Communication

Mechanisms for Solvent Tolerance in Bacteria*

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The development of tolerance in Pseudomonas putida DOT-T1 to toluene and related highly toxic compounds involves short- and long-term responses. The short-term response is based on an increase in the rigidity of the cell membrane by rapid transformation of the fatty acid cis-9,10-methylene hexadecanoic acid (C17:cyclopropane) to unsaturated 9-cis-hexadecenoic acid (C16:1,9 cis) and subsequent transformation to the trans isomer. The long-term response involves in addition to the changes in fatty acids, alterations in the level of the phospholipid polar head groups: cardiolipin increases and phosphatidylyethanolamine decreases. The two alterations lead to increased cell membrane rigidity and should be regarded as physical mechanisms that prevent solvent penetrance. Biochemical mechanisms that decrease the concentration of toluene in the cell membrane also take place and involve: (i) a solvent exclusion system and (ii) metabolic removal of toluene via oxidation. Mutants unable to carry out cis → trans isomerisation of unsaturated lipids, that exhibit altered cell envelopes because of the lack of the OprL protein, or that are unable to exclude toluene from cell membranes are hypersensitive to toluene.

Organic solvents with a \( \log P_{ow} \) value (logarithm of the partition coefficient of the target compound in a mixture of octanol/water) between 1.5 and 3 are extremely toxic to microorganisms, a characteristic that has been well documented for toluene (\( \log P_{ow} 2.5 \)) (1–4). De Smet et al. (2) demonstrated that toluene destabilizes the inner membrane of Gram-negative bacteria, causing a transition from a lamellar bilayer state to a hexagonal state, which results in the leakage of proteins, lipids, and ions and disruption of the cell membrane potential (1, 2). The consequent collapse of ATP synthesis together with 50% (\( \nu/\nu \)) toluene, despite the fact that this microorganism was not able to use this aromatic as a carbon source. This report was followed by three independent studies that described the isolation of three different \( P. \) putida strains that tolerated related organic solvents, e.g. styrene (6), xylene (7), and toluene (8). The toluene-tolerant isolate, called \( P. \) putida DOT-T1, metabolized toluene via the \( p \)-cresol pathway (8). The “unexpected” ability of these Pseudomonas strains to tolerate toxic solvents opens new avenues of research into cellular metabolism. In this study, we have explored the molecular basis for solvent tolerance by \( P. \) putida DOT-T1.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—\( P. \) putida DOT-T1 is a solvent-tolerant strain (8), whereas \( P. \) putida mt-2 is a toluene-sensitive strain (9).

Isolation of Toluene-sensitive Tn5 Mutants of \( P. \) putida DOT T1—About 2000 Tn5 transconjugants of \( P. \) putida DOT-T1 were obtained after mating this strain with \( E. \) coli (pG9S). The suicide plasmid pG9S bears Tn5, and mutagenesis was carried out as described before (10). Each individual Km\( ^{R} \) transconjugant was tested for its ability to grow on LB medium with 1% (\( \nu/\nu \)) toluene. Two clones that repeatedly failed to grow in the latter medium were found and called \( P. \) putida DOT-T1P4 and DOT-T1P94, respectively.

Analysis of Phospholipids—Phospholipids were extracted according to Bligh and Dyer (11). To measure fatty acids, phospholipids were saponified and esterified as described by Morrison and Smith (12), and, after gas chromatographic (GC)\(^{1} \) separation, the fatty acids were identified by mass spectrometry (MS). Monounsaturation position and geometry were chemically determined by using dimethyl disulfide derivatization and GC-MS (13).

To determine the nature of the head groups, cells were grown in the presence of 40 \( \mu \)Ci of \( [^{2}P] \)orthophosphate, and, after separation of the phospholipids as described by Ames (14), the radioactive spots were removed from the TLC plates and counted in a Packard Radiochemical counter.

Incorporation of \( [^{14}C] \)Trichlorobenzene into Cell Membranes—Exponentially growing cells were harvested by centrifugation, washed in LB, and suspended in 2.5 ml of LB to a cell density of about 150–200 cell protein/ml. Then the cells were incubated for 10 min at 30 °C and exposed to 5 \( \mu \)Ci of \( 1,2,4-[^{14}C] \)trichlorobenzene. After 10 min, 250 \( \mu \)l of the cell suspension was filtered through a 0.45-\( \mu \)m Millipore filter and washed with 2 ml of LB medium. The filters were dried, and the \( ^{14}C \) associated with the cell pellet (disintegrations/min) was determined in a Packard Radiochemical detector.

RESULTS AND DISCUSSION

Physical Barriers to Organic Solvents: Short- and Long-term Responses in Phospholipid Composition—We determined the fatty acid composition of phospholipids of \( P. \) putida DOT-T1 by GC-MS (11, 12). Table I shows the typical fatty acid content of phospholipids in \( P. \) putida DOT-T1 growing exponentially in LB medium in the absence of toluene (doubling time 60 min). The most abundant fatty acid was hexadecanoic acid (C16:0), representing up to about 45% of the total fatty acids, followed by cis-9,10-methylene hexadecanoic acid (C17:cyclopropane), which represented up to 30% of the total fatty acid content (Table I). The levels of these fatty acids and of those in Table I were relatively stable along the growth curve when this strain was grown in LB medium. To quantify the level of the polar head groups of phospholipids, bacteria were grown in LB medium in the absence of toluene (doubling time 60 min). This paper is available online at http://www-jbc.stanford.edu/jbc/
Armored Bacteria versus Organic Solvents

TABLE I

Phospholipid composition of P. putida strain DOT-T1 growing in the presence of organic solvents

Bacteria were grown in LB medium plus the indicated organic solvent at 1% (v/v) until the late exponential growth phase. ND, not determined.

| Solvent (log\(P_{o/w}\)) | None | Heptane (4.5) | n-Propylbenzene (3.6) | p-Xylene (3.2) | l-Octanol (2.9) | Toluene (2.5) |
|---------------------------|------|---------------|----------------------|---------------|----------------|---------------|
| **Fatty acids**            |      |               |                      |               |                |               |
| C14:0                     | 1    | 2             |                      |               |                |               |
| C16:1,9 cis               | 3    | 2             |                      |               |                |               |
| C16:1,9 trans             | 46   | 44            |                      |               |                |               |
| C17:cyclopropane          | 31   | 22            |                      |               |                |               |
| C18:2                     | 1    | 1             |                      |               |                |               |
| C18:1,9 cis ol            | 4    | 9             |                      |               |                |               |
| C18:1,11 cis vac          | 8    | 6             |                      |               |                |               |
| C18:1,11 trans vac        | 1    | 3             |                      |               |                |               |
| C18:0                     | 4    | 4             |                      |               |                |               |
| cis/trans                  | 7.5  | 1.9           |                      |               |                |               |
| Head groups               |      |               |                      |               |                |               |
| PE                        | 78   | 75            | 66                   | 10            | 3              | 63            |
| PG                        | 10   | 12            | 10                   | ND            | ND             | 12            |
| CL                        | 12   | 13            | 18                   | ND            | ND             | 25            |
| PE/PG + CL                | 3.5  | 3             | 2.4                  |               |                | 1.7           |

![Fig. 1. Short-term response of fatty acids in P. putida cells in the presence of 0.3% (v/v) toluene.](image)

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physically as described by Ames (14). It was found that in cells growing in the absence of solvents, about 75% of the polar head groups were in the form of PE, and the remaining head groups were distributed equally between PG and CL (Table I).

Cells grown in LB medium are differentially sensitive to the addition of toluene: 1% (v/v) toluene killed 99% of the cells, whereas the addition of 0.3% (v/v) toluene resulted in survival of more than 99% of the cells. It should be noted that 0.3% (v/v) toluene killed more than 99,9999% of the closely related solvent-sensitive strain P. putida mt-2. We therefore studied the short-term response of P. putida DOT-T1 to the addition of 0.3% (v/v) toluene by comparing phospholipid levels in cells before and after the addition of toluene. Cells responded to toluene by changing the amount of unsaturated C16:1 fatty acids and C17:cyclopropane (see Fig. 1). Immediately after the addition of the aromatic hydrocarbon, the level of C17:cyclopropane became negligible, with a concomitant increase in C16:1,9 cis, followed later by a decrease in this unsaturated form and an increase in the level of the trans isomer. Therefore the organism’s initial response to the presence of an organic solvent was to transform C17:cyclopropane into cis-hexadecenoic acid (C16:1,9 cis), which was immediately isomerized to the trans form. The transition from cis to trans in unsaturated hexadecenoic acid leads to increased membrane rigidity (15–17). Therefore this early response seems to be aimed at decreasing membrane fluidity, a reaction which also has been observed in other microbes exposed to toxic compounds (17–21).

For long-term studies, phospholipids were prepared from cells grown in the absence and in the presence of 1% (v/v) toluene, and \(^1\)H NMR and \(^31\)P NMR analyses were done. No C17:cyclopropane was detected in cells growing on toluene, and the cis:trans ratio was of about 1 in cells growing in the presence of toluene and 7.5% in cells growing in the absence of the aromatic hydrocarbon. Furthermore, alterations were observed in signals from protons located on carbon in C-O bonds, and \(^31\)P NMR analysis showed a shift in the intensity of different signals, suggesting changes in the amount of the different head groups. We then analyzed the phospholipid fatty acids from cells grown in the presence of 1% (v/v) toluene by GC-MS. In addition to the changes in C17:cyclopropane and C16:1 in the short-term assays, we found that the levels of cis-vaccenic acid (cis-octadec-11-enoic acid; C18:1,11 cis) and trans-vaccenic acid also increased with respect to the levels found in cells growing in the absence of solvent (Table I). In contrast, the concentration of cis-oleic acid (cis-octadec-9-enoic acid, C18:1,9 cis) remained unaltered. There was also a slight increase in the level of saturated octadecanoic acid (C18:0) (Table I).

In cells growing in the presence of toluene, the level of PG (12% of the total) did not change significantly, whereas the level of CL increased to as much as 22% of the total, and the level of PE decreased to about 65%. Head group composition is altered because after the cells are exposed to toluene more than 90% of the \(^31\)P incorporated into phospholipid head groups was devoted to CL synthesis, so that in the equilibrium the overall composition was modified. Similar changes in phospholipid composition have been observed in E. coli exposed to ethanol (20) or increased temperatures (21) and are known to result in increased membrane rigidity (15–17). These results support the hypothesis that, in the long-term, P. putida DOT-T1 responds to toluene by decreasing its membrane fluidity by altering both the phospholipid head groups and the amount of trans-fatty acids in phospholipids.

We also tested whether other organic solvents (heptane, log\(P_{o/w}\) 4.5; n-propylbenzene, log\(P_{o/w}\) 3.6; p-xylene, log\(P_{o/w}\) 3.2; and 1-octanol, log\(P_{o/w}\) 2.9) were able to induce responses similar to those mediated by toluene. Heptane had virtually no short-term effects, and n-propylbenzene led to moderate
about 1 in the presence of a solvent with a log varying from 7.5 for cells growing in the absence of solvent to the fatty acid composition of phospholipids changed with the short-term response. As expected, in long-term experiments polarity of the solvent, the stronger and more significant the that described above for toluene. Therefore, the higher the

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P.putida DOT-T1P4isaTn

Isomers—P.putida medium by altering the fluidity of the cell membrane.

culture medium (22). We concluded that in response to increases in the level of dissolved toluene in the
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C18:1,11 cis vac 16 26
C18:1,11 trans vac 0 0
C18:0 2 8

changes (the level of C17:cyclopropane decreased to 5%, and C16:1.9 cis plus 9 trans increased to 10% of the total (not shown)). In short-term assays, the addition of p-xylene or 1-oc-
tanol to cells growing on LB resulted in a response similar to that described above for toluene. Therefore, the higher the polarity of the solvent, the stronger and more significant the short-term response. As expected, in long-term experiments the fatty acid composition of phospholipids changed with the polarity of the solvent. In general, the lower the logPoW, the lower the content of C17:cyclopropane and the higher the concentrations of unsaturated fatty acids, with cis:trans ratios varying from 7.5 for cells growing in the absence of solvent to about 1 in the presence of a solvent with a logPoW of 3.6 or lower (Table I). In the long term, the level of PE decreased with the logPoW of the solvent, whereas the level of CL increased (Table I). In P. putida strain S12, the level of CL also increased in response to increases in the level of dissolved toluene in the culture medium (22). We concluded that P. putida DOT-T1 responds to the presence of solvents of different polarity in the medium by altering the fluidity of the cell membrane.

Toluene Sensitivity in a Mutant Unable to Produce trans Isomers—P. putida DOT-T1P4 is a Tn3 mutant of DOT-T1 that was isolated as a toluene-sensitive strain unable to grow on toluene supplied in the liquid phase at concentrations of 0.1% (v/v). However, the strain did grow in the presence of 1% (v/v) heptane. This strain was devoid of the trans isomers of the unsaturated C16:1 and C18:1 vaccenic fatty acids. These findings provide the first genetic evidence of the importance of cis → trans isomerization in the mechanism of tolerance to organic solvents (Table II).

Exclusion of Toluene from Cell Membranes—P. putida strain mt-2, which is not tolerant to toluene supplied in the liquid phase, is able to grow on toluene as the sole carbon source when this aromatic hydrocarbon is supplied via the vapor phase. We determined the fatty acid content of this strain when it grew in LB and LB plus toluene supplied via the vapor phase. In LB, the fatty acid content was very similar to that of DOT-T1 growing in the same medium (40% of the total fatty acid content was C16:0, and 27% was C17:cyclopropane). Upon transfer to a medium with toluene supplied via the vapor phase, the toluene-sensitive strain P. putida mt-2 was able to convert C17:cyclopropane into C16:1.9 cis, which it then isomerized to the trans isomer. This suggests that in addition to cis → trans isomerization, other mechanisms probably operate in response to the presence of toluene in the medium. This mechanism in P. putida DOT-T1 might involve the expenditure of energy, as we consistently observed: 1) that the yield of cultures of P. putida DOT-T1 grown on LB plus 1% (v/v) toluene was about 30–50% of that reached in LB medium alone, and 2) the higher the

 polarity of the solvent, the lower the yield of the culture.

A plausible hypothesis is that the naturally solvent-tolerant strain P. putida DOT-T1, in contrast with naturally solvent-sensitive strains, uses an energy-dependent solvent exclusion system that keeps toluene within a physiological range of concentrations in the membranes, as occurs in other microbes exposed to hydrophobic toxic compounds (23). To test this hypothesis, we measured the incorporation of the non-metabolizable toluene analog 1,2,4-[14C]trichlorobenzene in cell membranes of bacteria growing exponentially on LB and LB plus 1% (v/v) toluene. We found that after 10 min of incubation the amount of 1,2,4-[14C]trichlorobenzene incorporated in cells grown in the absence of the organic solvents was about 5-fold higher than in cells grown in the presence of the solvent (Table III). Similar assays were done with cells treated with 100 μM concentration of the uncoupler CCCP. In this case the amount of 14C incorporated in the cell membranes was similar regardless of the source of the bacteria. These results support the hypothesis that cells growing in the presence of a given organic solvent could use an energy-dependent exclusion system that may decrease the level of the solvent in the membranes.

P. putida DOT-T1P34, another toluene-sensitive derivative of solvent-tolerant strain DOT-T1, did not grow when toluene was supplied in the culture medium at 0.3% (v/v), but did grow, although slowly, when toluene was provided via the vapor phase (doubling time 5 h). In contrast with DOT-T1P4, this strain was able to carry out the conversions C17:cyclopropane → C16:1 cis → C16:1 trans when cells were exposed to toluene vapor. When we analyzed the incorporation of 1,2,4-[14C]trichlorobenzene as described above, we found that the level in cell membranes of bacteria grown on LB or LB plus toluene in the vapor phase was about 30- to 50-fold higher than in the wild-type cells grown under similar conditions (Table III). We therefore concluded that DOT-T1P34 is solvent-sensitive because of its inability to decrease the level of solvent in the membrane. This result supports the hypothesis that an exclusion mechanism for toluene is in operation.

**Table II**

| Fatty acid composition of phospholipids of P. putida strain DOT-T1P4 grown on LB medium with and without heptane |
|---|---|
| Fatty acid | Growth conditions |
| | LB | LB plus heptane |
| C14:0 | 2 | 1 |
| C16:1.9 cis | 5 | 10 |
| C16:1.9 trans | 0 | 0 |
| C16:9 | 44 | 46 |
| C17:cyclopropane | 27 | 4 |
| C18:2 | 1 | 1 |
| C18:1.9 cis ol | 1 | 3 |
| C18:1.11 cis vac | 16 | 26 |
| C18:1.11 trans vac | 0 | 0 |
| C18:0 | 2 | 8 |

**Table III**

| Incorporation of 1,2,4-[14C]trichlorobenzene into membranes of P. putida cells growing in the absence and in the presence of organic solvents |
|---|---|---|
| Culture conditions | Wild-type | DOT-T1P34 | DOT-OPRL |
| LB | 19,942 | 280,120 | 488,430 |
| LB plus toluene | 4,140 | 527,700 | 106,060 |

**OprL Mutants of P. putida DOT-T1 Are Toluene-sensitive**—Finally, short- and long-term responses require integrity of the cell envelope. We recently identified OprL (outer membrane protein L) as a protein required for the maintenance of cell envelope integrity in P. putida (24). We have generated in vitro oprL mutants (24) that have been used to create null OprL mutants of P. putida DOT-T1 by reverse genetics. These mutants are hypersensitive to toluene and are killed by concentrations as low as 0.1% (v/v). When exposed to toluene supplied via the vapor phase, the OprL mutant was able to carry out cis → trans isomerization of unsaturated lipids. However, its ability to reduce the level of aromatics in the membranes was curtailed: aromatics accumulated to 25- to 50-fold higher levels in comparison with the levels found in the membranes of the wild-type solvent-tolerant strain (Table III). This suggests that integrity of the cell surface structures is essential for the organic solvent exclusion system to function.
Metabolic Removal of Toluene Also Improves Toluene Tolerance—We found that 100% of *P. putida* DOT-T1 cells growing on LB plus 1% (v/v) toluene survived when transferred to LB medium without an organic solvent, or to LB with either 1% (v/v) 1-octanol or 1% (v/v) toluene. *P. putida* DOT-T1 cells growing on 1% (v/v) 1-octanol (log*P* _{OW} 2.9) do not express the toluene degradation pathway but do exhibit adaptive traits such as a high level of trans unsaturated fatty acids and are enriched in PE in phospholipid head groups (see Table I). When cells growing on 1% (v/v) 1-octanol were transferred to 1% (v/v) toluene (log*P* _{OW} 2.5), about 85% loss of viability resulted. This suggests that in addition to the organic solvent exclusion system described above, the removal of toluene via a metabolic pathway may also play a role in increasing survival in the presence of the organic solvent.

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