevated temperature may be related to the increased amount naphthalene in the vapor.

Observation of extracellular polysaccharide produced by strain CJ2. To examine the differences between resuspended cells and undispersed cells of strain CJ2, resuspended and undispersed CJ2 cells were investigated using a SEM. Undispersed CJ2 cells that were prepared in the conventional way for SEM (i.e. fixation by glutaraldehyde, ethanol dehydration, critical point drying and gold-palladium coating) showed that CJ2 cells were surrounded by dense EPS capsules (Fig. 2a). However, the SEM image of the resuspended CJ2 cells showed that the cells lost their EPS capsules, indicating that the EPS capsules were released easily from the cells just by dispersion. Similarly, bacterial EPS was known to be released from cells easily by blending [Kachlany *et al.*, 2001].

Discussion

It is generally considered that naphthalene is toxic to human and insects as well as microorganisms. However, pollutant-degrading bacteria are considered to be tolerant to pollutant toxicity at any rate due to their degrading ability [Park *et al.*, 2004; Park and Madsen, 2004]. *P. naphthalenivorans* strain CJ2 was isolated from a coal tar contaminated site on MSB media supplied with naphthalene vapor at 10°C, not at 20°C [Jeon *et al.*, 2003; 2004]. In this work the reason that strain CJ2 is not isolated on MSB agar with naphthalene vapor at 20°C was investigated. Our results showed that resupended CJ2 cells without EPS capsules formed colonies on MSB agar supplied with naphthalene vapor at 10°C with low naphthalene vapor pressure, but not at 20°C with high naphthalene vapor pressure. On the contrary, streaked cells with EPS capsules grew on MSB agar supplied with naphthalene vapor at 10°C, 20°C, and even 25°C. Therefore, it is concluded that EPS materials can protect strain CJ2 cells from the naphthalene toxicity by forming capsules that

can be a diffusion barrier of naphthalene vapor. The toxicity tests of *P. putida* NCIB 9816-4 revealed that strain NCIB 9816-4 is more tolerant than strain CJ2 to naphthalene toxicity and can grow well on MSB agar with high naphthalene vapor pressure without capsules. Thus, these results partially can explain why *Pseudomonads* have been isolated dominantly from the coal tar-contaminated site (site 24) in which strain CJ2 is responsible for naphthalene degradation [Jeon *et al.*, 2003]. Pollutant-degrading bacteria can be sensitive at the concentrations of pollutants generally used for bacteria isolation at laboratory. Therefore, the results reported here suggest that uncultured pollutant-degrading bacteria can be isolated just by decreasing pollutant toxicity.

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References

Bakermans C and Madsen EL (2002) Diversity of 16S rDNA and naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters. *Microb Ecol* **44**, 95-106.

Herrick JB, Stuart-Keil KG, Ghiorse WC, and Madsen EL (1997) Natural horizontal transfer of a naphthalene dioxygenase gene between bacteria native to a coal tar-contaminated field site. *Appl Environ Microbiol* **63**, 2330-2337.

Jeon CO, Park W, Padmanabhan P, DeRito PC, Snape JR, and Madsen EL (2003) Discovery of a previously undescribed bacterium with distinctive dioxygenase that is responsible for *in situ* biodegradation in contaminated sediment. *Proc Nat Acd Sci USA* **100**, 13591-13596.

Jeon CO, Park W, Ghiorse WC, and Madsen EL (2004) *Polaromonas naphthalenivorans* sp. nov., a naphthalene-degrading bacterium from naphthalene-contaminated sediment. *Int J Syst Evol Microbiol* **54**, 93-97.

Jeon CO, Park M, Ro HS, Park W, and Madsen E L (2006) The naphthalene catabolic (*nag*) genes of *Polaromonas naphthalenivorans* CJ2: Evolutionary implications for two gene clusters and novel regulatory control. *Appl Environ Microbiol* **72**, 1086-1095.

Kachlany SC, Levery SB, Kim JS, Reuhs BL, Lion LW, and Ghiorse WC (2001) Structure and carbohydrate analysis of the exopolysaccharide capsule of *Pseudomonas putida* G7. *Environ Microbiol* **3**, 774-784.

Kalogeraki VS and Winans SC (1997) Suicide plasmids containing promoterless reporter genes can stimultaneously disrupt and create fusions to target genes of diverse bacteria. *Gene* **188**, 69-75.

Madsen EL (2000) Nucleic-acid characterization of the identity and activity of subsurface microorganisms. *Hydrogeology J* **8**, 112-125.

Madsen EL, Sinclair JL, and Ghiorse WC (1991) *In situ* biodegradation: Microbiological patterns in a contaminated aquifer. *Science* **252**, 830-833.

Park W, Jeon CO, and Madsen EL (2002) Interaction of NahR, a LysR-type transcriptional regulator, with the alpha subunit of RNA polymerase in the naphthalene degrading bacterium, *Pseudomonas putida* NCIB 9816-4. *FEMS Microbiol Lett* **213**, 159-165.

Park W, Jeon CO, Hohnstock-Ashe AM, Winans SC, Zylstra GJ, and Madsen EL (2003) Identification and characterization of the conjugal transfer region of the pCg1 plasmid from naphthalene-degrading *Pseudomonas putida* Cg1. *Appl Environ Microbiol* **69**, 3263-3271.

Park W, Jeon CO, Cadillo H, Derito C, and Madsen EL (2004) Survival of Naphthalene-Degrading *Pseudomonas putida* NCIB 9816-4 in Naphthalene-Amended Soils: Toxicity of Naphthalene and its Metabolites. *Appl Microbiol Biotechnol* **64**, 429-435.

Park W and Madsen EL (2004) Characterization in *Pseudomonas putida* Cg1 of nahR and its role in bacterial survival in soil. *Appl Microbiol Biotechnol* **66**, 209-216.

Radajewski S, Ineson P, Parekh NR, and Murrell JC (2000) Stable-isotope probing as a tool inmicrobial ecology. *Nature* **403**, 646-649.

Serdar CM and Gibson DT (1989) Studies of nucleotide sequence homology between naphthalene-utilizing strains of bacteria. *Biochem Biophys Res Commun* **164**, 772-779.

Shuttleworth KL and Cerniglia CE (1995) Environmental aspects of PAH biodegradation. *Appl Biochem Biotechnol* **54**, 291-302.

Simon MJ, Osslund TD, Saunders R, Ensley BD, Suggs S, Harcourt A, Suen WC, Cruden DL, Gibson DT, and Zylstra GJ (1993) Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. *Gene* **127**, 31-37.

Stuart-Keil KG, Hohnstock AM, Drees KP, Herrick JB, and Madsen EL (1998) Plasmids responsible for horizontal transfer of naphthalene catabolism genes between bacteria at a coal tar-contaminated site are homologous to pDTG1 from *pseudomonas putida* NCIB 9816-4. *Appl Environ Microbiol* **64**, 3633-3640.

Wilson MS, Bakermans C, and Madsen EL (1999) *In situ*, real-time catabolic gene expression: extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater. *Appl Environ Microbiol* **65**,

80-87.

Wilson MS and Madsen EL (1996) Field extraction of a transient intermediary metabolite indicative of real time *in situ* naphthalene biodegradation. *Environ Sci Technol* **30**, 2099-2103.

Wilson MS, Herrick JB, Jeon CO, Hinman DE, and Madsen EL (2003) Horizontal transfer of *phnAc* dioxygenase genes within one of two phenotypically and genotypically distinctive naphthalene-degrading guilds from adjacent soil environments. *Appl Environ Microbiol* **69**, 2172-2181.

Zhang H, Kallimanis A, Koukkou AI, and Drainas C (2004) Isolation and characterization of novel bacteria degrading polycyclic aromatic hydrocarbons from polluted Greek soils. *Appl Microbiol Biotechnol* **65**, 124-131.