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REVIEW

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Bacteria tolerant to organic solvents

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Abstract The toxic effects that organic solvents have on whole cells is an important drawback in the application of these solvents in environmental biotechnology and in the production of fine chemicals by whole-cell biotransformations. Hydrophobic organic solvents, such as toluene, are toxic for living organisms because they accumulate in and disrupt cell membranes. The toxicity of a compound correlates with the logarithm of its partition coefficient with octanol and water (log P_{ow}). Substances with a log P_{ow} value between 1 and 5 are, in general, toxic for whole cells. However, in recent years different bacterial strains have been isolated and characterized that can adapt to the presence of organic solvents. These strains grow in the presence of a second phase of solvents previously believed to be lethal. Different mechanisms contributing to the solvent tolerance of these strains have been found. Alterations in the composition of the cytoplasmic and outer membrane have been described. These adaptations suppress the effects of the solvents on the membrane stability or limit the rate of diffusion into the membrane. Furthermore, changes in the rate of the biosynthesis of the phospholipids were reported to accelerate repair processes. In addition to these adaptation mechanisms compensating the toxic effect of the organic solvents, mechanisms do exist that actively decrease the amount of the toxic solvent in the cells. An efflux system actively decreasing the amount of solvents in the cell has been described recently. We review here the current knowledge about exceptional strains that can grow in the presence of toxic solvents and the mechanisms responsible for their survival.

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Introduction

Extremophiles are adapted to live under conditions of extreme temperature, pH, salinity, or pressure. Some organic solvents, as pollutants originating from human activities, also create extreme environmental conditions. However, some solvents have already been present in the environment for a long time as a result of natural biosynthesis (Jüttner and Henatsch 1986; Nonino 1997). The naturally produced solvents are present in low concentrations. They can be mineralized by microbial activities, and many of the metabolic pathways involved have been elucidated for various organisms and for a great number of compounds (Dagley 1986; Gibson and Subramanian 1984; Smith 1990, 1994).

One of the major problems encountered in the application of these microbial mineralization processes in wastewater and waste gas treatment or in bioremediation is the low stability of the desired activity as the result of inactivation of the cells, which is caused by the toxic effects that have been described for several pollutants (Shirai 1987; Jenkins et al. 1987; Sikkema and de Bont 1991; Barr and Aust 1994).

Moreover, currently there is an interest in the performance of biotransformations using or aided by organic solvents (Nikolova and Ward 1993; Salter and Kell 1995). Organic solvents are already used widely in the application of biotransformations with enzymes (Carrea et al. 1995). The use of organic solvents has also several advantages in the application of whole-cell systems. The solvents can increase the concentration of poorly water-soluble substrates or products. Using a second phase of an organic solvent, products can be extracted continuously from the aqueous reaction system. This process enables not only the reduction of inhibitory effects caused by the product but also a much easier recovery with positive effects on the costs for the downstream processing. In addition, the solvents or com-

pounds of similar structure are also interesting as substrates for some applications. However, problems resulting from the toxicity of hydrophobic organic solvents for whole cells are still an important drawback for the application of these compounds in biocatalysis (Salter and Kell 1995).

Because of the toxicity problems in the applications described, microorganisms that can adapt to and survive the presence of organic solvents up to a second phase are of great interest. In this review, we focus on the toxic effects of organic solvents, on unique solvent-tolerant bacterial strains, and on the mechanisms behind their resistance.

Effects of organic solvents on microorganisms

Organic solvents, like alcohols, aromats, or phenols, are classical antimicrobial agents (Hugo 1978; Lucchini et al. 1990). Therefore, they have been widely used as disinfectants, food preservatives, tools for the permeabilization of cells, and also narcotic agents (de Smet et al. 1978; Davidson and Branden 1981; Naglak et al. 1990; Sikkema et al. 1992). The antimicrobial action of a solvent correlates with its hydrophobicity as expressed by the logarithm of the partition coefficient of the compound in a mixture of *n*-octanol and water (log P_{ow}). Organic solvents with log P_{ow} values between 1 and 5, such as toluene, are highly toxic for microorganisms. This influence of hydrophobicity on toxicity can be found in different solvent classes, e.g., aromats, alcohols, phenols, and alkanes (Laane et al. 1987; Rezessy-Szabo et al. 1987; Sierra-Alvarez and Lettinga 1991; Osborne et al. 1990; Sikkema et al. 1994).

It has been established that there is a systematic relationship between values of log P_{ow} in the range between 1 and 5 and the partitioning of solvents in membrane buffer systems (Osborne et al. 1990; Sikkema et al. 1992, 1994). Hence, the log *P* value is a suitable parameter that describes the accumulation of these solvents in membranes. The accumulation of the solvent toluene into bacterial membranes could be made visible by electron microscopy (de Smet et al. 1978; Aono et al. 1994a). These results demonstrate that the membrane in which the solvents accumulate is the main target of the toxic effect. This of course does not rule out additional sides of toxic action, as these may be caused by the specific properties of a molecule.

The mechanisms of the membrane toxicity have been reviewed by Sikkema et al. (1995). To understand the mechanisms that allow microorganisms to survive in the presence of the solvents, the following toxic effects on the membranes have to be taken into consideration. First, the accumulation of organic solvents leads to an a specific permeabilization of the cell membranes. In *Escherichia coli*, it was observed that potassium ions and ATP are released after treatment with phenol (Heipieper et al. 1991). For the solvent toluene, leakage from the cell of macromolecules such as RNA, phospholipids, or proteins could be demonstrated (Jackson and de Moss 1965; Woldringh 1973). This permeabilization is the result of considerable damage of the cytoplasmic membrane, whereas the outer membrane is still intact (de Smet et al. 1978). Other studies with bacterial and artificial membranes revealed an increase in the membrane surface area and a passive flux of protons and other ions across the membrane because of the presence of solvents (Fay and Farias 1977; Leão and Uden 1984; Uribe et al. 1985; Cartwright et al. 1986; Monti et al. 1987; Sikkema et al. 1992, 1994). This flux of ions dissipates the proton motive force (Δp), and affects both the proton gradient (ΔpH) and the electrical potential ($\Delta\Psi$) (Cartwright et al. 1986; Sikkema et al. 1994). Therefore, the second mechanism of the membrane toxicity of organic solvents is to diminish the energy status of the cell. In addition to a decreased proton motive force, the ATP synthesis can become impaired, a partial inhibition of the ATPase activity can be observed, and proteins engaged in the energy-transducing process are affected (Bowles and Ellefson 1985; Uribe et al. 1990). Third, besides the proteins engaged in energy transduction, the accumulation of solvents into a membrane also affects the function of other proteins embedded in the membrane. In *E. coli*, toluene leads to a total inactivation of the galactose permease system (Jackson and de Moss 1965), and in *Saccharomyces cerevisiae* the proton-potassium translocation is blocked (Uribe et al. 1985). Fourth, a further important aspect of the membrane structure, the fluidity, which is defined as the reciprocal of viscosity, is affected by organic solvents (Sikkema et al. 1994). An increased fluidity of membranes results in changes in stability, structure, and interactions within the membrane (Yuli et al. 1981; Zheng et al. 1988). Additionally, membrane-active compounds can affect the hydration characteristics of the membrane surface (Shimooka et al. 1992) and the thickness of the membrane (Seeman 1972). It can be concluded that once a solvent has dissolved in a membrane, it will disturb the integrity of the membrane and hence its function as a barrier, as a matrix for enzymes, and as an energy transducer.

Strains of bacteria tolerant to organic solvents

Despite the general toxic effects of organic solvents, some microbial strains tolerate high concentrations of compounds such as toluene. This surprising observation was first made by Inoue and Horikoshi in 1989 for a *Pseudomonas putida* strain, IH-2000, which grows in the presence of a second phase of toluene (Inoue and Horikoshi 1989; Inoue et al. 1991). This strain is not able to metabolize the toluene. Soon afterward, other researchers confirmed this initial observation. Other *Pseudomonas putida* strains were shown to grow in a two-phase solvent-water system containing toluene. These strains were isolated on xylene, styrene, or toluene (Cruden et al. 1992; Weber et al. 1993; Ramos et al. 1995). They all are able to grow in the presence of a second phase of various solvents such as xylene, styrene, and toluene, but benzene as a second phase is not tolerated. These *P. putida* strains were all isolated from a normal soil environment. Similarly, other solvent-tolerant strains of *Pseudomonas* belonging to other species have been

obtained. Strains of *P. aeruginosa*, *P. fluorescens*, and, recently, *P. mendocina* were isolated (Nakajima et al. 1992; Aono et al. 1992; Ogino et al. 1994, 1995; Ikura et al. 1997). Attempts to isolate solvent-tolerant strains from more extreme environments, like the deep sea, also resulted in the isolation of solvent-tolerant representatives belonging to other genera. A *Flavobacterium* was reported to grow in the presence of a second phase of benzene (5%) (Moriya and Horikoshi 1993a), and apparently is even more tolerant to solvents than the strains belonging to the genus *Pseudomonas*.

From the deep-sea environment, gram-positive strains were also isolated that are solvent tolerant. Strains belonging to the genus *Bacillus* were described that survived a second phase of benzene (Moriya and Horikoshi 1993b; Abe et al. 1995). The authors contribute this remarkable property of benzene tolerance to the source from which the strains were isolated. A benzene-tolerant *Rhodococcus* strain was isolated from a contaminated site in Australia on benzene as growth substrate (Paje et al. 1997). However, more recently we showed gram-positive bacteria that are tolerant to benzene or toluene can also be isolated from normal soil environments. Five strains belonging to the genus *Bacillus* were isolated that were able to withstand a second phase of toluene (Isken and de Bont 1998).

Another way to obtain solvent-tolerant strains is to increase the resistance of nontolerant strains by mutations. In this way, mutant strains with enhanced solvent tolerance properties were obtained of *Pseudomonas putida* PpG1 (Shima et al. 1991) and of *Pseudomonas aeruginosa* PAO1161 (Komatsu et al. 1994). Interestingly, *Escherichia coli* K12 also could be mutated to yield strains that are more solvent tolerant (Aono et al. 1991). This topic of enhanced solvent tolerance in *E. coli* is discussed in detail in this issue by Aono.

However, the tolerance of a particular strain to a solvent is not always tested for in a clear-cut way. The medium composition, the cultivation conditions, and also the history of the inoculum have an effect on the ability of an organism to grow in the presence of a solvent. For the sake of convenience, we consider here organisms solvent tolerant that have been shown to be able to grow in the presence of a second phase of toluene. The solvent-tolerant strains have been compiled in Table 1. From the table it is apparent that the potential for solvent resistance is much higher in gram-negative than in gram-positive strains. This observation is in agreement with the observation that gram-negative bacteria appear to be less sensitive to lipophilic compounds than gram-positive bacteria (Harrop et al. 1989; Inoue and Horikoshi 1991; Vermuë et al. 1993). This fact possibly may be explained by the presence of the additional outer membrane in gram-negative bacteria.

Adaptation mechanism

Research has begun to uncover the mechanisms responsible for the unique property of solvent tolerance since the first solvent-tolerant strain was isolated. Because the membrane is the main target of the toxic action of solvents, it is not surprising that, in the first paper about solvent-tolerant bacteria, changes in the membrane composition were already predicted to play a crucial role in the mechanisms contributing to solvent tolerance (Inoue and Horikoshi 1989). Some possible mechanisms involved in solvent tolerance as considered by various researchers are shown in Fig. 1. Indeed, several adaptive changes in the structure of the membrane have been observed in reaction to the accumulation of organic solvents in the membranes of microorganisms. Such adaptations have often been studied with non-solvent-tolerant microorganisms and with less toxic solvents such as ethanol (Ingram 1986, 1990). However, we discuss here only those mechanisms likely to be involved as defense mechanism in the solvent-tolerant bacteria.

Adaptation at the level of the cytoplasmic membrane

In the cytoplasmic membrane, changes at the level of the lipids and proteins have been observed. These adaptations reestablish the stability and fluidity of the membrane once it is disturbed by the solvent (Weber and de Bont 1996). In

Table 1. Strains tolerant to organic solvents

Organism	Several solvents tolerated	Isolation source	Isolation solvent	Reference
P. putida IH-2000	Heptanol, toluene	Soil	Toluene	Inoue and Horikoshi 1989
P. putida Idaho	Dimethylphthalate Toluene		Xylene	Cruden et al. 1992
P. aeruginosa ST-001	Heptanol, toluene	Soil	Xylene	Aono et al. 1992
P. putida S12	Dimethylphthalate Toluene	Soil	Styrene	Weber et al. 1993
Flavobacterium DS-711	Benzene, toluene	Deep sea	Benzene	Moriya and Horikoshi 1993b
Bacillus DS-994	Benzene, toluene	Deep sea	Benzene	Moriya and Horikoshi 1993b
P. aeruginosa LST-03	Toluene	Soil	Cyclohexane	Ogino et al. 1994
P. putida DOT-T1	Toluene	Water	Toluene	Ramos et al. 1995
Pseudomonas LF-3	Toluene	Soil	Styrene	Yoshida et al. 1997
P. mendocina LF-1	Dimethylphthalate	Soil	Styrene	Ikura et al. 1997
P. mendocina K08-1	Toluene			
Rhodococcus strain 33	Benzene	Soil	Benzene	Paje et al. 1997

P., *Pseudomonas*.

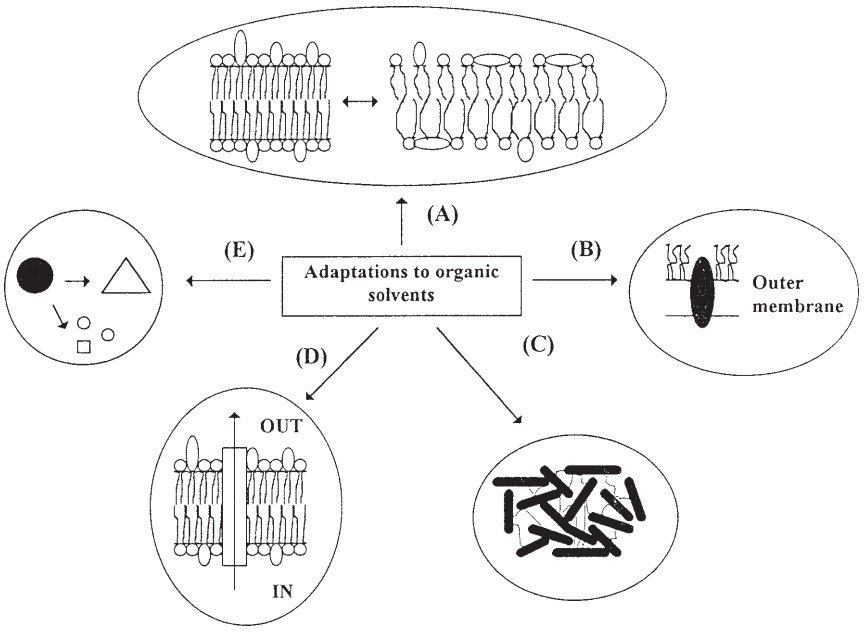

Fig. 1A–E. Schematic presentation of adaptation mechanisms that protect cells against the toxic effects of organic solvents. **A** Changes in the structure of the cytoplasmic membrane. **B** Modification of the LPS or porines of the outer membrane. **C** Reduction of cell wall hydrophobicity. **D** Active export of the solvents. **E** Transformation of the solvent. The scheme is a modification of the one presented by Sikkema et al. (1995)

principle, several mechanisms are possible and may vary from strain to strain. Mechanisms discussed here are (i) the degree of saturation of the fatty acids, (ii) *cis/trans* isomerization of unsaturated fatty acids, (iii) composition of phospholipid headgroups, and (iv) dynamics of phospholipid turnover.

It has been observed that solvents cause a shift in the ratio of saturated to unsaturated fatty acids. Relatively polar solvents such as ethanol or acetone cause an increase, while relatively apolar solvents such as benzene cause a decrease in the amount of unsaturated fatty acids in the membrane (Ingram 1976, 1977). For some solvent-tolerant *Pseudomonas putida* (Weber et al. 1994; Pinkart et al. 1996) and in the case of a solvent-tolerant strain of *P. aeruginosa* (Isken, unpublished results), such an increase in the saturation degree was demonstrated during adaptation to the presence of toluene. Alterations in the saturation degree of the fatty acids change the fluidity of the membrane and in this way compensate for the effects caused by solvents. This stabilization of the membrane fluidity is known as "homeoviscous adaptation" (Shinitzky 1984).

A recently discovered alternative mechanism for changing the fluidity of the membrane is the isomerization of the *cis* bond of an unsaturated fatty acid into the *trans* configuration (Heipieper et al. 1992). This conversion is caused by an energy-independent isomerase (Diefenbach and Keweloh 1994; Holtwick et al. 1997). The isomerization increases the membrane ordering (Fig. 2) and consequently decreases the membrane fluidity (Diefenbach et al. 1992; Chen et al. 1995a; Keweloh and Heipieper 1996). The *cis* to *trans* isomerization also takes place in solvent-tolerant strains after the exposure to toluene and other solvents (Weber et al. 1994; Heipeiper et al. 1995). The amount of *trans*-unsaturated fatty acids in solvent-tolerant bacteria

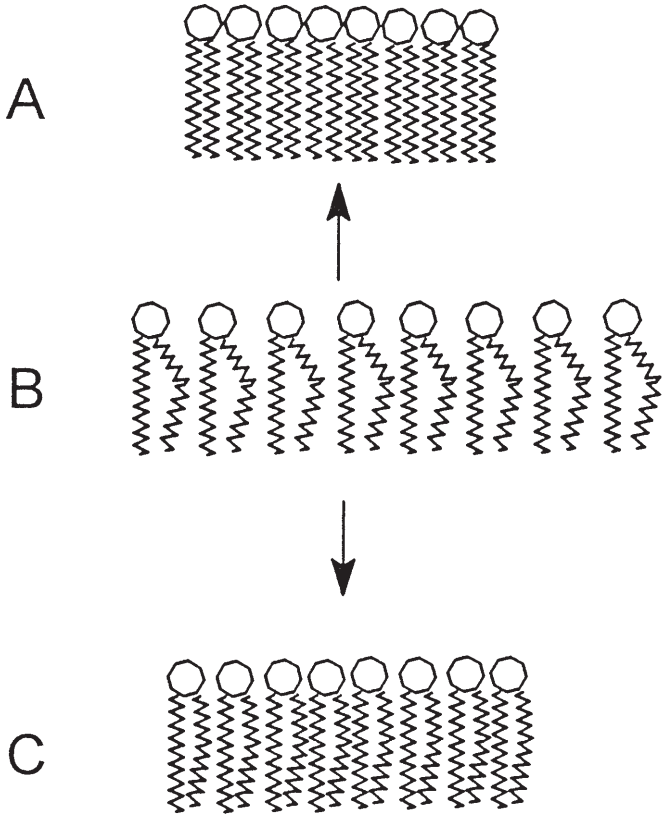

Fig. 2A–C. Different conformations of phospholipids and their effect on the structuring of the lipid bilayer. **A** Saturated fatty acids; **B** *cis*-unsaturated fatty acids; **C** *trans*-unsaturated fatty acids

corresponds to their survival in the presence of a second phase of toluene (Weber et al. 1994)

The enzyme responsible for the *cis* to *trans* isomerization in non-solvent-tolerant strains has been partially purified. It appears that the enzyme is periplasmatic and acts both on phospholipids and on free fatty acids (Chen et al. 1995b; Chen 1996). The enzyme was difficult to purify because its activity in extracts is relatively low. A different approach was followed by Holtwick et al. (1997), who obtained mutants of *P. putida* P8. An isomerase-negative mutant was obtained, and a DNA fragment that complemented the mutation was isolated and cloned. The DNA sequence showed no significant homologous regions when the deduced amino acid sequence was compared with other proteins.

Ramos et al. (1997) postulated that this *cis/trans* isomerization is only the second part of the two-step transformation of cyclopropane fatty acids into *trans*-unsaturated fatty acids and that a lower amount of cyclopropane fatty acids supports the survival in the presence of solvents. To prove that the *cis/trans* isomerization is necessary for the survival of solvent-tolerant strains, studies were performed with mutants lacking the ability to perform these isomerizations. A transposon mutant of the solvent-tolerant *Pseudomonas putida* DOT-T1 is both solvent sensitive and unable to perform the isomerization (Ramos et al. 1997). However, the *cis/trans*-isomerization is unlikely to be the only necessary adaptation mechanism to organic solvents because strains are known that can perform the isomerization and are still solvent sensitive (Pinkart et al. 1996; Ramos et al. 1997). The *cis/trans* isomerization has also been reported as a response to starvation (Guckert et al. 1986) and in the presence of antibiotics (Isken et al. 1997) or heavy metals (Heipieper et al. 1996). This indicates that the *cis/trans* ratio may be part of a general stress response of microorganisms.

Apart from the fatty acid composition, the headgroups of lipids also alter during solvent adaptation. In solvent-tolerant *P. putida* strains, the relative amount of diphosphatidylglycerol (cardiolipin) increases during adaptation to the solvent toluene (Weber and de Bont 1996; Ramos et al. 1997). Such a change in the headgroups had also been found in *Escherichia coli* mutants with an increased resistance to solvents (Clark and Beard 1979). Recently, *Pseudomonas putida* Idaho was shown to adapt differently. The amount of phosphatidylethanolamine increases in this solvent-tolerant strain (Pinkart and White 1997). In general, the regulation of the headgroup composition is said to control the phase preference of the lipids. In this way, the effect of solvents on the fluidity, the volume, and the density of the lipids is compensated (Weber and de Bont 1996).

Not only is the composition of the membranes important, but the dynamics of biosynthesis of membrane compounds may also play an important role in solvent tolerance. Pinkart and White (1997) demonstrated that in the solvent-tolerant *P. putida* Idaho the rate of phospholipid synthesis increases after exposure to xylene. The total amount of total phospholipids increases in this strain. A solvent-sensitive control strain, *P. putida* MW1200, has a much lower turnover of lipids and reduced phospholipid content after exposure to xylene. Therefore, it is likely that *P. putida* Idaho is better equipped to repair damaged membranes than the solvent-sensitive strain.

Apart from changes in the composition of the cytoplasmic membrane and in the dynamics of the formation of phospholipids, alterations in the protein content have been observed as a response to solvents. Until now, however, such changes in the protein content have been studied in non-solvent-tolerant strains (Dombek and Ingram 1984; Ingram 1986; Keweloh et al. 1990). In addition, lipid-soluble compounds were shown to play a role in adaptation to solvents. *Zymomonas mobilis* increases the amount of hopanoids as response to ethanol (Bringer et al. 1985), and in *Staphylococcus aureus* the tolerance to oleic acid correlates with carotenoid production (Chamberlian et al. 1991). Adaptation mechanisms observed in nontolerant organisms might also pay a role in solvent tolerance.

Adaptation at the level of the outer membrane

As mentioned, gram-negative bacteria are less sensitive to solvents than gram-positive organisms. However, no differences between gram-positive and gram-negative bacteria were observed with regard to the critical concentration of molecules dissolved in the cytoplasmic membrane (Vermuë et al. 1993). Hence, the differences in solvent tolerance must be based on other alterations.

In contrast to gram-positive bacteria, gram-negative bacteria have an additional outer membrane. The outer membrane was shown to be engaged in promoting solvent tolerance. Ions such as Mg^{2+} or Ca^{2+} stabilize the organization of the outer membrane and contribute to a higher resistance of solvent-tolerant *Pseudomonas* strains toward toluene (Inoue et al. 1991; Ramos et al. 1995; Weber and de Bont 1996). After adaptation to toluene, solvent-tolerant *Pseudomonas putida* S12 cells become less hydrophobic (Weber and de Bont 1996). Recently, it was shown that a reduction of the cell hydrophobicity correlates with changes in the lipopolysaccharide (LPS) content (Aono and Kobayashi 1997). Indeed, the LPS composition of solvent-tolerant *P. putida* Idaho changes as a result of the presence of solvents (Pinkart et al. 1996).

Apart from changes in the LPS, the porines that are embedded in the outer membrane have been related to solvent tolerance. On the one hand, mutants of *P. putida* DOT-T1 lacking the porine OmpL are hypersensitive to solvents, possibly because of the missing stabilization of the envelope integrity by OmpL (Ramos et al. 1997). On the other hand, the absence of the porine OmpF in *Pseudomonas aeruginosa* (Li et al. 1995) leads to a higher tolerance toward solvents. Such an increase in solvent tolerance caused by the absence of a porine was also obtained in non-tolerant *Escherichia coli* (Aono and Kobayashi 1997). The authors of these studies suggested that organic solvent molecules are able to pass through the proteins. Therefore, mutants lacking these porines have a higher tolerance to solvents.

Adaptation at the level of the cell wall

Bacteria with hydrophobic cell walls have been shown to have a higher affinity for hydrophobic compounds (van Loosdrecht et al. 1990; Jarlier and Nikaido 1994). Therefore, modifications in these cell walls lowering the hydrophobicity may provide a higher tolerance to solvents. To our knowledge this has not been studied with solvent-tolerant strains so far.

Adaptation caused by transformation of solvents

Resistance to antibiotics is often based on the degradation of these antimicrobial compounds into less- or nontoxic products. Because many of the toxic solvents can be degraded by microorganisms, tolerance could be mediated by such a degradation. Possibly, the benzene-tolerant *Rhodococcus* strain described recently may partly depend on this mechanism because this strain was shown to be an effective degrader of benzene (Paje et al. 1997). Additionally, transformations were predicted to play a role in solvent tolerance in *E. coli* (Ferrante et al. 1995). However, many of the solvent-tolerant strains mentioned are able to cope with a broad range of solvents up to a second phase, which often cannot be transformed at all by these strains. Hence, degradation may mediate the resistance of some strains to specific solvents, but it cannot be the main mechanism contributing to the tolerance to a broad range of solvents.

Adaptation caused by active excretion of solvents

The degradation or transformation of a toxin as described here is only one mechanism to decrease the amount of toxins in the cell or membrane actively. An alternative way to decrease the concentration of a toxin is the removal of a compound from the cell by active excretion. Such efflux systems are well known for lipophilic cytotoxic agents, such as antibiotics (Nikaido 1994, 1996; Paulsen et al. 1996; George 1996). Many of the energy-driven export systems play an important role in drug resistance as they are able to pump out a wide range of compounds having no common chemical structure. The only common feature is that most of these compounds are charged amphiphilic molecules. It was shown that genes coding for the proteins engaged in such an export can be induced by structurally unrelated hydrophobic compounds (Lewis et al. 1994).

Interestingly, the adaptation of solvent-tolerant *P. putida* S12 to toluene results in an increased resistance to various chemically and structurally unrelated antibiotics, such as tetracycline, chloramphenicol, nigericin, polymyxin B, or piperacilline (Isken et al. 1997). This effect is shown in Fig. 3. However, this effect can be explained in different ways. First, the enhanced antibiotic resistance can be the result of an active export of these antibiotics. Second, this enhanced resistance can be the result of other defense mechanisms induced during the adaptation to toluene. Indeed, we demonstrated that the amount of toluene accumulated in *P. putida* S12 is dependent on energy (Isken and de Bont 1996). In cells adapted to toluene, the presence of different energy inhibitors resulted in significantly higher amounts of toluene accumulating in *P. putida* S12 (Fig. 4). This strain is not able to transform the toluene. Therefore, we concluded that the amount of toluene in the cell is kept

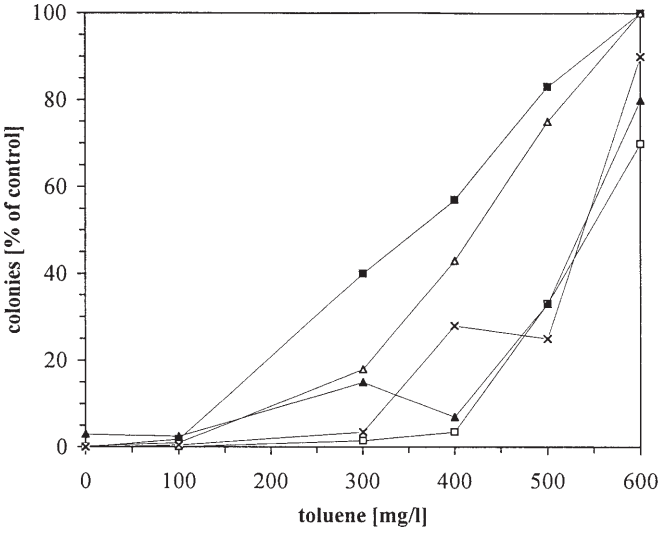

Fig. 3. Effect of the adaptation of *Pseudomonas putida* S12 to toluene on the survival in the presence of antibiotics. Cells were grown in a chemostat under defined conditions in the presence of various concentrations of toluene. The colony-forming units (CFU) in the presence and absence of antibiotics were determined. The CFU of cells growing in the presence of 15 mg/l piperacillin (*X*), 20 mg/l chloramphenicol (*solid squares*), 1 mg/l tetracycline (*open squares*), 20 mg/l nigericin (*open triangles*), and 1 mg/l polymyxin B (*solid triangles*) are relative to a control grown in the absence of antibiotics

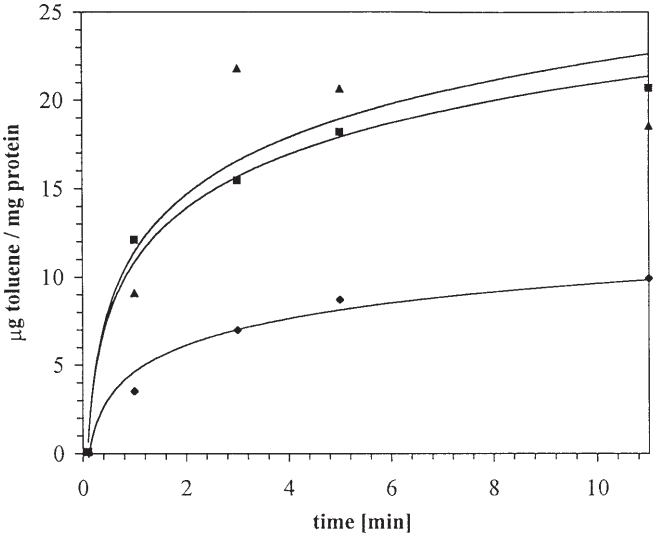

Fig. 4. Effects of the presence of energy-coupling inhibitors on the accumulation of [^{14}C]toluene in toluene-adapted cells of *Pseudomonas putida* S12. Cells not inhibited (*diamonds*) were compared with cells to which the respiratory chain inhibitor potassium cyanide at 20 mM (*triangles*) or the proton conductor carbonyl cyanide *m*-chlorophenylhydrazone at 0.25 mM (*squares*) was added via the washing buffer before addition of [^{14}C]toluene

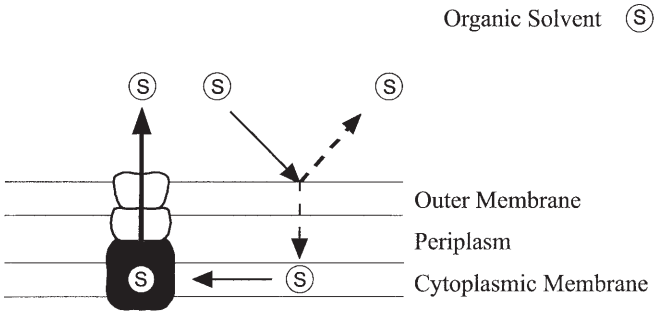

Fig. 5. Proposed structure and mechanism of the solvent efflux system in *Pseudomonas putida* S12. The scheme is a modification of that presented by Kieboom et al. (1998)

at a relative low level by action of an active efflux system. Since then, active efflux as a mechanism contributing solvent tolerance has also been observed in another solvent-tolerant *P. putida* strain (Ramos et al. 1997). The active efflux of toluene was observed by us at the whole-cell level. The question remained open as to whether this was a new kind of export system or if it has features in common with the well-known antibiotic efflux pumps. Attempts to clone the genes encoding the putative pump were carried out using solvent-sensitive transposon mutants (Kieboom et al. 1998). The region of the genome containing the transposon insertion was cloned, and the relevant nucleotide sequence was determined and compared to known sequences. The gene responsible for the export of toluene in *P. putida* S12 showed homology with other well-known export systems responsible for the active efflux of antibiotics out of the cell. The homology of this gene called *srpABC* with proton-dependent efflux pumps, such as the *acrAB* operon in *E. coli*, suggests that the solvent efflux is dependent on the proton motive force. The participation of the multidrug efflux pump AcrAB, encoded within the *acrAB* operon, in the solvent tolerance of *E. coli* recently was demonstrated by White et al. (1997). Recently, during the International Congress on Extremophiles '98 a pouter was presented (Hirayama et al. 1998) revealimgasimilar situation as for P. putida 512. Genes involued in soluent transport had been located in another strain of Pseudomonas. A mechanism explaining how the efflux of solvents by SrpABC may take place is suggested in Fig. 5.

Role of general stress response

The large number of adaptation mechanisms described here suggest that solvent tolerance is not mediated by one mechanism only. It is likely that a combination of different mechanisms contribute together to the solvent tolerance; this includes the presence of a large stress response system, such as the one known for heat shock, which is induced or activated by the solvents. Indeed, the induction of a large number of proteins by toxins was demonstrated in *E. coli* where the presence of pollutants (Blom et al. 1992) or the uncoupler 2.4-dinitrophenol (Gage and Neidhardt 1993) leads to the induction of 53 or 39 different proteins, respectively. When *Clostridium acetobutylicum* initiates the solvent transformation, various known heat-shock proteins are expressed (Pich et al. 1990). In *P. putida* KT2442 the expression of approximately 100 proteins is affected by the presence of 2-chlorophenol (Lupi et al. 1995).

As mentioned, the adaptation to solvents does not only enhance the resistance to other solvents (Heipieper and de Bont 1994), but also to heavy metals (Heipieper et al. 1996) and antibiotics (Isken et al. 1997) in the solvent-tolerant strain *Pseudomonas putida* S12. Such a correlation was earlier found in *E. coli* where the overexpression of stress response genes enhanced tolerance to various environmental factors (Aono et al. 1995; Nakajima et al. 1995a,b; Asako et al. 1997). The question remains whether genes that mediate solvent resistance and the functions of which are still unknown, such as *ostA* (Aono et al. 1994b), can be embedded in the cascade system of stress response.

Concluding remarks

Although organic solvents are highly toxic for living organisms because they accumulate in and disrupt cell membranes, more and more bacterial strains have been obtained that can adapt to and survive these antimicrobial agents. Initially, most solvent-tolerant strains isolated belong to the genus *Pseudomonas*. In the meantime, however, other genera have also been shown to include solvent-tolerant strains.

The survival of the well-studied *Pseudomonas* strains is based on their ability to induce or activate a broad range of different adaptation mechanisms. Many of these mechanisms can also be found in nontolerant bacteria, or they are known as a defense to other antimicrobial compounds. In adapting to organic solvents, the tolerant organisms alter the structure of their cell envelope. They change the saturation degree of the fatty acids, the *trans/cis* ratio of the unsaturated fatty acids, or the phospholipid headgroup composition of the membrane lipids. An enhanced phospholipid turnover increases their ability to repair membrane damage, and the transformation of the toxic solvent may contribute tolerance. Furthermore, the solvent-tolerant *Pseudomonas* possess an active efflux system pumping the solvent out of the cell. This efflux system has features in common with the multidrug efflux pumps studied in detail for antibiotics.

It is obvious that only a combination of mechanisms allows the survival of the unique solvent-tolerant strains. The regulation of such a diverse response system may be connected to a general stress response. This is likely, as the tolerance to organic solvents correlates to the resistance toward other harmful environmental factors in the solvent-tolerant strains studied. Because most of the strains described so far need an adaptation period to cope with the solvent stress, it is more appropriate to call them solvent tolerant than extremophile.

In the future, use of the solvent-tolerant strains offers new perspectives in environmental biotechnology, and this will simplify the application of organic solvents in whole-cell biotransformation. Application of the solvent-tolerant strains in environmental biotechnology can enhance the stability of mineralization processes as the drawbacks caused by toxic effects play a minor role. In the fine chemistry area, many biocatalytic applications of whole cells are suboptimal because of the formation of toxic products or problems in recovery of the product. For both problems, a continuous extraction of the product can be a solution. Using solvent-tolerant strains the number of applicable extraction solvents increases toward more hydrophilic ones. Therefore, products can be recovered that cannot be extracted in the already used hydrophobic solvents. In addition, the so-called toxic products are already less toxic for these strains.

Apart from the application of the solvent-tolerant strains in whole-cell systems, these strains may become a source for new enzymes. Examples of new solvent-stable proteases or lipases produced by solvent-tolerant strains have already been reported (Ogino et al. 1994, 1995). As a consequence, we expect the number of possible applications of these unique solvent-tolerant bacteria to increase in the near future.

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