Short Communication

Isolation of Toluene-resistant Mutants from *Pseudomonas putida* PpG1 (ATCC 17453)

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Toluene, like many organic solvents, is highly biotoxic and kills most microorganisms at low concentrations. It is often used therefore to sterilize microbial cultures and to permeabilize bacterial cells in the assay of enzymes. The physiological basis of such phenomena, however, remains poorly characterized. Recently, Inoue and Horikoshi reported the discovery of a variant strain, *Pseudomonas putida* IH-2000, which grew in culture media containing a separate phase of toluene or other solvents, e.g., cyclohexane, xylenes, ethylbenzene, *o*-dichlorobenzene, or heptanol, at more than 50% (v/v).¹⁾

To analyze the toluene resistance, Inoue *et al.* have isolated toluene-sensitive mutants from *P. putida* IH-2000 and cloned a 10-kilobase DNA fragment that restored the toluene resistance of this strain.¹⁾ For the same purpose, we attempted the other way, the isolation of toluene-resistant mutants from strains that were originally toluene-sensitive. The analysis of these mutants will give us more essential information to characterize toluene resistance of bacteria.

In this paper we describe isolation of toluene-resistant mutants from *P. putida* PpG1 (ATCC 17453)^{2,3)} and partial characterization of the toluene resistance of these mutants.

About fifty strains isolated from soil or obtained from ATCC were tested for resistance

to organic solvents added to liquid media. Among them, three *Pseudomonas* strains from soil and two ATCC strains, *P. putida* (ATCC 17453) and *P. putida* (ATCC 12633)^{2,4)} were resistant to *p*-xylene but not to toluene, because toluene is more biotoxic than *p*-xylene in these tests. For isolation of toluene-resistant mutants, these five *p*-xylene-resistant *Pseudomonas* strains were cultivated and the cells that had spontaneously mutated to toluene resistance were selected.

Pseudomonas strains were cultivated in culture tubes containing 3 ml of L-broth (Tryptone, 10 g/l; yeast extract, 5 g/l; sodium chloride, 5 g/l) shaken reciprocally at 120 rpm at 30°C. The overnight cultures were seeded into 3 ml of fresh media at the optical density (600 nm) of 0.05, and 0.6 ml of toluene was added to each tube. After three days of cultivation, microbial growth was observed in the culture of one of the five strains, *P. putida* PpG1. The toluene resistant strain thus obtained was purified by single colony selection. For genetic analysis of toluene resistance mutations, we isolated auxotrophic mutants PpG1-7 (His⁻) and PpG1-9 (Ilv⁻) from *P. putida* PpG1 and again toluene-resistant mutants were isolated from PpG1-7 and PpG1-9 by the same method. The frequency of such mutants was estimated as 10⁻⁸–10⁻⁹. These mutants were designated as PpG1-7T (His⁻, toluene resistant) and PpG1-9T (Ilv⁻, toluene resistant), respectively, and used for further analysis.

The toluene-resistant mutants PpG1-7T or PpG1-9T were streaked on L-agar plates, toluene was poured onto the surface of plates to approximately 2 mm depth, and the plates were incubated for 48 hr at 30°C. Colony formation was observed under the toluene layer in both mutants but no colony formation of the parental strain *P. putida* PpG1 was observed (Fig. 1). The cells picked from each colony were viable when transferred to media containing no organic solvent. Growth of the toluene-resistant mutant PpG1-7T in the presence of 20% (v/v) of toluene in liquid media was also observed. After three to four days of

Fig. 1. Plate Cultures of PpG1-7T (A) and Parent PpG1 (B).

The plates were overlayed with (right) or without (left) toluene and incubated at 30°C for 48 hr. The mutant PpG1-7T could form discrete colonies under the toluene layer.

cultivation at 30°C shaken in a culture tube, the optical density (600 nm) increased above 1.0 and became constant. The content of toluene was not critical to the bacterial growth, because the aqueous phase was equilibrated with toluene in such conditions. Divalent cations such as Mg^{2+} were reported to stabilize membrane structure and to maintain normal morphology and growth of bacterial cells,⁵⁻⁷⁾ but no facilitative effect for cell growth was observed in the presence of Mg^{2+} (2–50 mM) compared to the absence of Mg^{2+} . The same result was obtained with PpG1-9T (data not shown). After several weeks of cultivation on L-agar plates without toluene, these mutants still had the toluene-resistant phenotype, sug-

gesting that these mutations are very stable.

In conclusion, we isolated toluene-resistant mutants from *P. putida* (ATCC 17453). Further analysis of these mutants will give us more information on toluene resistance in bacteria.

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