Short Communication

Preparation of Organic Solvent-Tolerant Mutants from Pseudomonas aeruginosa Strain PAO1161

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Received May 2, 1994

Most organic solvents (OSs) are toxic and inhibit growth of microorganisms even at low concentrations. Therefore, they are used to sterilize microbial cultures and to cultivate in a sterile condition. However, the physiological basis of such phenomena is poorly understood. Although there are some microorganisms that can utilize a number of OSs as their sole carbon and energy sources, OSs must be provided as a vapor or at a very low concentration to avoid growth inhibition.

Inoue and Horikoshi have reported the isolation of a toluene-tolerant bacterium (Pseudomonas putida strain IH-2000) and its index of relative solvent toxicity. They used, as a toxicity index of solvents, the log $P$, defined as the common logarithm of a partition coefficient ($P$) of a given solvent between equimolar $n$-octanol and water. Some microorganisms are especially OS-tolerant and can grow even in the presence of $p$-xylene (log $P$ 3.1), toluene (log $P$ 2.8), or benzene (log $P$ 2.0). Hence, they are useful for biosolvent digestion or for bioremediation of OS-contaminated sites.

Some industrial biotransformation processes use water-insoluble organic compounds in a two-liquid-phase system consisting of water and OS. When such compounds are used as substrates, large quantities of water are required. This water consumption is a major factor in the fermentation industry. The development of OS-tolerant microbial catalysts for use in bioreactors might solve this problem. Toluene-tolerant mutants were isolated from Pseudomonas putida PpG1 (ATCC 17453), which was originally $p$-xylene-tolerant. In addition, $n$-hexane-tolerant mutants were isolated from Escherichia coli K-12 by 1-methyl-3-nitrosoguanidine (NTG) mutagenesis, and their utilization as hosts for genetic manipulation is now underway.

In this paper, we report the effective preparation of $n$-hexane and $p$-xylene-tolerant mutants by repetitive subculturing from Pseudomonas aeruginosa strain PAO1161. P. aeruginosa strain PAO1161 is one of the derivatives of the Pseudomonas type strain PAO1, which has lost its restriction system, and is available as a Pseudomonas ssp. host. From this strain, we obtained two $n$-hexane-tolerant microorganisms that are interesting for the elucidation of the biological mechanisms of OS-tolerance.

We developed $n$-hexane and $p$-xylene-tolerant mutants of P. aeruginosa strain PAO1161 on the basis of the high log $P$ for these solvents. PI medium (supernatant of a suspension of 45 g of Pseudomonas Isolation Agar (Difco) in 1 liter of deionized water) was used for cultivating strains of Pseudomonas aeruginosa unless otherwise stated. The supernatant of the PI medium was sterilized by filtration. Strains were incubated in test tubes containing 2 ml of PI medium at 30°C with shaking at 200 rpm. Growth was monitored turbidimetrically at 660 nm. Initially, $n$-hexane-tolerant mutants were developed because this solvent has a higher log $P$ than $p$-xylene. Overnight cultures of the parental strain PAO1161 were inoculated into 2 ml of fresh medium containing 5, 10, 20% (v/v) $n$-hexane. After 24 h of cultivation, growth was observed in one of the test tubes containing 5% (v/v) $n$-hexane. A culture from this test tube seeded a medium containing 20% (v/v) $n$-hexane. After 24 h of incubation, the OD$_{660}$ increased above 1.0. Thus, the $n$-hexane-tolerant mutant obtained was designated as PAK101 and used to look for further mutations.

This mutant strain PAK101 was inoculated into 2 ml of fresh medium containing 5, 10, 20% (v/v) $p$-xylene. After 24 h of cultivation, growth was observed in one of the test tubes containing 5% (v/v) $p$-xylene. This culture was inoculated into a medium containing 20% (v/v) $p$-xylene.

Table Solvent-Tolerance of PAH Strains and Related Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>$n$-Hexane log $P$</th>
<th>$p$-Xylene log $P$</th>
<th>Toluene log $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1161</td>
<td>3.9</td>
<td>3.1</td>
<td>2.8</td>
</tr>
<tr>
<td>PAK101</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PAK102</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Abbreviations used are as follows: +, growth; -, no growth.

Cells were streaked on the medium, overlaid with the solvent, and incubated at 30°C for 24 h.

A

B

Fig. Plate Cultures of Mutant Strain PAK102 (A) and Wild Strain PAO1161 (B).

Plates were overlaid with (right) or without (left) $p$-xylene and incubated at 30°C for 24 h.

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Abbreviation: OS, organic solvent.
After 24 h of incubation, dense growth was observed. This p-xylene-tolerant mutant was designated as PAK102. Table shows OS-tolerance of strains used in this paper.

This mutant strain PAK102 and its parental strain PAO1161 were streaked on PI-agar plates. Plates were overlaid with p-xylene to approximately 2 mm depth, and incubated for 48 h at 30°C. Mutant strain PAK102 could form colonies under the p-xylene layer, but strain PAO1161 could not (Fig.). This strain PAK102 was also able to grow in PI medium containing 50% (v/v) p-xylene. This study demonstrates that OS-tolerant bacteria were effectively prepared by repetitive subculturing, and these mutant strains still had an OS-tolerant phenotype after a week of cultivation on PI-agar plates without OS, suggesting that these mutations are stable. Although many genes encoding a variety of enzymes have been isolated from Pseudomonas spp., some of which seem to be very useful in biotransformation processes, there are no Pseudomonas spp. hosts available even in water-OS two-liquid-phase systems. These mutants are expected to be useful as Pseudomonas spp. hosts in biotransformations, even in water-OS two-liquid-phase systems. Further analysis of these mutants will give us much more information about the biological mechanisms of OS-tolerance.

Acknowledgments. We thank Dr. M. Fukuda of Nagaoka University of Technology for the gift of P. aeruginosa strain PAO1161. We also thank Dr. T. J. McGenity for critical reading of the manuscript.

References