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NOTES

Pseudomonas putida Which Can Grow in the Presence of Toluene

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A *Pseudomonas putida* strain able to grow in the presence of more than 50% toluene was isolated from soil. The strain was tolerant of other toxic solvents, including aliphatic hydrocarbons, alicyclic hydrocarbons, aromatic hydrocarbons, alcohols, and ethers. The stability of the solvent tolerance of strain IH-2000 was stimulated by addition of Mg^{2+} and Ca^{2+} to the medium containing toluene.

Most organic solvents are generally biotoxic and prohibit growth of microorganisms even at low concentrations, i.e., less than 2 to 3% in culture media. Toluene is a highly toxic solvent and at a concentration of 0.1% will kill most microorganisms. Therefore, toluene has been used for many years by microbiologists to sterilize microbial cultures and to maintain solutions in a sterile condition. Furthermore, because toluene is effective in extracting lipids from cell membranes, it has been used as an unmasking agent in the assay of a variety of enzymes, e.g., β-galactosidase (3), arabinose isomerase (2), and alkaline phosphatase (7). It has been reported that some microorganisms, such as *Pseudomonas putida* (10), *Pseudomonas aeruginosa* (5), a *Pseudomonas* sp. (1), an *Achromobacter* sp. (10), and a *Nocardia* sp. (8), can assimilate toluene. However, these bacteria tolerated toluene concentrations of less than 0.3%. Microorganisms which can grow in the presence of more than 0.3% toluene have never been found previously.

Solvent-tolerant microorganisms have numerous potential commercial applications in industrial biotransformation processes which involve the use of organic substrates with low solubility in water. When such compounds are used as substrates, large quantities of water are required, and this water consumption is a major cost factor in the fermentation industry. The development of solvent-tolerant microbial catalysts for use in bioreactors might solve this problem. Also, from the academic point of view, such microorganisms are an interesting subject for elucidation of the biological mechanisms of solvent tolerance.

Previously we reported the isolation of the toluene-tolerant bacterium (strain IH-2000) and the index of relative solvent toxicity (4). This article deals with preliminary characterization of this strain.

For culture of strain IH-2000, modified LB medium consisting of 10 g of Bacto-Tryptone (Difco Laboratories), 5 g of yeast extract (Difco), 5 g of NaCl, and 10 mM $MgSO_4 \cdot 7H_2O$ in 1 liter of deionized water was used unless otherwise noted. The pH of the liquid medium was adjusted to 7.0. CS basal medium, consisting of 1 g of NH_4NO_4, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 0.2 g of KCl at pH 7.2, was used to test for utilization of hydrocarbons. Cultures were generally grown in test tubes containing 5 ml of medium with shaking at 30°C. Cell growth was determined by measuring the optical density at 660 nm (OD_{660}). Analysis

TABLE 1. Summary of characteristics of strain IH-2000

Morphological characteristics	
Form	Rods
Size (μm)	0.7–1.0 by 2.0–4.0
Gram stain	Negative
Motility	+
Flagellum	>1, polar
G+C DNA (mol%)	62.5
Cultural characteristics	
Aerobiosis	+
Growth at 41°C	−
pH range	5.5–9.0
Biochemical characteristics	
Production of pigment	
Fluorescent (diffusible)	+
Pyocyanine	−
Carotenoids	−
Levan formation from sucrose	−
Arginine dehydrolase	+
Oxidase reaction	+
Catalase reaction	+
O-F test	Oxidative
Denitrification	−
Hydrolysis of gelatin	−
Hydrolysis of poly-β-hydroxybutyrate	−
Hydrolysis of Tween 80	−
Utilization of carbon sources	
Glucose	+
Trehalose	−
2-Ketogluconate	+
meso-Inositol	−
Geraniol	−
L-Valine	+
β-Alanine	+
DL-Arginine	+
Benzylamine	+
Hippurate	+
L-Tryptophan	−
L-Kynurenine	−
Anthranilate	−
Galactose	−
Aminovalerate	+

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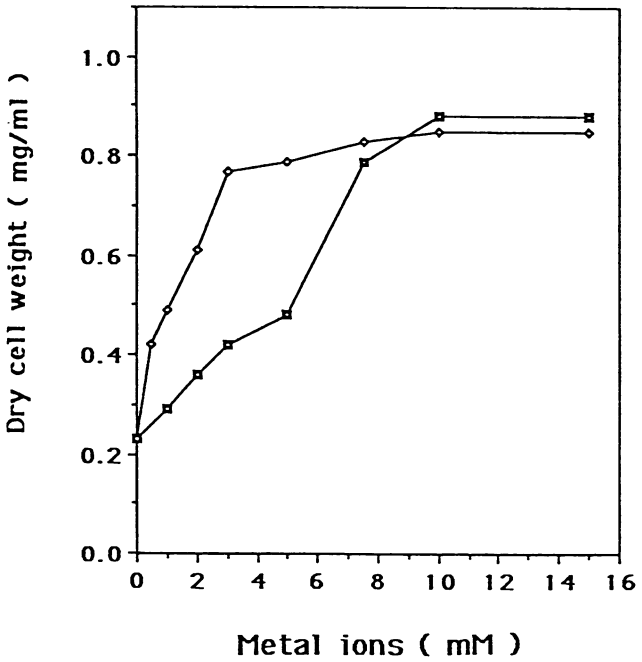

FIG. 1. Influence of metal ion concentration on growth. Growth was determined after incubation for 48 h at 30°C in LB medium containing $MgSO_4 \cdot 7H_2O$ or $Ca(NO_3)_2 \cdot 4H_2O$ and 30% (vol/vol) toluene. Cell dry weight was calculated on the basis of the predetermined correlation of 0.60 mg (dry weight)/ml as equivalent to 1 OD_{660} unit. Symbols: □, Mg^{2+} ions; ◇, Ca^{2+} ions.

of G+C content of DNA was done by the method of Tamaoka and Komagata (9).

Table 1 shows the morphological and biochemical characteristics of strain IH-2000. From these results, determined in accordance with standard methods (6), the isolate was identified as a strain of *Pseudomonas putida* biovar A by its ability to grow in the presence of toluene.

Each hydrocarbon tested, such as toluene, *p*-xylene, cyclohexane, hexane, and octane, was added to CS basal medium at a 0.5% (vol/vol) final concentration, and cultures were incubated at 30°C for 48 h. Strain IH-2000 could not grow in the CS medium containing hydrocarbons and could not utilize hydrocarbons as an energy source.

We tried to isolate a solvent-tolerant plasmid and TOL plasmid from strain IH-2000, but no plasmid was detected.

The influence of various metal ions on stabilization of toluene tolerance was investigated. Growth was determined after incubation for 48 h at 30°C in LB medium containing various metal ions (Cu^{2+}, Rb^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Ba^{2+}, Al^{3+}, Sn^{2+}, Pb^{2+}, V^{2+}, Mo^{7+}, W^{6+}, Mn^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+}) at 2 or 5 mM concentration and 30% toluene per culture. Of the ions tested, Mg^{2+} and Ca^{2+} were the most effective for stabilization of toluene tolerance. The cell yield was increased about twofold and threefold when Mg^{2+} and Ca^{2+}, respectively, were added to the LB medium containing toluene, compared with the control without metal ions. Other metal ions repressed growth of this strain at a concentration of 2 mM and inhibited growth at 5 mM.

Figure 1 shows the influence of different concentrations of Mg^{2+} and Ca^{2+} ions on growth of strain IH-2000. Growth increased in the presence of more than 2 mM Mg^{2+} and in the presence of more than 0.5 mM Ca^{2+}. The optimal concentration of Mg^{2+} was more than 7.5 mM, and that of Ca^{2+} was 3.0 mM. Addition of Ca^{2+} was more effective than Mg^{2+} for stability of solvent tolerance. For the different solvents, such as styrene, *p*-xylene, and cyclohexane, these metal ions were as effective for solvent tolerance as toluene. The growth of strain IH-2000 was repressed by addition of different solvents in medium without metal ions. These observations suggested that metal ions might be effective in improving solvent tolerance in living cells. It is very interesting that only Mg^{2+} and Ca^{2+} among the metal ions tested improved solvent tolerance.

To investigate the influence of different concentrations of toluene on growth of strain IH-2000, this strain was incubated in a test tube shaker for 24 h at 30°C in modified LB medium supplemented with toluene at concentrations ranging from 0.1 to 200% (vol/vol). The strain has a high tolerance for toluene and grew without significant evidence of inhibition over a wide range of toluene concentrations. The colonies which grew on nutrient agar plates overlaid with toluene remained viable for several weeks.

TABLE 2. Solvent tolerance of strain IH-2000

Inhibition of growth and solvents in group	Growth[a] at solvent concn (vol/vol):		
	50.0%	5.0%	2.5%
No inhibition			
Hydrocarbons (pentane, hexane, heptane, octane, isooctane, nonane, decane, dodecane, 2-pentane, 2-hexane, 1-octene, 1-dodecene, 1,3-pentadiene, 1,7-octadiene, cyclopentane, methylcyclopentane, cyclohexane, methylcyclohexane, butylcyclohexane, cyclooctane, styrene, toluene, *p*-xylene, ethylbenzene, propylbenzene, chlorobenzene, *o*-dichlorobenzene, bromobenzene)	+	+	+
Alcohols (heptanol, octylalcohol, decylalcohol)			
Ethers (butylether, hexylether, diphenylether, benzylether, methoxytoluene)			
Slight inhibition			
Alcohols (methanol)	−	± or +	+
Ethers (butylvinylether)			
Ketones (2-heptanone)			
Miscellaneous (acetal, dimethylformamide, dimethyl sulfoxide)			
Moderate inhibition			
Alcohols (ethanol)	−	−	± or +
Ketones (acetone, cyclohexanone)			
Inhibition			
Hydrocarbons (benzene, fluorobenzene, nitrobenzene)	−	−	−
Alcohols (propanol, butanol)			
Ethers (diethylether, propylene oxide)			
Ketones (methylethylketone, 2-pentanone, 2-hexanone)			
Miscellaneous (chloroform, ethylacetate, acetonitrile)			

[a] Symbols: +, growth (≥0.3 mg [dry weight]/ml); ±, slight growth; −, no growth.

The tolerance of strain IH-2000 was tested in liquid cultures containing 50, 5, and 2.5% of the appropriate solvent. This strain tolerated other toxic solvents in addition to toluene, including saturated and unsaturated aliphatic hydrocarbons, alicyclic hydrocarbons, aromatic hydrocarbons, alcohols, and ethers. Interestingly, this strain did not tolerate some solvents, such as benzene, fluorobenzene, nitrobenzene, propanol, butanol, diethylether, propylene oxide, chloroform, and ethylacetate. For ketones, this strain could not grow in media containing 50% solvent, but could grow at a concentration of 2.5% solvent, such as acetone and cyclohexanone. Table 2 summarizes the solvent tolerance properties of strain IH-2000. These observations indicated that solvent tolerance is a stable phenotypic property of strain IH-2000. We believe that certain unique cell surface properties of strain IH-2000 permit growth under such harsh conditions. It will be very interesting to compare the cell surface components of this strain with those of other strains.

This strain has considerable potential for application in bioreactors. It is possible that strain IH-2000 can be altered for use in bioreactors by genetic engineering, such as by transformation with plasmids which confer degradation or biotransformation properties. At present, we are investigating the mechanisms of solvent tolerance from two aspects, the cell surface components and the genetic basis.

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REFERENCES

1. **Claus, D., and N. Walker.** 1964. The decomposition of toluene by soil bacteria. J. Gen. Microbiol. **36:**107–122.
2. **Dobrogosz, W. J., and R. D. DeMoss.** 1963. Induction and repression of L-arabinose isomerase in *Pediococcus pentosaceus*. J. Bacteriol. **85:**1350–1365.
3. **Herzenberg, L. A.** 1959. Studies on the induction of β-galactosidase in a cryptic strain of *Escherichia coli*. Biochim. Biophys. Acta **31:**525–539.
4. **Inoue, A., and K. Horikoshi.** 1989. A *Pseudomonas* thrives in high concentration of toluene. Nature (London) **338:**264–266.
5. **Kitagawa, M.** 1956. Studies on the oxidation mechanism of methyl group. J. Biochem. **43:**553–564.
6. **Krieg, N. R., and J. G. Holt.** 1984. Gram-negative aerobic rods and cocci, p. 140. *In* R. G. E. Murray and D. J. Brenner (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
7. **Levinthal, C., E. R. Signer, and K. Fetherhof.** 1962. Reactivation and hybridization of reduced alkaline phosphatase. Proc. Natl. Acad. Sci. USA **48:**1230–1238.
8. **Raymond, R. L., V. W. Jamison, and J. O. Hudson.** 1967. Microbial hydrocarbon co-oxidation. I. Oxidation of mono- and dicyclic hydrocarbons by soil isolates of the genus *Nocardia*. Appl. Microbiol. **15:**857–865.
9. **Tamaoka, J., and K. Komagata.** 1984. Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol. Lett. **25:**125–128.
10. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and xylene by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the Tol plasmid. J. Bacteriol. **124:**7–15.