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Adaptation of *Pseudomonas putida* S12 to High Concentrations of Styrene and Other Organic Solvents

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***Pseudomonas putida* S12 could adapt to grow on styrene in a two-phase styrene-water system. Acetate was toxic for *P. putida* S12, but cells were similarly able to adapt to higher acetate concentrations. Only by using these acetate-adapted cells was growth observed in the presence of supersaturating concentrations of toxic nonmetabolizable solvents such as toluene.**

It has been established repeatedly that aromatic solvents with a log P_{OW} (partition coefficient between octanol and water) below 4.0, such as styrene, toluene, and tetralin, are toxic for microorganisms even at low concentrations (5, 15). These solvents can accumulate in the cytoplasmic membrane of bacteria, causing impairment of membrane functions and expansion of the cell membrane, resulting in leakage of cellular metabolic products (15). Nevertheless, two *Pseudomonas* species which grew on yeast-peptone-glucose medium in the presence of 50% toluene have been isolated (1, 10). Recently, it has been shown that *Pseudomonas putida* Idaho is not only resistant to *p*-xylene in a two-phase system but can even use *p*-xylene at these high concentrations as the carbon and energy source (4).

By using styrene as the sole carbon and energy source in subsaturating concentrations, we previously isolated 14 bacteria which were thought not to grow at styrene concentrations exceeding the solubility of styrene in water (7). Surprisingly, we found that one of the isolates (*Pseudomonas* strain S12) is able to adapt to higher concentrations of styrene, resulting in growth on styrene in a two-phase styrene-water system.

Identification. Strain S12 was previously identified as a *Pseudomonas* species (7). The organism was further identified by its growth and biochemical characteristics as a *P. putida* species (12). The identification was confirmed by fatty acid analysis of the strain with the microbial identification system of MIDI (Newark, Del.) (11).

Adaptation to supersaturating solvent concentrations. *P. putida* S12 was precultured at 30°C on phosphate-buffered (pH 7.0) mineral salts medium (6) with styrene as the carbon source. Growth was assessed by determining CO_2 evolution. Exponential growth (0.6 h^{-1}) was observed without an appreciable lag phase (Fig. 1) at an initial amount of 2 μl of styrene in the incubation system (25 ml of liquid medium in a total volume of 250 ml). This initial amount of styrene in the incubation system, on the basis of its partition coefficient, results in a concentration of 0.3 mM in the water phase, which is well below the water-saturating level of 1.5 mM (2). Growth of the organism was completely inhibited by raising the initial amount of styrene to supersaturating amounts (0.25 ml/25 ml). Surprisingly, however, the culture started to evolve CO_2 after about 20 h (Fig. 1). Subsequently, cells grown on this high concentration of styrene were used to inoculate fresh medium (25 ml) with either 2 μl or 0.25 ml of styrene. No significant lag time was observed for both conditions, and the growth rates (0.6 h^{-1}) on the basis of the CO_2 profiles were identical. Adapted cells could also be obtained by following the above procedure but by starting from a single colony of *P. putida* S12 grown on acetate agar plates.

P. putida S12 also grew on supersaturating concentrations of octanol or heptanol as the sole carbon and energy source. Use of these solvents at supersaturating amounts also resulted in lag times of approximately 20 h. This resistance to high concentrations of a solvent, which is also used as a carbon and energy source, is similar to the resistance to *p*-xylene observed in *P. putida* Idaho (4).

Growth of *P. putida* S12 adapted to 1% (vol/vol) styrene was studied in more detail by determining dry weight and viable counts as well as CO_2 evolution. Figure 2 shows a typical growth curve of *P. putida* S12 growing at 1% (vol/vol) styrene. After a short lag period, exponential growth was observed. In the stationary phase, a rapid decline in the viable cell count was observed.

Solvent tolerance. Whether a culture of the organism would develop tolerance to a solvent if it were growing on a nontoxic substrate in the presence of a nonmetabolizable solvent was also tested. Unadapted *P. putida* S12 cells were grown on several carbon sources, and 1% (vol/vol) toluene was added to the exponentially growing cultures (optical density at 660 nm, ≈0.4). The culture bottles (250 ml) containing 25 ml of medium were closed with Mininert valves (Phase Separations, Waddinxveen, The Netherlands) to prevent evaporation. Only cultures growing on either acetate or propionate eventually continued to grow in the presence of toluene. No growth in the presence of toluene was observed with either glucose, fructose, glycerol, ethanol, arginine, alanine, succinate, lactate, or pyruvate as the carbon source. In medium lacking solvents, *P. putida* S12 could grow on these carbon sources. With acetate as the carbon source, whether unadapted cells could grow in the presence of several other solvents was subsequently tested (Table 1). After a lag time, growth in the presence of solvents with a log P_{OW} of 2.3 or higher was observed. Similar resistance to solvents has been observed in *P. putida* IH-2000 and *Pseudomonas aeruginosa* ST-001 (1, 10).

Survival after the addition of toluene. The effect of a solvent on unadapted cells was further studied by exposing

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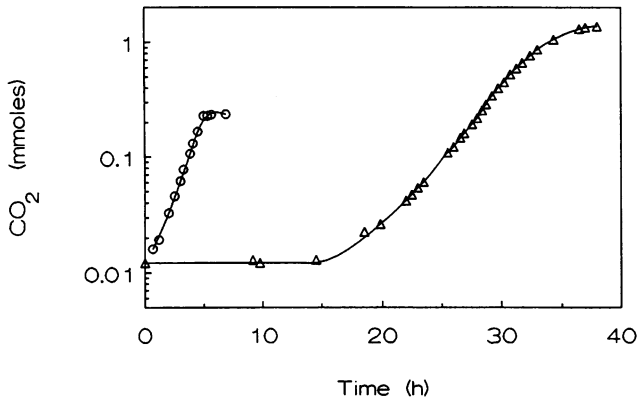

FIG. 1. Growth of *P. putida* S12 on styrene at two different concentrations. Cells were precultured with 0.008% (vol/vol) styrene, and CO_2 production was measured at low (○) and high (△) initial styrene concentrations of 0.008% (vol/vol) and 1% (vol/vol), respectively.

either acetate-grown or glucose-grown cells to toluene. Numbers of viable cells in exponentially growing cultures were determined just before toluene additions (1%, vol/vol) as well as 1 h after additions. Only 0.002% of the initial cells were still viable 1 h after the addition of 1% (vol/vol) toluene to cells growing on acetate (60 mM). Growth of the culture eventually resumed, as was confirmed by measuring CO_2 concentrations after 48 h. However, when glucose medium was used, no viable cells were detected 1 h after the addition of toluene and no growth within 48 h was observed. Whether cells originating from a culture grown on acetate in the presence of toluene would retain their resistance to toluene when grown in the absence of a solvent was subsequently tested. If such cells were grown on acetate for about 10 generations in the absence of toluene, about 10% of the cells survived the addition of toluene. However, when cells from the solvent-adapted acetate culture were transferred to and grown in glucose medium for 10 generations, no survivors were observed 1 h after the addition of toluene.

Toxicity of acetate. The response of *P. putida* S12 to the presence of a toxic solvent is quite remarkable. Cells either precultured at subsaturating concentrations of the toxic styrene or at elevated acetate concentrations were able to acquire resistance to the solvent. The undissociated acids of acetate and propionate are toxic for microorganisms and are generally used as food preservatives (3). Therefore, whether acetate is also toxic for *P. putida* S12 was tested. Growth of glucose-precultured cells at different concentrations of acetate was measured, and it was observed that growth of the cells was inhibited at acetate concentrations higher than 40 mM (Fig. 3). When cells had reached the stationary phase and were transferred to fresh medium with the same respective acetate concentration, no growth inhibition was observed. When toluene (1%, vol/vol) was added to such cultures in the beginning of their exponential growth phase, growth after 48 h was only observed at initial acetate concentrations of 40 mM or higher.

Sheu and Freese (14) have reported quite similar inhibition profiles for *Bacillus subtilis*. This organism grew at half its maximum growth velocity at 80 mM acetate (pH 6.5),

TABLE 1. Growth of *P. putida* S12 on solvents as sole carbon source, or in presence of solvent with acetate as carbon source

Solvent	Log P_{OW}[a]	Growth[b] on:	
		Acetate in presence of solvent (1%, vol/vol)	Solvent (1%, vol/vol)
Decane	5.6	+	−
Propylbenzene	3.6	+	−
Hexane	3.5	+	−
Cyclohexane	3.2	+	−
Ethylbenzene	3.1	+	−
p-Xylene	3.0	+	−
Styrene	3.0	+	+
Octanol	2.9	+	+
Toluene	2.5	+	−
Heptanol	2.4	+	+
Dimethylphthalate	2.3	+/−	−
Fluorobenzene	2.2	−	−
Benzene	2.0	−	−

[a] Log P_{OW} values were calculated according to the method of Rekker and de Kort (13).

[b] Symbols: +, growth with >0.5 mmol of CO_2 produced after 48 h; +/−, growth with >0.5 mmol of CO_2 produced after 120 h; −, no growth (<0.1 mmol of CO_2 produced after 120 h).

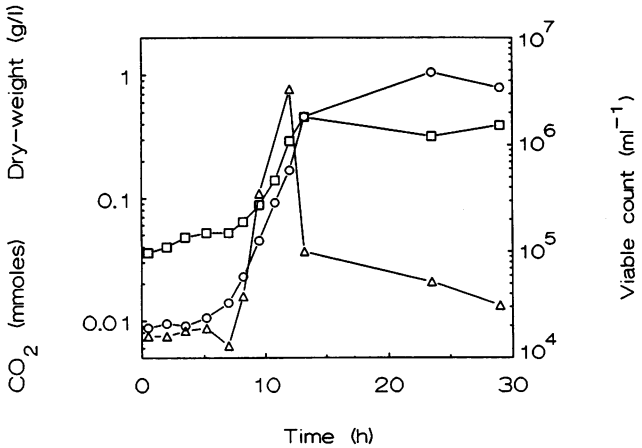

FIG. 2. Growth of *P. putida* S12 on 1% (vol/vol) styrene in 25 ml of mineral salts medium. ○, total amount of CO_2 produced; □, dry weight; △, viable count (cells per milliliter).

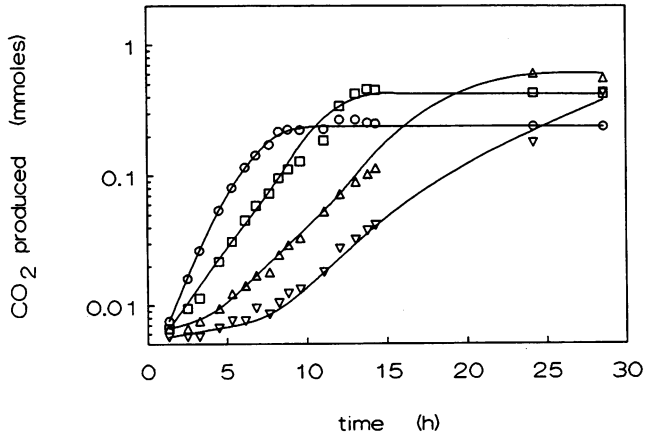

FIG. 3. Growth of *P. putida* S12 at various acetate concentrations at pH 7.0. ○, 20 mM; □, 40 mM; △, 60 mM; ▽, 80 mM.

whereas for *P. putida* S12, this point was reached at 60 mM acetate (pH 7.0). At 60 mM acetate (pH 7.0) and 80 mM acetate (pH 6.5), the concentrations of the undissociated acetic acids are 0.3 and 1.4 mM, respectively. The results of Sheu and Freese (14) suggest that microorganisms grown in the presence of short-chain fatty acids have a lower membrane fluidity. Due to this reduced membrane fluidity of acetate-grown cells, a small number of *P. putida* S12 cells apparently can survive and adapt to toluene. A similar situation is expected for cells growing on subsaturating concentrations of styrene, whereas glucose-grown cells, possessing a normal membrane fluidity, are all killed by the addition of toluene. Several studies have demonstrated that bacteria can change their lipid composition when grown in the presence of organic solvents (8, 9). Currently, we are studying whether such changes are important in the adaptation of *P. putida* S12 to high concentrations of styrene as the growth substrate or to other solvents when growing on acetate.

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