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Active Efflux of Toluene in a Solvent-Resistant Bacterium

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We investigated the mechanisms behind the organic-solvent resistance of the solvent-tolerant strain *Pseudomonas putida* S12. By use of ^{14}C-labeled toluene, we obtained evidence that an energy-dependent export system may be responsible for this resistance to toluene.

Solvent-resistant bacteria. Toluene and many other organic solvents kill microorganisms because they accumulate in and disrupt cell membranes (13). However, Inoue and Horikoshi (7) isolated a toluene-resistant *Pseudomonas putida* strain which grew in a two-phase toluene-water system. This surprising observation has been confirmed by others (1, 12, 15), and a search has started to uncover the mechanisms behind this remarkable solvent tolerance. One approach focused on physicochemical changes in the membrane, and it was found that rigid *trans*-unsaturated fatty acids are incorporated in membranes of solvent-resistant cells (6, 13). However, this incorporation of *trans*-unsaturated fatty acids most likely is not the only response to toxic solvents as was also demonstrated by Pinkart et al. (11). Recently, mutants of *Escherichia coli* showing resistance to some organic solvents were described (2, 9). Despite these efforts, no complete picture is available of the physiological basis of the adaptation of some organisms to toxic organic solvents.

Solvent-resistant strain *P. putida* S12. Strain S12 was isolated on toxic styrene, and it grew in the presence of a second phase of this and other organic solvents (5, 15). Other solvents, such as toluene, were not utilized as carbon sources but were tolerated by the bacterium as a second phase when growing on compounds like glucose (14). Solvents are toxic to bacteria because they accumulate in bacterial cytoplasmic membranes, where they have considerable and, with solvents like toluene, devastating effects on the structural and functional properties of these membranes (13). Therefore, studies with strain S12 so far have focused on physicochemical changes at the level of membranes in order to explain the ability of the solvent-resistant bacteria to survive in such a hostile environment. *P. putida* S12 was indeed one of the first solvent-resistant strains for which the exceptional ability to increase the rigidity of its membrane by isomerizing *cis*- into *trans*-unsaturated fatty acids was reported (14).

Alternative mechanisms in coping with solvent stress. Physicochemical changes may be important in coping with solvent stress, but they are defensive in the sense that the organism has to make the best of a situation with high concentrations of solvents in its membrane. The reduction of the influx of solvents into the membrane by the creation of a low-permeability barrier, maybe at the lipopolysaccharide level, will also have its limitation because of the intrinsic properties of the lipophilic solvents and their tendency to accumulate in the lipid bilayer. A more assertive approach of the cells would be to prevent or at least reduce accumulation of solvents in the membrane. A conceivable method to reduce the actual concentration of a solvent in the membrane in a dynamic process would be actively transporting the solvent molecules out of the lipid bilayer into the aqueous phase. To date, such active efflux systems for small and uncharged hydrophilic molecules have not yet been described.

We now have investigated if an active solvent-efflux system is present in the solvent-resistant bacterium *P. putida* S12. The export of [^{14}C]toluene was studied because toluene is not metabolized or transformed by this strain.

Cultivation of the organism. *P. putida* S12 was grown in a

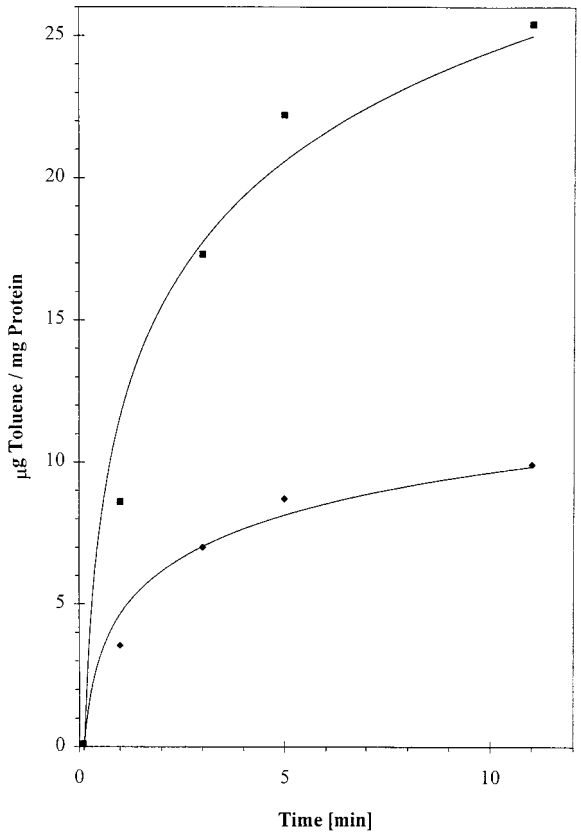

FIG. 1. Accumulation of [^{14}C]toluene in *P. putida* S12 organisms that have either adapted (◆) or not adapted (■) to toluene.

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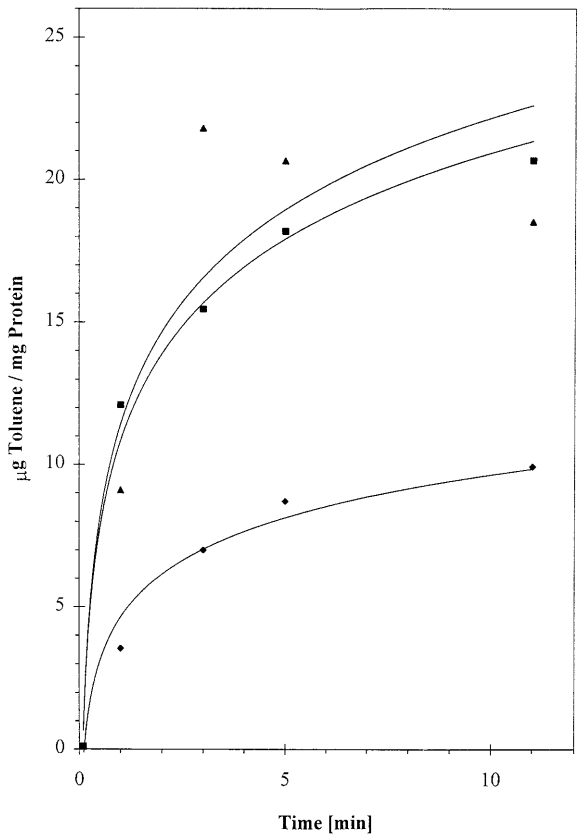

FIG. 2. Effects of the presence of energy-coupling inhibitors on the accumulation of [^{14}C]toluene in toluene-adapted cells of *P. putida* S12. Cells not inhibited (◆) were compared with cells to which the respiratory chain inhibitor potassium cyanide at 20 mM (▲) or the proton conductor carbonyl cyanide *m*-chlorophenylhydrazone at 0.25 mM (■) was added via the washing buffer prior to the addition of [^{14}C]toluene.

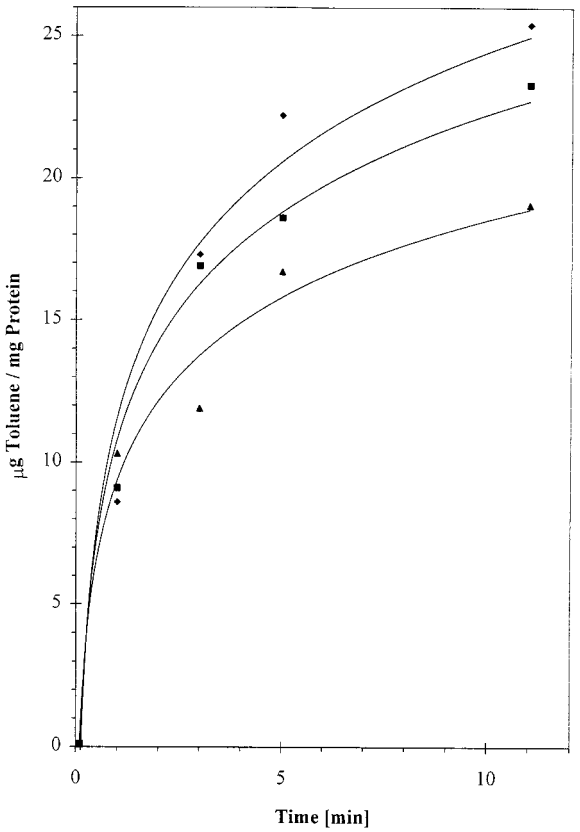

FIG. 3. Effects of the presence of energy-coupling inhibitors on the accumulation of [^{14}C]toluene in nonadapted cells of *P. putida* S12. Cells not inhibited (◆) were compared with cells to which the respiratory chain inhibitor potassium cyanide at 20 mM (▲) or the proton conductor carbonyl cyanide *m*-chlorophenylhydrazone at 0.25 mM (■) was added via the washing buffer prior to the addition of [^{14}C]toluene.

chemostat on glucose (30 mM) in a mineral medium (4) as described before (14) and either in the presence or in the absence of 510 mg of toluene liter^{-1}. In this way, adapted and nonadapted cells having otherwise similar characteristics were obtained. Cells harvested at steady state were washed in 50 mM phosphate buffer (pH 7.0) containing glucose (30 mM) to energize the cells, resuspended in the same buffer to a concentration of approximately 4 g of protein liter^{-1}, and stored on ice until used.

Accumulation assays. Assays were conducted in an Eppendorf Thermo-mixer at 30°C and 600 rpm in 1-ml incubation mixtures containing 0.1 ml of the cell suspension, 0.15 ml of washing buffer containing glucose to give a final concentration of 15 mM, and 0.75 ml of 500 mg of [^{14}C]toluene liter^{-1} in the washing buffer (uniformly ring-labeled toluene was obtained from American Radiolabeled Chemicals Inc., St. Louis, Mo., with a specific activity of 55 mCi mmol^{-1} and it was diluted with cold toluene to approximately 40,000 cpm sample^{-1}). Closed vials were used to limit evaporation. The given values represent the average of triplicate measurements and varied from the mean by not more than 15%. Every experiment was reproduced independently three times. Cells and buffer were preincubated for 2 min at assay conditions before starting the experiment by adding the [^{14}C]toluene solution. At time intervals of 0, 2, 4, and 10 min, cells were spun down for 1 min with a MicroCen 13 table centrifuge at room temperature. In independent experiments it was assessed that ample oxygen supply to the cells was warranted during the whole procedure. The supernatant fraction was carefully separated, and the pellet was washed in 0.75 ml of nonlabelled 500-mg liter^{-1} toluene in wash buffer and spun down again for 1 min. The supernatant obtained was again removed. The radioactivity retained in the two supernatants and in the washed pellet was measured in a liquid scintillation counter (model 1600TR; Packard Instruments Co., Downers Grove, Ill.). The percentage of the total radioactivity recovered in the pellet was used to calculate the concentration of toluene in the cell per milligram of protein. The protein content of whole cells was measured by following the method of Biuret as described by Mokrasch and McGilvery (8), with bovine serum albumin (Sigma, St. Louis, Mo.) as the standard.

Accumulation of toluene in cells. Cells grown both in the presence and in the absence of toluene were used to determine the accumulation of toluene over time (Fig. 1). The amount of toluene accumulating in the adapted bacteria was twice as low as in the nonadapted bacteria. We then determined if this observation was due to the existence of an energy-dependent transport system for toluene. The influx of [^{14}C]toluene was measured in the absence and presence of either the respiratory chain inhibitor potassium cyanide or the proton conductor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). These two inhibitors are structurally not related. Thus, the production of any artifact by aspecific interactions by a particular chemical structure is minimized.

In adapted cells the presence of either inhibitor resulted in

significantly higher amounts of toluene accumulating in *P. putida* S12 (Fig. 2). In experiments with cells not adapted to toluene, no effect of the inhibitors on the amount of toluene accumulating in cells was seen (Fig. 3).

Conclusions from the experiments. The results as presented in Fig. 1 to 3 cannot be explained by the degradation of toluene by the bacterium. *P. putida* S12 does not grow on toluene. Moreover, we have not been able to demonstrate any transformation of toluene by incubating cell suspensions in the presence of various concentrations of toluene (detection limit, 1.0 nmol of toluene degraded min^{-1} mg of $protein^{-1}$). To explain the results recorded during the first minute in Fig. 1 and 2, transformation reactions should result in a turnover of approximately 50 nmol of toluene min^{-1} mg of $protein^{-1}$.

However, active efflux of toluene from the cell can explain the results observed. Our results will need confirmation by in vitro work to unequivocally assess the existence of an active efflux system in solvent-resistant bacteria. Furthermore, our whole-cell experiments do not allow us to speculate on the nature of the efflux system. Possibly, the toluene-exporting system has features in common with systems studied in prokaryotes and eukaryotes for lipophilic cytotoxic agents, such as anticancer drugs and antibiotics (3, 10). These compounds are, however, charged amphiphilic molecules, while toluene is a small uncharged hydrophobic compound.

A toluene-exporting system in combination with physiochemical adaptations of the membranes of *P. putida* strains offers an attractive explanation for the mechanisms of solvent resistance in these organisms.

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