Physiology of solvent tolerance in *Pseudomonas putida* S12

Promotor: dr. ir. J.A.M. de Bont
Hoogleraar in de Industriële Microbiologie

Physiology of solvent tolerance in

Pseudomonas putida S12

Sonja Isken

Proefschrift
ter verkrijging van de graad van doctor, op gezag van de rector magnificus
van Wageningen Universiteit, dr. ir. L. Speelman, in het openbaar te
verdedigen op vrijdag 6 oktober 2000 des namiddags om vier uur in de Aula.

Isken, Sonja

Physiology of solvent tolerance in *Pseudomonas putida* S12 / Sonja Isken
Thesis Wageningen University. With ref. - with summary in Dutch and German.

ISBN 90-5808-225-3

The research described in this thesis was financially supported in the framework of an industrial relevant research program of the Association of Biotechnology Centres in the Netherlands (ABON).

BIBLIOTHEEK
LANDBOUWUNIVERSITEIT
WAGENINGEN

Contents

1.	General introduction	7
2.	Bacteria tolerant to organic solvents	11
3.	Active efflux of toluene in a solvent-resistant bacterium	25
4.	Effect of solvent adaptation on the antibiotic resistance in *Pseudomonas putida* S12	31
5.	Physiological characterization of the solvent efflux system of *Pseudomonas putida* S12	43
6.	Effect of organic solvents on the yield of solvent-tolerant *Pseudomonas putida* S12	51
7.	Concluding remarks	63
	References	69
	Summary	79
	Samenvatting	81
	Zusammenfassung	83
	Nawoord	85
	Curriculum vitae	87
	Bibliography	88

NN08201, 2852

STELLINGEN

1. Bij de bewering dat tijdens adaptatie aan tolueen cyclopropaanvetzuren via *cis*- in *trans*- onverzadigde vetzuren worden omgezet, laten Ramos et al. ten onrechte de kennis over cyclopropaanvetzuren in micro-organismen buiten discussie.
Ramos JL, Duque E, Rodriguez-Herva JJ, Godoy P, Haïdour A, Reyes F, Fernandez-Barrero A (1997) Mechanisms for solvent tolerance in bacteria. J Biol Chem 272:3887-3890

2. Een gen zou niet "*solvent-resistant gene*" genoemd moeten worden als het genproduct de giftigheid van oplosmiddelen juist kan versterken.
Ferrante AA, Augliera J, Lewis K, Klibanov AM (1995) Cloning of an organic solvent-resistance gene in Escherichia coli: the unexpected role of alkylhydroperoxide reductase. Proc Natl Acad Sci 92:7617-7621

3. Net zoals een hoospomp in z'n eentje een kajak niet zeevaardig maakt, brengt een efflux - pomp in z'n eentje geen oplosmiddeltolerantie tot stand.

4. Menig extremofiel houdt niet van uitersten.

5. Bij *Helianthus annuus* en *Pinus sylvestris* is stress de oorzaak voor tolueenemissie.
Heiden AC, Kobel K, Komenda M, Koppmann, Shao M, Wildt J (1999) Toluene emission from plants. Geophys Res Letters 26:1283-1286

6. Niet alleen grote boten kunnen een soliton veroorzaken.
Hamer M (1999) Solitary killers. New Scientist 163:18-20

7. Verlaging van het vetgehalte in voedsel wordt vaak met een slechtere smaak betaald.
Sinki G, Labuda I (1999) The flavor industry's response to health trends. Perfumer & Flavorist 24:13-17

8. De menselijke geschiedenis is veelal door de kleinste organismen bepaald.
Dixon B (1994) Power unseen: how microbes rule the world. WH Freeman, Spektrum Oxford, New York, Heidelberg

9. Het verschil tussen Duitsland en Nederland is groter dan vele Duitsers denken en kleiner dan menig Nederlander hoopt.

10. Man darf ruhig dumm sein, man muß sich nur zu helfen wissen.
Großonkel Otto

11. Mensch gedenke der Zeit, denn heute ist morgen schon gestern.

Stellingen behorende bij het proefschrift "Physiology of solvent tolerance in *Pseudomonas putida* S12". Sonja Isken, Wageningen, vrijdag 6 oktober 2000.

1
GENERAL INTRODUCTION

ORGANIC SOLVENTS IN BIOTECHNOLOGY

Production and use of various organic solvents is widespread among industrial processes. In biotechnology, solvents like aromatics, alcohols or alkanes, play a role in the biotechnological production of fine chemicals as well as in environmental biotechnology.

During the production of fine chemicals, organic solvents are already used widely in the application of biotransformations with enzymes (Carrea et al. 1995). The use of organic solvents also has several advantages in the application of whole cell systems (Nikolova and Ward 1993, Salter and Kell 1995, León et al. 1998). Solvents can increase the available amount of poorly water-soluble substrates for the cells. Using a second phase of an organic solvent the products of a biotransformation can be extracted continuously from the aqueous reaction system. This 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, solvents or structurally similar compounds 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, Angelova and Schmauder 1999).

Because of the widespread industrial use of organic solvents, they have been present in the environment since the early industrial revolution. Their toxic and even carcinogenic effects make them a potential threat to human health. However, microorganisms are often able to transform organic solvents, often leading to complete mineralization of these compounds. Although many microorganisms degrade organic solvents at low concentrations, various solvents can become toxic at slightly higher concentrations (Volsky et al 1990, Blum and Speece 1991). This can cause problems in bioremediation, waste gas and wastewater treatment (Barr and Aust 1994).

The toxicity of organic solvents plays a decisive role in the biotechnological production of fine chemicals as well as in environmental microbiology.

Toxicity of organic solvents

Organic solvents, like alcohols or aromatics, are classical antimicrobial agents (Hugo 1978, Lucchini 1990). They have been widely used as disinfectants, food preservatives, to permeabilize cells and also as narcotic agents (de Smet et al. 1978, Davidson and Branden 1981, Naglak et al. 1990, Sikkema et al. 1992).

Several studies have shown that 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 $P_{o/w}$). Organic solvents with log $P_{o/w}$ values between 1 and 5, like toluene, are highly toxic for microorganisms (Laane et al. 1987, Rezessy-Szabo et al. 1987, Sierra-Alvarez and Lettinga G 1991, Osborne et al. 1990, Sikkema et al. 1994). Such organic solvents accumulate in the membranes of living organisms. This accumulation leads to specific permeabilization of the cell membranes, increased membrane surface area, diminished energy status of the cell, damaged function of proteins embedded in the membrane, increased fluidity of membranes and decreased membrane stability. 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. These mechanisms of the solvent toxicity were reviewed by Sikkema et al. (1995).

Solvent tolerant bacteria

As mentioned above, the toxicity of organic solvents to whole cells is an important drawback in the application of these solvents in the production of fine chemicals employing whole-cell biotransformations and in environmental biotechnology. Therefore, microorganisms, which can adapt to and survive the presence of organic solvents as a second phase, are of great interest. Such microorganisms have been isolated in the last decade (Inoue and Horikoshi 1989, Cruden et al. 1992, Weber et al. 1993, Ramos et al. 1995). Most of these bacteria belong to the Gram negative genus *Pseudomonas*. Since the discovery of the first solvent-tolerant strain efforts have been made to elucidate the mechanisms responsible for the solvent-tolerance.

Outline of this thesis

In this thesis several aspects of the physiology of the solvent-tolerant strain *Pseudomonas putida* S12 are discussed. Toluene is the model solvent used in most studies described in this thesis. In **chapter 2** an overview is given of various reports on solvent-tolerant strains. The mechanisms involved in the tolerance of these strains to organic solvents are discussed. One of these mechanisms present in *P. putida* S12, an active efflux of toluene, is described in

chapter 3. It has long been known that aspecific active efflux systems are involved in the defense of microorganisms against antibiotics. As demonstrated in **chapter 4**, cells adapted to survive high toluene concentrations also exhibit an enhanced resistance to a broad range of such antibiotics. However, the substrate specificity and energy dependency of the toluene efflux system was investigated (**chapter 5**) and antibiotics were shown not to be substrates for the toluene efflux system. These results are discussed in terms of general stress response. Finally, in **chapter 6**, effects of various organic solvents on the growth yield and maintenance requirements of *P. putida* S12 are described. It is demonstrated that effects of different solvents on the cells do not depend on the chemical composition of a solvent but on its concentration reached in the bacterial membrane. Hence, results found for toluene can be extrapolated to other solvents as well.

2
BACTERIA TOLERANT TO ORGANIC SOLVENTS

SONJA ISKEN AND JAN A. M. DE BONT

Published in: Extremophiles (1998) 2:229-238

ABSTRACT

The toxic effects that organic solvents have on whole cells are 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 as 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 $P_{O/W}$). Substances with a log $P_{O/W}$ 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 speeding up repairing processes. In addition to these adaptation mechanisms compensating the toxic effect of the organic solvents, mechanisms do exist which actively decrease the amount of the toxic solvent in the cells. An efflux system actively decreasing the amount of solvents in the cell was described recently. We review the current knowledge about exceptional strains that can grow in the presence of toxic solvents and the mechanisms responsible for their survival.

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 et al. 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 due to 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 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 compounds 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 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 correlate with its hydrophobicity as expressed by the logarithm of the partition coefficient of the compound in a mixture of *n*-octanol and water (log $P_{O/W}$). Organic solvents with log $P_{O/W}$ values between 1 and 5, like toluene, are highly toxic for microorganisms. This influence of the hydrophobicity on the toxicity can be found in different solvent-classes, e.g. aromats, alcohols, phenols, alkanes (Laane et al. 1987, Rezessy-Szabo et al. 1987, Sierra-Alvarez and Lettinga G, 1991, Osborne et al. 1990, Sikkema et al. 1994).

It has been established that there is a systematic relationship between values of log $P_{O/W}$ 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 cause does not rule out additional sides of toxic action, as they may be caused by the specific properties of a molecule.

The mechanisms of the membrane toxicity were reviewed by Sikkema et al. (1995). To understand the mechanisms that allow microorganisms to survive 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 aspecific 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 DeMoss 1965, Woldringh 1973). This permeabilization is the result of considerable damages 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 matrix for enzymes and as 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 afterwards, 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* but 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, resulted also in the isolation of solvent-tolerant representatives belonging also 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 also Gram-positive strains were isolated that are solvent tolerant. Strains belonging to the genus *Bacillus* were described which 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 and/or toluene can also be isolated from normal soil environments. We isolated five strains belonging to the genus *Bacillus* which were able to withstand a second phase of toluene (Isken and de Bont, 1998a).

Another way to obtain solvent-tolerant strains is to increase the resistance of non-tolerant strains by mutations. In this way, mutants 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, also *Escherichia coli* K12 could be mutated to yield strains that are more solvent tolerant (Aono et al. 1991).

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 here consider 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 2.1. 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.

Table 2.1 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 1993a
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

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 Figure 2.1. 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 will discuss here only those mechanisms likely to be involved as defense mechanism in the solvent-tolerant bacteria.

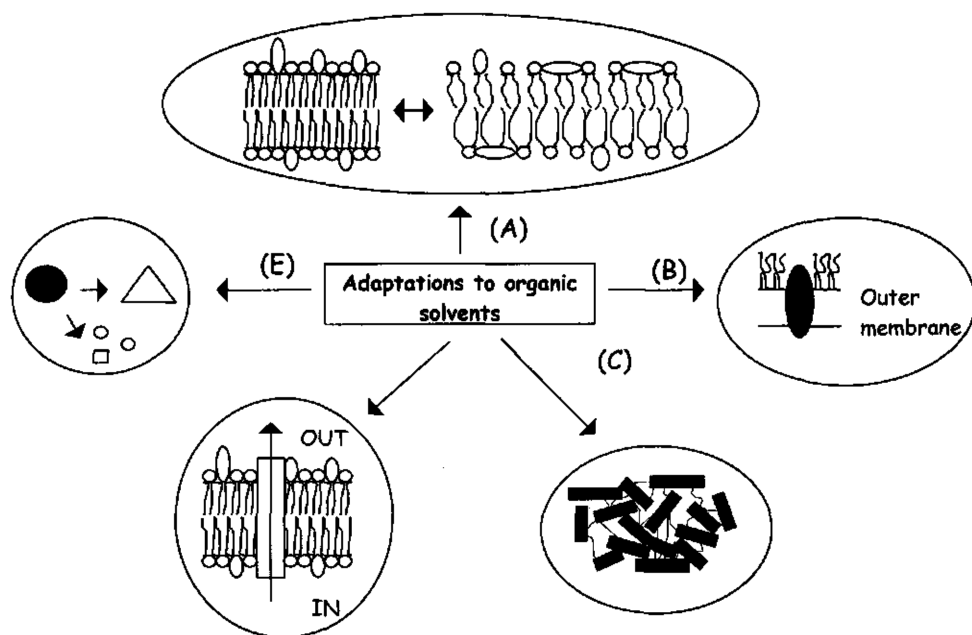

Figure 2.1 Schematic presentation of adaptation mechanisms that protect cells against the toxic effects of organic solvents. (A) Changes in the structure of the cytoplasmic membrane. (B) Modification of the LPS or porines of the outer membrane. (C) Reduction of cell wall hydrophobicity. (D) Active export of the solvents. (E) Transformation of the solvent. The scheme is a modification of the one presented by Sikkema et al. (1995).

Adaptation on 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 membranes once it is disturbed by the solvents (Weber and de Bont 1996). In principle, several mechanisms are possible and may vary from strain to strain. Mechanisms to be 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 the phospholipids turnover.

It has been observed that solvents cause a shift in the ratio 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).

An 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 (Figure 2.2) and consequently decreases the membrane fluidity (Diefenbach et al. 1992, Chen Q et al. 1995a, Keweloh and Heipieper 1996). The *cis/trans* isomerization takes also place in solvent-tolerant strains after the exposure to toluene and other solvents (Weber et al. 1994, Heipieper et al. 1995). The amount of *trans*-unsaturated fatty acids in solvent-tolerant bacteria corresponds to their survival in the presence of a second phase of toluene (Weber et al. 1994).

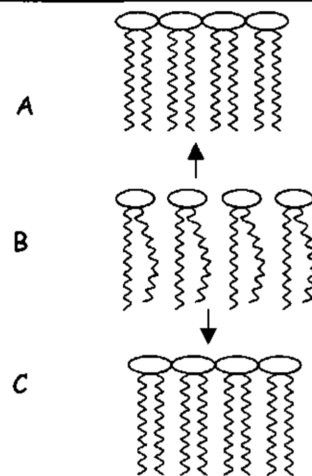

Figure 2.2 Different conformations of phospholipids and their effect on the structuring of the lipid bilayer. (A) saturated fatty acids; (B) *cis*-unsaturated fatty acids; (C) *trans*-unsaturated fatty acids.

The enzyme responsible for the *cis/trans* isomerization in non-solvent-tolerant strains has been purified recently (Holwick et al. 1999, Pedrotta and Witholt 1999). It appeared the enzyme is periplasmatic and acts in vivo both on phospholipids and on free fatty acids (Chen Q et al. 1995b, Chen Q 1996). In vitro the purified isomerase acted only on free unsaturated fatty acids (Pedrotta and Witholt 1999). Holtwick et al (1997) obtained isomerase-negative mutants of *P. putida* P8. 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. Close to the N terminus of the predicted polypeptide of the *cis/trans* isomerase gene (*cti*) a cytochrome c-type heme-binding motif was found (Holtwick et al. 1999). As a consequence a possible mechanism of heme-catalyzed *cis/trans* isomerization of unsaturated fatty acids is discussed nowadays.

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 cyclopropan fatty acids support 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 this isomerization. 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 which 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 *Pseudomonas putida* strains the relative amount of diphosphatidylglycerol (cardiolipin) increases during the 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 the composition of the membranes is important, but the dynamics of biosynthesis of membrane compounds may also play an important role in solvent tolerance. Recently, Pinkart and White (1997) demonstrated that in the solvent-tolerant *Pseudomonas 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 turn-over of lipids and a reduction of the 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, such changes in the protein content have been studied in non-solvent-tolerant strains (Dombek and Ingram 1985, 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 (Chamberlain et al. 1991). Adaptation mechanisms observed in non-tolerant organisms might also play a role in solvent tolerance.

Adaptation on the level of the outer membrane

As mentioned above, 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 the Gram-positive bacteria the Gram-negative bacteria have an additional outer membrane. The outer membrane was shown to be engaged in promoting solvent-tolerance. Ions, like Mg^{2+} or Ca^{2+}, stabilize the organization of the outer membrane and contribute to a higher resistance of solvent-tolerant *Pseudomonas* strains towards 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 lipopolysaccaride (LPS) content (Aono and Kobayashi 1997). Indeed, the LPS composition of solvent-tolerant *Pseudomonas putida* Idaho changes as 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 with 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 towards solvents. Such an increase in solvent tolerance due to 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 porines. Therefore, mutants lacking these porines have a higher tolerance to solvents. Recently, this theory was disproved for

Escherichia coli (Asako et al. 1999). The organic solvent tolerance was shown to be independent of OmpF levels in the membrane. Earlier found relations between solvent sensitivity and the level of OmpF were caused by variations in transcriptional activators.

Adaptation on the level of the cell wall

Bacteria with hydrophobic cell walls were 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 non-toxic 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 since 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 above 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 mechanisms 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 above 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 out of 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 *Pseudomonas putida* S12 to toluene results in an increased resistance to various chemically and structurally unrelated antibiotics (Isken et al. 1997). 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). This strain is not able to transform the toluene. Therefore, we concluded that the amount of toluene in the cell is kept 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 *Pseudomonas putida* strain (Ramos et al. 1997). The active efflux of toluene was observed by us at the whole cell level. The question remained open if we had to deal with 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 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 suggests that the solvent efflux is dependent on the proton motive force. Similar genes encoding for solvent efflux systems were described in other strains of *Pseudomonas putida* (Ramos et al. 1998, Fukumori et al. 1998, Kim et al. 1998, Mosqueda and Ramos 2000). The participation of the multidrug efflux pump AcrAB in the solvent tolerance of *Escherichia coli* was demonstrated by White et al. (1997). A mechanism how the efflux of solvents by SrpABC in *Pseudomonas putida* S12 may take place is suggested in Figure 2.3.

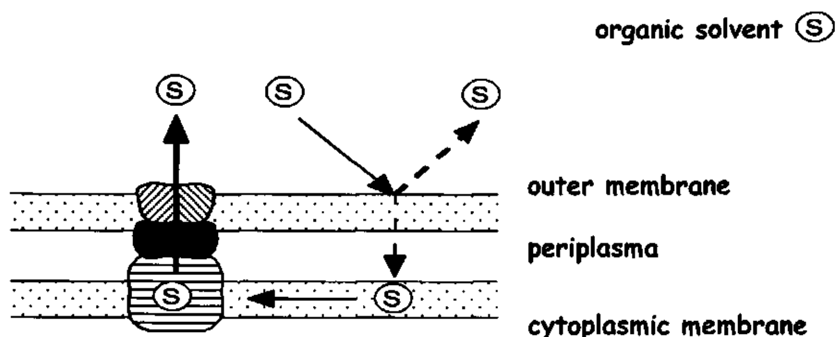

Figure 2.3 Proposed structure and mechanism of the solvent efflux system in *Pseudomonas putida* S12. The scheme is a modification of the one presented by Kieboom et al. (1998a).

ROLE OF GENERAL STRESS RESPONSE

The large number of adaptation mechanisms described above suggests 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 which mediate solvent-resistance and which functions are still unknown, like *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 as they accumulate in and disrupt cell membranes, more and more bacterial strains have been obtained which can adapt to and survive these antimicrobial agents. Initially, most solvent-tolerant strains isolated belong to the genus of *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 non-tolerant 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 and/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 the 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 towards 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 the use of the solvent-tolerant strains offers new perspectives in environmental biotechnology, and it will simplify the application of organic solvents in whole cell biotransformation. The 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 the 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 towards more hydrophilic ones. Therefore, products can be recovered which 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 for new solvent-stable proteases or lipases produced by solvent-tolerant strains can already been found in literature (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.

3

ACTIVE EFFLUX OF TOLUENE IN A SOLVENT-TOLERANT BACTERIUM

SONJA ISKEN AND JAN A. M. DE BONT

Published in: Journal of Bacteriology (1996) 178:6056-6058

ABSTRACT

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.

INTRODUCTION

Solvent resistant bacteria

Toluene and many other organic solvents kill microorganisms because they accumulate in and disrupt cell membranes (Sikkema et al. 1995). However, Inoue and Horikoshi (1989) isolated a toluene-resistant *Pseudomonas putida* strain which grew in a two-phase toluene-water system. This surprising observation has been confirmed by others (Cruden et al. 1992, Weber et al. 1993, Ramos et al. 1995) 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 (Heipieper et al. 1994, Weber et al. 1994). However, this incorporation of *trans*-unsaturated fatty acids most likely is not the only response to toxic solvents as also demonstrated by Pinkart et al. (1996). Recently, mutants of *Escherichia coli* showing resistance to some organic solvents were described (Ferrante et al. 1995, Nakajima et al. 1995). 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 (Hartmans et al. 1990, Weber et al. 1993). 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 (Weber et al. 1994). 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 (Sikkema et al. 1995). 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 (Weber et al. 1994).

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 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.

MATERIALS AND METHODS

Cultivation of the organism

P. putida S12 was grown in a chemostat on glucose (30 mM) in a mineral medium (Hartmans et al. 1989) as described before (Weber et al. 1994) and either in the presence or in the absence of 510 mg/l of toluene. 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/l of protein and stored on ice until used.

Accumulation assays

Assays were conducted in an Eppendorf Thermo-mixer at 30^0C 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/l of ^{14}C-toluene 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/l 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 (1956), with bovine serum albumin (Sigma, St. Louis, Mo.) as the standard.

RESULTS AND DISCUSSION

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 (Figure 3.1). 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.

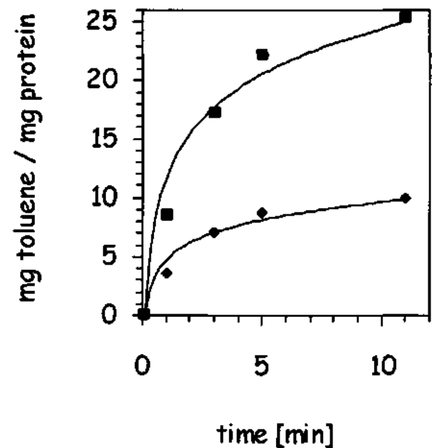

Figure 3.1 Accumulation of ^{14}C-toluene in *P. putida* S12 organisms that have either adapted (◆) or not adapted (■) to toluene.

In adapted cells the presence of either inhibitor resulted in significantly higher amounts of toluene accumulating in *P. putida* S12 (Figure 3.2). In

experiments with cells not adapted to toluene, no effect of the inhibitors on the amount of toluene accumulating in cells was seen (Figure 3.3).

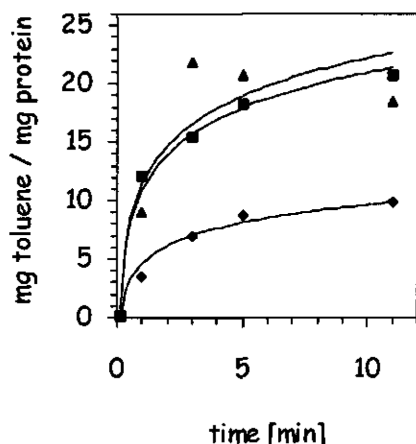

Figure 3.2 Effects of the presence of energy-coupling inhibitors on the accumulation of ^{14}C-toluene in toluene-adapted cells of *P. putida* S12. Cells not inhibited (◆) were compared with cells to which the respiratory chain inhibitor potassium cyanide at 20 mM (▲) or the proton conductor carbonyl cyanide *m*-chlorophenylhydrazone at 0.25 mM (■) was added via the washing buffer prior to the addition of ^{14}C-toluene.

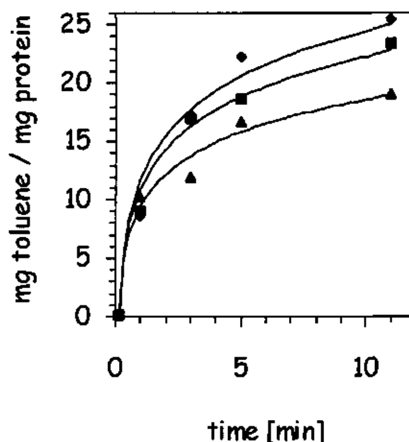

Figure 3.3 Effects of the presence of energy coupling inhibitors on the accumulation of ^{14}C-toluene in non-adapted cells of *P. putida* S12. Cells not inhibited (◆) were compared with cells to which the respiratory chain inhibitor potassium cyanide at 20 mM (▲) or the proton conductor carbonyl cyanide *m*-chlorophenylhydrazone at 0.25 mM (■) was added via the washing buffer prior to the addition of ^{14}C-toluene.

Conclusions from the experiments

The results as presented in Fig. 3.1 to 3.3 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. 3.1 and 3.2, 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 (Gottesman and Pastan 1993, Nikaido 1994). 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.

4
EFFECT OF SOLVENT ADAPTATION ON THE ANTIBIOTIC RESISTANCE IN *PSEUDOMONAS PUTIDA* S12

SONJA ISKEN, PEDRO M. A. C. SANTOS AND JAN A. M. DE BONT

Published in: Applied Microbiology and Biotechnology (1997) 48:642-647

ABSTRACT

The effect of the adaptation to toluene on the resistance to different antibiotics was investigated in the solvent-resistant strain *Pseudomonas putida* S12. We followed the process of the solvent adaptation of *P. putida* S12 by cultivating the strain in the presence of increasing concentrations of toluene and studied the correlation of this gradual adaptation to the resistance towards antibiotics. It was shown that the tolerance to various, chemically and structurally not related antibiotics with different targets in the cell, increased during this gradual adaptation. The survival of *P. putida* S12 in the presence of antibiotics like tetracycline, nigericin, polymyxin B, piperacillin or chloramphenicol increased within 30 and 1000 fold after the adaptation to 600 mg/l of toluene. However, cells grown in the absense of any solvents lost their adaptation to toluene even when grown in the presence of antibiotics. Results are discussed in terms of physico-chemical properties of membranes as affected by the observed *cis/trans* isomerization of unsaturated fatty acids as well as in term of active efflux of molecules from the cytoplasmic membrane.

INTRODUCTION

Many organic solvents such as toluene are toxic to organisms because they accumulate in and disrupt cell membranes (Sikkema et al. 1995). However, in recent years exceptional strains have been isolated which grow in the presence of a second phase of solvents earlier believed to be lethal (Cruden et al. 1992, Inoue and Horikoshi 1989, Ramos et al. 1995, Weber et al. 1993).

Different mechanisms contributing to the solvent tolerance of these strains have been suggested. Firstly, alterations in the cytoplasmic membrane suppressing the effects of solvents on the membrane stability were described. As the reaction to solvent stress *cis*-unsaturated fatty acids in the membrane lipids were transferred into *trans*-unsaturated fatty acids (Chen et al. 1995, Heipieper et al. 1992, Heipieper et al. 1995, Heipieper et al. 1996, Weber et al. 1994). Because of the steric differences between the two configurations of the unsaturated fatty acids, this isomerization reduces the membrane fluidity and acts against the increase in fluidity caused by the organic solvent (Diefenbach et al. 1992, Heipieper et al. 1994a, Sikkema et al. 1995, Weber et al. 1994). Another adaptive response in membranes of solvent-resistant *P. putida* was a changed headgroup composition of the membrane lipids (Weber and de Bont 1996).

Secondly, an altered lipopolysaccharide composition in the outer membrane may play a role in limiting the rate of diffusion of solvents into the cytoplasmic membrane. In the solvent-resistant strain *P. putida* Idaho xylene affected the composition of the lipopolysaccharides of the outer membrane (Pinkart et al. 1996). In addition, it could be shown that the outer membrane of *P. putida* S12 became less hydrophobic in response to toluene (Weber and de Bont 1996). Thirdly, we have been studying if the solvent-resistant strain *P. putida* S12 possesses an active efflux system exporting toluene out of the cell (Isken and de Bont 1996). By use of ^{14}C-labelled toluene, it was shown that an energy-dependent export system is indeed present in toluene-adapted cells. At the moment, this active efflux system is poorly understood. However, it may have features in common with the multidrug efflux pumps for antibiotics which have been studied in detail (Nikaido 1994a, Nikaido 1996). These energy-driven export systems play a major role in drug resistance, and they are able to pump out a wide range of compounds having no common chemical structure. Therefore, it is of great interest to determine if the tolerance of the solvent-resistant *P. putida* strains to organic solvents correlates with their antibiotic resistance.

In this paper we report the tolerance of toluene-adapted cells of *P. putida* S12 to antibiotics and vice versa and show a direct relation between solvent adaptation and resistance to antibiotics.

MATERIALS AND METHODS

Microorganism and culture conditions

Pseudomonas putida S12 was previously isolated as a styrene-degrading organism (Hartmans et al. 1990) growing in the presence of a second phase of various organic solvents, even if the solvent, like toluene, cannot be used as carbon source (Weber et al. 1993). The strain was cultivated in a minimal medium as described by Hartmans et al. 1989 with 15 mM glucose as the sole carbon source. For cultivation on solidified medium 3.5 g/l yeast extract and 15g/l agar were added. For batch cultures cells were grown in 100-ml shaken cultures in a horizontally shaking water bath at 30°C.

Continuous-culture experiments were performed in a chemostat with 0.8 litre working volume at 30^{0}C, pH 7.0, 600 rpm and at a dilution rate of 0.2 h^{-1}. Different toluene concentrations were supplied to the chemostat via the gas phase by passing a part of the airflow through a column (15 cm) filled with toluene. The total airflow was kept constantly at 320 ml/min.

Effect of the presence of antibiotics on the growth rate

An inoculum of 5% (v/v) overnight culture was transferred to fresh medium. After 3 h of exponential growth, the antibiotics were added. All the antibiotics (tetracycline, piperacillin, nigericin, chloramphenicol, penicillin G, ampicillin and polymyxin B) were obtained from Sigma Chemicals (St. Louis, Mo.). Cell growth was measured by monitoring the turbidity (optical density at 560 nm) of cell suspensions in a spectrophotometer. For antibiotics with an effect on the optical density we also followed the growth by measuring the CO_2 production via headspace analysis in a gas chromatograph (Packard, the Netherlands, model 427 with Hayesep Q column, Chrompack, the Netherlands).

Growth inhibition caused by the antibiotics was measured by comparing the differences in growth rates, μ (h^{-1}), of poisoned cultures with that of a control culture as described before (Heipieper et al. 1995). The LD_{50}'s were defined as the antibiotic-concentrations allowing half-maximal growth rates and the MIC's as the lowest antibiotic-concentrations which caused no increase in optical density within 5 h after the antibiotic was added.

Survival in the presence of antibiotics

The survival in the presence of antibiotics was determined by measuring the number of colonies forming units (CFU) in the presence and absence of different concentrations of antibiotics. Therefore, we plated 0.1 ml of suitable dilutions' in 0.9% (w/v) saline on agar-plates containing different concentrations of antibiotics. The same antibiotics as for the measurement of the growth rate-effect were

used and added to the medium after cooling down. After plating cells were incubated for 20 hours at 30^{0}C and the CFU were determined.

Survival in the presence of toluene

The survival in the presence of toluene was determined by measuring the number of colonies forming units (CFU) before and after incubating cells 1 h at 30°C in the presence of a second phase of toluene (1%(v/v)). The CFU were determined by plating 0.1 ml of suitable dilutions in 0.9% (w/v) saline on agar-plates. The agar plates were incubated for 20 hours at 30^{0}C and the CFU were counted.

Loss of adaptation

Adapted *Pseudomonas putida* S12 were obtained by cultivating cells in a chemostat in the presence of 600 mg/l of toluene. The loss of the adaptation to toluene was studied by transferring cells into fresh media and incubating them at 30°C in the presence and absence of different antibiotics or solvents. After 3h of growth the survival of these cells in the presence of a second phase of toluene was measured as described above and compared to the survival determined at the time of inoculation.

Determination of fatty acid composition

Cells of 50 ml suspension were centrifugated 3h after addition of the antibiotics and washed with phosphate buffer (50mM, pH 7.0). The lipids were extracted with chloroform/methanol/water as described by Bligh and Dyer 1959. Fatty acid methyl esters were prepared by a 15 min incubation at 95 ^{0}C in boron trifluoride/methanol using the method of Morrison and Smith 1964. The fatty acid methyl esters were extracted with hexane.

Fatty acid methyl ester analysis was performed using gas chromatography (GC) (capillary column: CP-Sil 88; 50 m; temperature program from 160 to 220 ^{0}C; flame ionisation detector). The instrument used was a CP-9000 gas chromatograph (Chrompack-Packard). The fatty acid methyl esters were identified with the aid of standards. The relative amounts of the fatty acids were determined from the peak areas of the methyl esters using a Chromatopac C-R6A integrator (Shimadsu, Kyoto, Japan). Replicate determinations indicated that the relative error (standard deviation/mean) x 100% of the values was 2-5%. The *trans/cis* ratio of unsaturated fatty acids was defined as the ratio between the amount of the two *trans*-unsaturated fatty acids and the two *cis*-unsaturated fatty acids of this bacterium as described before (Heipieper et al. 1995).

RESULTS

Effect of antibiotics on growth of non-adapted Pseudomonas putida S12

We investigated the effect of various antibiotics on *P. putida* S12 not adapted to toluene and showed that penicillin G and ampicillin did not affect the growth of *P. putida* S12, but five other antibiotics did. Cultures continued to grow exponentially after the addition of antibiotics, but with the exceptions of penicillin G and ampicillin, at reduced growth rates. The extent of these reductions in growth rates was a function of the concentration of the antibiotics (Figure 4.1). For all tested antibiotics the data collected in Figure 4.1 were used to determine the LD_{50} and MIC concentrations allowing half-maximal growth rates and preventing growth, respectively (Table 4.1).

Table 4.1 Effect of various antibiotics on non-adapted *Pseudomonas putida* S12

antibiotic	LD_{50}[a] [mg/l]	MIC[b] [mg/l]
tetracycline	0.8	3.0
chloramphenicol	21	40
nigericin	14	20
polymyxin B	3	15
piperacillin	8.5	30
penicilin G	>200	-
ampicillin	>250	-

[a] LD_{50} was defined as the concentration allowing half-maximal growth rate μ[hours^{-1}] within 3 hours

[b] MIC was defined as the lowest concentration not resulting in any CO_2 production and in any increase in optical density within 5 hours in liquid media

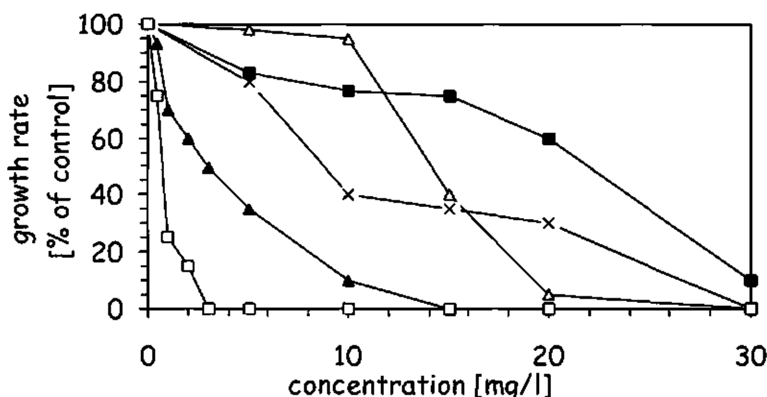

Figure 4.1 Effect of different antibiotics on the growth rate of non-adapted *Pseudomonas putida* S12 growing in liquid medium. Different concentrations of the antibiotics piperacillin (X), chloramphenicol (■), tetracycline (□), nigericin (△) and polymyxin B (▲) were added to exponentially growing cultures. The growth rates after the addition of the antibiotics are given relative to a control growing in the absence of antibiotics.

Effect of antibiotics on the membrane composition of growing cells

The effect of antibiotics on the fatty acid composition of cells was determined 3h after adding different concentrations of the antibiotics to exponentially growing liquid cultures. No significant changes in the level of saturation of fatty acids were observed (data not shown). Two of the five compounds tested had an effect on the formation of *trans* unsaturated fatty acids. Nigericin and polymyxin B led to an increase in the *trans/cis* ratio of the unsaturated fatty acids (Figure 4.2).

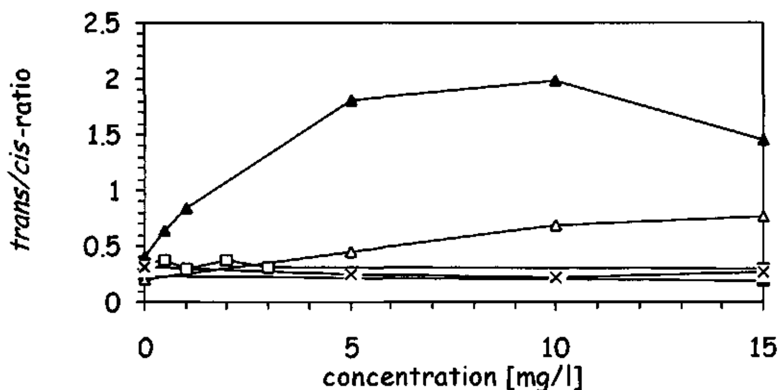

Figure 4.2 Effect of different concentrations of the antibiotics piperacillin (X), chloramphenicol (■), tetracycline (□), nigericin (△) and polymyxin B (▲) on the *trans/cis* ratio of the unsaturated fatty acids of non-adapted *Pseudomonas putida* S12 growing exponentially in batch cultures. The *trans/cis* ratio was determined after 3h incubation in the presence of the antibiotics.

Effect of adaptation to toluene on antibiotic resistance of P. putida S12

The stepwise adaptation of *P. putida* S12 to increasing concentrations of toluene was investigated with chemostat cultures. Cells were taken from steady states at various concentrations of toluene and were used to determine the CFU, the fatty acid composition and the survival in the presence of different antibiotics on agar plates. The adaptation to increasing concentrations of toluene led to an increase in the *trans/cis*-ratio of the unsaturated fatty acids (Figure 4.3A). For the investigations of the survival in the presence of antibiotics, we used the MIC concentrations determined on solid medium for non-adapted cells which were below those observed for liquid media in Table 4.1 (data not shown). For all antibiotics tested, survival expressed as the percentage of CFU in the presence of an antibiotic related to the CFU in the absence of any growth inhibitors increased dramatically during the adaptation procedure (Figure 4.3B).

The survival of the cells in the presence of tetracycline, chloramphenicol and piperacillin increased more than a factor of 1,000 after adapting the cells to 600 mg/l toluene. For nigericin and polymyxin B these factors were 500 and 30, respectively. Enhanced resistance was also observed for other antibiotic concentrations ranging between the LD_{50} and the MIC (results not shown).

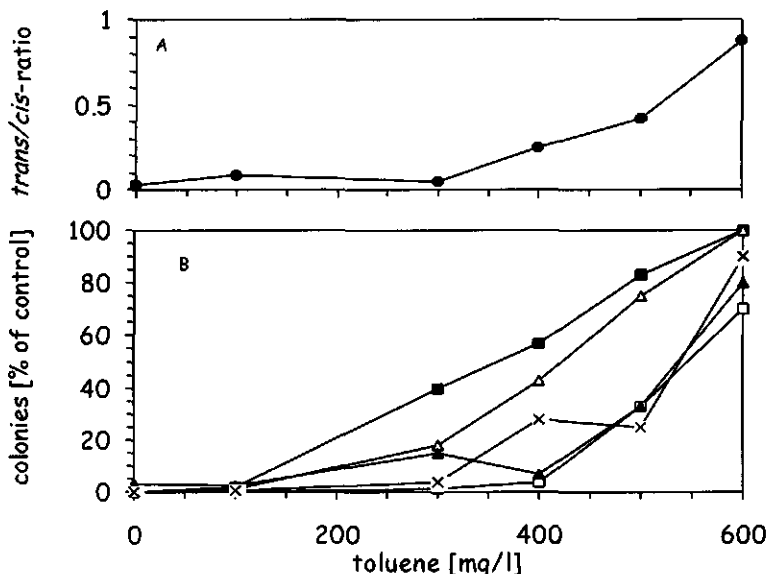

Figure 4.3 Effect of the adaptation of Pseudomonas putida S12 to toluene on the fatty acid composition and on the survival in the presence of antibiotics. Cells were grown in a chemostat under defined conditions in the presence of various concentrations of toluene. The trans/cis-ratios (•) of these chemostat grown cells are shown. (B) 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 (■), 1 mg/l tetracycline (□), 20 mg/l nigericin (△) and 1 mg/l polymyxin B (▲) is given relative to a control grown in the absence of antibiotics. These concentrations present the MIC's of non-adapted cells on solid medium.

Loss of the adaptation

P. putida S12 was grown in a chemostat in the presence of 600 mg/l of toluene. After reaching the steady state these adapted cells were transferred into fresh media and cultivated in the presence and absence of solvents or antibiotics for 3 h. After this, the survival in the presence of a second phase of toluene was determined. The survival in the presence of a second phase of toluene remained constant for cells cultivated in the presence of an organic solvent, but it dropped dramatically within 3h for cells cultivated in the absence of any solvent. This decrease of the survival rate was even stronger when cells grew in the presence of an antibiotic. The decrease of the survival in the presence of a second phase of toluene after 3h of cultivation in the presence and absence of different antibiotics and solvents is shown in Fig. 4.4.

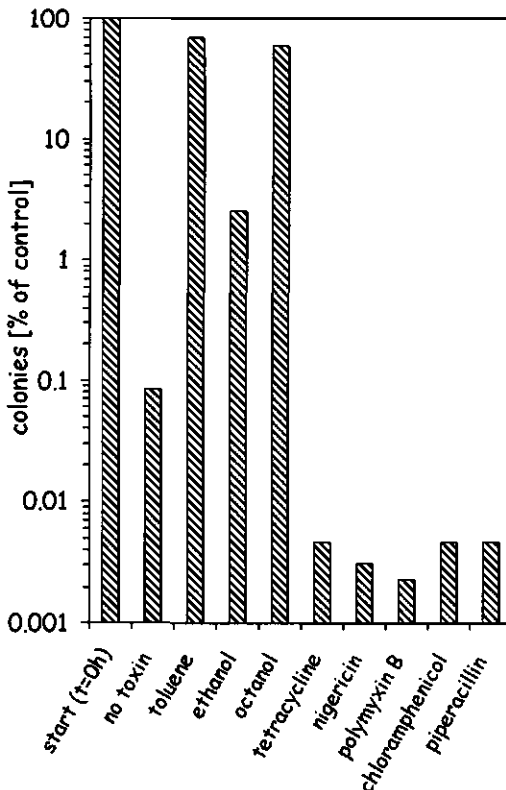

Figure 4.4 Effect of medium supplements on the loss of the adaptation of *Pseudomonas putida* S12 to toluene. Cells which had been adapted to 600 mg/l of toluene in a chemostat were transferred into fresh media supplemented with no toxin, 500 mg/l toluene, 31 g/l ethanol, 156 mg/l octanol, 1 mg/l tetracycline, 20 mg/l nigericin, 1 mg/l polymyxin B, 20 mg/l chloramphenicol or 15 mg/l piperacillin. After 3h in the presence of these different supplements a second phase of toluene was added. The CFU's after exposure to toluene is given as the percentage with respect to the CFU's before this exposure. The percentage of the cells which grew in the presence of 600 mg/l toluene in the chemostat and that could survive the exposure to a second phase of toluene is also given (start,t=0h).

DISCUSSION

When grown in a mineral medium supplied with glucose, *Pseudomonas putida* S12 was inhibited by various antibiotics that affect most Gram-negative bacteria. The tested antibiotics differ in their mode of action as well as in their chemical structures and physical properties. Chloramphenicol and tetracycline are both inhibitors of the protein-biosynthesis. However, chloramphenicol can be chemically classified as a derivative of phenylethylamine while tetracycline has a structure with four substituted rings. Furthermore, we used the antibiotics nigericin and polymyxin B, two ionophors destroying the electric or ionic gradients across the membrane. Nigericin consists of a non-cyclic chain of substituted heterocycles that gets into the active cyclic form due to the interaction with potassium ions while polymyxin B, belonging to the group of polypeptide antibiotics, is cyclic by itself. The fifth antibiotic used, piperacillin, is a charged derivate of penicillin. In contrast to other β-lactam antibiotics piperacillin can effectively inhibit Gram-negative bacteria.

Pseudomonas putida S12 adapted to toluene showed a response to the five antibiotics which was different from the cells grown in the absence of toluene. To allow the careful control of the amount of toluene to which cells are exposed, *P. putida* S12 was cultivated in a chemostat at varying toluene concentrations. Interestingly, the inhibition caused by any of the antibiotics dropped during this gradual adaptation to toluene. This increase in antibiotic-resistance was dependent on the dose of toluene to which the cultures were exposed to before. Therefore, the solvent toluene must induce or activate one or more mechanisms responsible for the antibiotic resistance. As the used antibiotics have different action targets in the cell and were structurally and chemically not related, it is more likely that more and aspecific mechanisms are engaged. These induced or activated changes could be at the level of the composition of the cytoplasmic membrane, at the level of the outer membrane, or might involve an active efflux system.

At the level of changes in the cytoplasmic membrane, we recorded the *cis/trans* isomerization of unsaturated fatty acids. The ionophores, nigericin and polymyxin B, have their target in the membrane. Therefore, adaptive membrane changes alter their target and make them presumably less effective. Indeed, both antibiotics induced the *cis* to *trans* isomerization whereas other antibiotics did not. This finding is supported by the earlier observation that the *cis/trans* isomerization system is induced by compounds affecting the fluidity and structure of bacterial membranes (Chen et al. 1995, Heipieper et al. 1994a, Keweloh and Heipieper 1996). Additionally, the role of the *cis/trans* isomerization in antibiotic resistance is also stressed by the observation that cells are more resistant to

antibiotics when they have been adapted to toluene concentrations which were shown to induce this isomerization (Figure 4.3). In fact, these changes on the level of the membranes reduce the permeability of the membrane (Heipieper et al. 1994a, Pinkart et al. 1996, Sikkema et al. 1995). Therefore, not only the ionophores but also the other antibiotics may be diminished in reaching their target. In contradiction to this speculation, it was shown that the membrane adaptation of the solvent-tolerant strain *Pseudomonas putida* Idaho did not effect the MIC of the antibiotic difloxacin although this MIC was lower in the solvent-sensitive strain *Pseudomonas putida* MW1200 (Pinkart et al. 1996).

Recently, we reported the presence of an active export system for the solvent toluene in *P. putida* S12 (Isken and de Bont 1996). The nature of this transport system is not clear, but it may have features in common with well studied aspecific export systems for antibiotics (Nikaido 1994a; Nikaido 1996). The question arises whether toluene induces a multidrug export system for antibiotics in *P. putida* S12 or whether the export system for toluene is aspecific and can export some antibiotics as well.

The first possibility can be supported by the observation that genes coding for proteins that are engaged in the export of hydrophobic uncouplers are inducable by structural unrelated hydrophobic compounds (Lewis et al. 1994). In our experiments, toluene may act as such an inducer for an antibiotic export system. In this case the strain would contain one or more other drug export systems in addition to the toluene export system studied recently.

If the second speculation was true, antibiotics should induce or activate the export system, and as a consequence, cells grown in the presence of high concentrations of antibiotics should be tolerant to toluene. Indeed, this is not the case. However, it is likely that the presence of the export system alone is not sufficient to archive solvent resistance and that the induction or activation of additional other adaptation mechanisms at the inner or outer membrane are required.

The importance of the combination of different mechanisms to prevent drug access to a bacterial cell in terms of permeability barriers and active efflux has been discussed earlier (Nikaido 1994a). In that study the importance of low-permeability barriers which surround bacteria is stressed although the most effective permeability barrier cannot completely shut out the influx of small molecules. Therefore, the most effective prevention of drug access connects the enhanced permeability barrier with active efflux systems. Similar considerations would hold here as we tested five different antibiotics with similar dose-response behaviour depending on the adaptation of the cells to toluene.

However, the adaptation of *P. putida* S12 to toluene does not only enhance the resistance to antibiotics, but also towards other solvents such as ethanol and towards heavy metals (Heipieper and de Bont 1994; Heipieper et al. 1996). This demonstrates the presence of a broad stress response system. This system is

readily activated by solvents but not by antibiotics. The induction of large amounts 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) led to the induction of 53 or 39 different proteins, respectively. Similar results were also observed with other genera. When *Clostridium acetobutylicum* initiates the solvent transformation various known heat shock proteins were expressed (Pich et al. 1990). In *Pseudomonas putida* KT2442 the expression of about 100 proteins was effected due to the presence of 2-chlorophenol (Lupi et al. 1995). Therefore, it is not surprising that a broad spectrum of mechanisms will be induced during the solvent adaptation. In recent years, genes involved in the tolerance of *Escherichia coli* to organic solvents have been studied (Aono et al. 1994; Ferrante et al. 1995; Nakajima et al. 1995a; Nakajima et al. 1995b). The expression of some of these genes led to slightly increased MIC values for several antibiotics and heavy metals (Aono et al. 1995, Nakajima et al. 1995a; Nakajima et al. 1995b). These genes are also responsible for regulation and are engaged in the stress response system.

5

PHYSIOLOGICAL CHARACTERIZATION OF THE SOLVENT EFFLUX SYSTEM OF *PSEUDOMONAS PUTIDA* S12

SONJA ISKEN AND JAN A. M. DE BONT

Accepted for publication in: Applied Microbiology and Biotechnology

ABSTRACT

The *active efflux system contributing to the solvent tolerance of Pseudomonas putida* S12 was characterized physiologically. The mutant *P. putida* JK1, which lacks the active efflux system, was compared with the wild type organism. None of 20 known substrates of common multi drug resistant pumps had a stronger growth inhibiting effect on the mutant than on the wild type. The amount of ^{14}C-toluene accumulating in *P. putida* S12 increased in the presence of the solvent xylene and in the presence of uncouplers. Thereby, the effect of uncouplers confirms the proton-dependency of the efflux system in *P. putida* S12. Other compounds, potential substrates for the solvent pump, did not affect the accumulation of ^{14}C-toluene. These results show that the efflux system in *P. putida* S12 is specific for organic solvents and does not export antibiotics or other known substrates of multi drug resistant pumps.

INTRODUCTION

Several *Pseudomonas putida* strains are tolerant to a wide variety of structurally unrelated hydrophobic solvents that are lethal for most other organisms (Inoue and Horikoshi 1989, Cruden et al. 1992, Weber et al. 1993, Ramos et al. 1995). These strains, able to grow in two-phase water-solvent systems, all have evolved a number of resistance mechanisms to the toxic effects of solvents (Ramos et al. 1997, Isken and De Bont 1998b, De Bont 1998). Thereby, an important mechanism is the active efflux of solvents from the cell. By using ^{14}C-labeled solvents, it was shown that an energy-dependent export system is present in toluene-adapted cells of *Pseudomonas putida* (Isken and de Bont 1996, Ramos et al. 1997). The genes of the efflux systems present in different solvent-tolerant *Pseudomonas* strains show homology to transporters involved in the extrusion of hydrophobic antibiotics from the membrane (Kieboom et al. 1998a, Ramos et al. 1998, Fukumori et al. 1998, Kim et al. 1998). Indeed, the antibiotic resistance of mutants lacking a solvent efflux system decreased in some cases (Ramos et al. 1998, Fukumori et al. 1998).

In *Pseudomonas putida* S12 the adaptation to toluene resulted in an increased resistance to various chemically and structurally unrelated hydrophobic antibiotics (Isken et al. 1997). It was not tested if this enhanced resistance of toluene-adapted cells to antibiotics is due to the induction of a specific resistance mechanism for combating antibiotics or if the solvent pump is able to accept antibiotics as substrates. Therefore, we have now determined whether the solvent efflux system in *P. putida* S12 can export antibiotics and thus also acts as a multidrug resistant pump.

MATERIALS AND METHODS

Microorganisms and culture conditions

P. putida S12 (ATTC 700801) was isolated as a styrene-degrading organism (Hartmans et al. 1990). This strain is able to grow in the presence of a second phase of various organic solvents even if these solvents, like toluene, cannot be metabolized (Weber et al. 1993). *P. putida* JK1 is a solvent-sensitive transposon mutant of *P. putida* S12. This mutant has interrupted *srpABC* genes, which code for the energy-dependent solvent-efflux-system (Kieboom et al. 1998a). Both strains were routinely grown in a minimal medium as described by Hartmans et al. (1989) with 15 mM glucose as the sole source of carbon and energy. 3.5 g yeast extract / l and 15 g agar / l were added for cultivation on solidified, complete medium. For the mutant strain 50 mg kanamycin / l was always added to the growth medium.

Continuous-cultivation was performed in a chemostat in the presence of toluene as described before (Isken et al. 1999).

Growth inhibition by substrates of multi drug resistant pumps

We used a plate diffusion test for the quantitative determination of the growth inhibition by known substrates of multi drug resistant pumps. Agar plates with complete medium were inoculated with a homogenous bacterial layer of *P. putida* S12 or *P. putida* JK1. Whatman No.1 filter disks (diameter 1 cm) containing 10 μl ethanolic stock solution of the compound tested were applied to the surface of the plates. After incubation for 24 hours at 30 ^0C we determined the diameter of the inhibition zone.

Accumulation of toluene in P. putida S12

Uptake experiments were performed based on the method described before (Isken and De Bont 1996). All cells used during these accumulation experiments were adapted to toluene in continuous culture. Assays were conducted at 30^0C in 1 ml incubation mixtures containing washed and energized cells to a final concentration of 0.4 g protein / l and 15 mM glucose. Inhibition or competition experiments were conducted in the presence of 1 mM EDTA when the molecular weight of the compound tested was higher than 600. In experiments with vanadate the phosphate buffer was replaced by MOPS-buffer supplemented with 10 mM $MgSO_4$. Cells were preincubated for 2 min at assay conditions. Then unlabeled inhibitors or competitors were added to the cell suspension and incubated for 2 more minutes. The assay was started by adding ^{14}C-toluene diluted with cold toluene to approximately 40,000 cpm per sample. We used a concentration of 1 mM of ^{14}C-toluene investigating competition with 1 mM of xylene and a concentration of 4 mM of ^{14}C-toluene investigating the effect of antibiotics and potential inhibitors of transport systems. After 10 min, cells were spun down for 1 min, washed and spun down again for 1 min. 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, USA). 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 method of Lowry et al. (1951) was used to determine the protein concentration using bovine serum albumin as standard. The given values represent the average of triplicate measurements and varied from the mean by not more than 15%. Every experiment was independently reproduced three times.

Chemicals

Uniformly ring-labeled toluene was obtained from American Radiolabeled Chemicals Inc., St. Louis, USA, with a specific activity of 55 mCi $mmol^{-1}$. Prior to utilization, 100 mM of vanadate (Aldrich, Zwijndrecht, The Netherlands) was set to pH 10 $\pm$ 0.1 with hydrochloric acid. The yellow solution was incubated at 100 ^0C until it became colorless. The concentration of vanadate was determined by

measuring the optical density at 265 nm and using an extinction coefficient of 2925 $M^{-1}cm^{-1}$ (Poolman, personal communication). All other chemicals were commercially available and used without any further purification.

RESULTS

Growth in the presence of potential substrates of multidrug efflux pumps

The effect of various known substrates of multidrug efflux pumps (Nikaido 1996, Paulsen et al. 1996) on the growth of *P. putida* S12 and its *srp*-negative mutant JK1 was studied using a plate diffusion test. With this approach, a concentration gradient of the compound tested is built up around one spot on the plate. Thus, differences in the size of the inhibition zone correspond to different growth inhibiting concentrations. We either applied concentrations that showed growth inhibition in previous experiments with *P. putida* S12 or the maximum solubility concentration of the tested compound. As shown in Table 5.1, the growth inhibition by all compounds tested was similar for the wild type and the mutant.

Table 5.1 Growth inhibition of *P. putida* S12 and of the solvent-sensitive mutant JK1 by various compounds known to be substrates of common multidrug efflux pumps. Plates were inoculated and the test compound was applied on Whatman No.1 filters (diameter 1 cm) that were positioned on the plate. Inhibition was assessed as the diameter of halo forming in cm after 24 hours of incubation.

compound	c_{stock} [g/l]	*P. putida* S12	*P. putida* JK1
control	pure ethanol	1.0	1.0
nigericin	1.5	1.2	1.2
valinomycin	1.1	1.4	1.4
polymyxin B	0.1	1.8	1.9
monensin	13.5	1.2	1.2
tetracycline	8	4.8	4.9
chloramphenicol	7	1.2	1.2
piperacillin	1.2	2.8	3.0
gramicidin D	2	1.6	1.4
CCCP	3.5	1.4	1.4
N-phenylnaphtylamine	10	1.6	1.6
Rhodamin G6	6.7	1.8	1.7
Rhodamin 123	1	1.2	1.2
Acridinorange	0.1	2.0	1.4
Acridinflavine	0.8	1.2	1.2
Doxorubin	0.6	1.2	1.2
Laudan	0.4	2.4	2.4
Prodan	0.7	1.6	1.5
ß-estradiol	1	1.8	1.8
progesterone	6.5	1.2	1.2
deoxycorticosterone	4	1.2	1.2

Substrate specificity of the solvent efflux system

The above results only give an indication of the substrate specificity of the solvent efflux pump of *P. putida* S12. More specific results for the substrate specificity can be obtained in competition experiments using ^{14}C-toluene in combination with the tested compound. Therefore, the solvent xylene and antibiotics with different chemical structures and sites of action were tested for their ability to compete with ^{14}C-toluene for transport.

Competition experiments with xylene were used as a positive control. In these experiments the occurrence of a second phase of the solvents was prevented by using 1 mM of xylene and only 1 mM of ^{14}C-toluene instead of the 4 mM of ^{14}C-toluene used in the standard assay. The concentration of ^{14}C-toluene in the cells was 2.2 μg ^{14}C-toluene / mg protein in the absence of xylene while in the presence of this competitor the concentration was 3.5 μg ^{14}C-toluene / mg protein. Therefore, the accumulation of ^{14}C-toluene similarly should increase in the presence of compounds that compete with toluene for the pump.

For the investigation of the competition with antibiotics, we used the minimum inhibitory concentration of the antibiotics as previously determined on solid medium for non-adapted cells (Isken et al. 1997). These concentrations were applied in order to make sure that an excess of the antibiotic was present in the assay. Furthermore, cells received an EDTA treatment to facilitate the entrance of antibiotics into the cells. This treatment had no effect on the accumulation of ^{14}C-toluene in the absence of antibiotics (data not shown). The results of the competition experiments are summarized in Figure 5.1. The presence of the antibiotics valinomycin, nigericin and polymyxin B that all act as uncouplers of the proton motive force lead to a higher concentration of ^{14}C-toluene in the cell. None of the other antibiotics tested affected the accumulation of toluene. In addition, reserpine, a known potent inhibitor of the multidrug resistant P-glycoprotein, and *ortho*-vanadate, an inhibitor of ATP driven transport systems, did not affect the accumulation of ^{14}C-toluene.

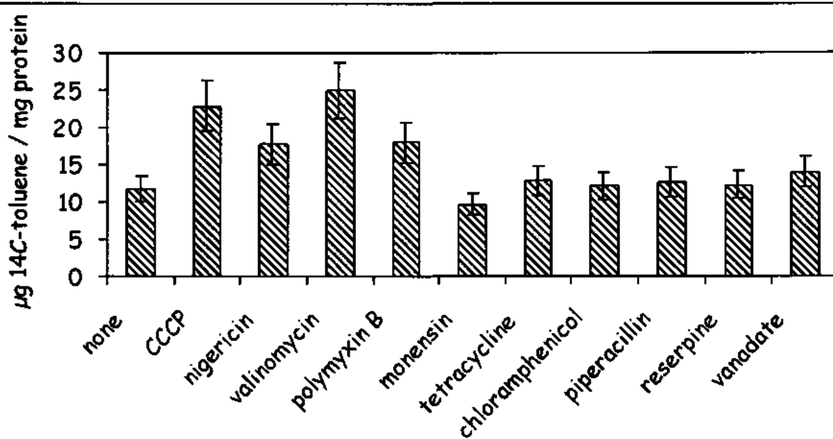

Figure 5.1 Accumulation of ^{14}C-toluene by toluene-adapted cells of *P. putida* S12 in the presence of unlabeled antibiotics and uncouplers. 20 mg/l nigericin, 200 mg/l valinomycin, 2 mg/l polymyxin B, 100 mg/l monensin, 1 mg/l tetracycline, 20 mg/l chloramphenicol, 15 mg/l piperacillin, 10 mg/l reserpine or 92 mg/l *ortho*-vanadate was added 2 min prior to the addition of ^{14}C-toluene (4 mM). The accumulation of ^{14}C-toluene in the cell was determined after 10 minutes. As a control the accumulation in the presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP) at 50 mg/l was determined.

DISCUSSION

Solvent efflux systems play an important role in constituting solvent tolerance to *Pseudomonas putida* (Isken and De Bont 1996, Ramos et al. 1997, Kieboom et al. 1998a). Different genes encoding for solvent efflux systems have been isolated which show homology with genes of known proton-dependent efflux systems (Kieboom et al. 1998a, Ramos et al. 1998, Fukumori et al. 1998). The energy dependency of transport systems can be characterized physiologically by use of inhibitors (Bolhuis et al. 1994). Indeed in the case of *P. putida* S12, we demonstrate that only compounds affecting the proton motive force can inhibit the active efflux of ^{14}C-toluene. Thus, also physiological studies confirm that the toluene export system in *P. putida* S12 is dependent on the proton motive force. This energy dependence of the export system may explain at least a part of the observed decrease in the cell growth efficiency (Isken et al. 1999).

The substrate specificity of solvent efflux pumps has been related to different growth inhibition of wild type and solvent-sensitive mutants (Kieboom et al. 1998a, Li et al. 1998, Fukumori et al. 1998, Kim et al. 1998). *P. putida* JK1, a mutant with inactive solvent efflux genes, is sensitive to several organic solvents

that are tolerated by the wild type *P. putida* S12 (Kieboom et al. 1998a). Now, we tested the growth of *P. putida* S12 and *P. putida* JK1 in the presence of 20 compounds that are known substrates of multidrug resistant pumps (Nikaido 1996, Paulsen et al. 1996). None had a stronger growth inhibiting effect on the mutant than on the wild type. However, changes in the medium composition can influence the results of growth studies. Therefore, we measured the competition of various compounds with ^{14}C-toluene to determine whether they are exported by the toluene export system in *P. putida* S12.

Every compound that is a substrate for the export system should compete with ^{14}C-toluene during the accumulation assay. Indeed, the solvent xylene does. However, the only antibiotics affecting the accumulation of ^{14}C-toluene in *P. putida* S12 are ionophores destroying the proton motive force. As discussed above, the effect of these antibiotics results from uncoupling rather than from competition. All other types of antibiotics show no competition with ^{14}C-toluene. Based on both the competition experiments and the growth studies, we conclude that antibiotics are no substrates of the solvent efflux system in *P. putida* S12.

As the solvent efflux pump in *P. putida* S12 does not accept antibiotics as substrates, other mechanisms must contribute to the reported relation between solvent tolerance and antibiotic resistance (Isken et al. 1997). Maybe organic solvents induce mechanisms involved in antibiotic resistance. Indeed, environmental pollutants like organic solvents select bacteria with antibiotic resistant phenotype in *P. aeruginosa* (Li and Poole 1999) and solvents induce large amounts of proteins in *P. putida* KT2442, a strain developing solvent-tolerant variants (Lupi et al. 1995, Fukumori et al. 1998). We can conclude that a number of different mechanisms may contribute to the correlation between antibiotic resistance and solvent tolerance, but the solvent efflux system of *Pseudomonas putida* S12 is not involved in antibiotic resistance.

6
Effect of Organic Solvents on the Yield of Solvent-tolerant *Pseudomonas putida* S12

Sonja Isken, Antoine Derks, Petra F.G. Wolffs and Jan A.M. de Bont

Published in: Applied and Environmental Microbiology (1999) 65: 2631-2635

Abstract

Solvent-tolerant micro-organisms are useful in biotransformations with whole cells in two-phase solvent-water systems. The results presented here describe effects organic solvents have on the growth of these organisms. The maximal growth rate of *Pseudomonas putida* S12, 0.8 h^{-1}, was not affected by toluene in batch cultures but in chemostat culture the solvent decreased the maximal growth rate by nearly 50%. Toluene, ethylbenzene, propylbenzene, xylene, hexane and cyclohexane reduced the biomass yield and this effect depended on the concentration of a solvent in the bacterial membrane and not on its chemical structure. The dose-response of solvents on yield was linear up to a concentration of approximate 200 mM of solvent in the bacterial membrane, both in the wild type and in a mutant lacking an active efflux system for toluene. Above this critical concentration the yield in the wild type remained constant at 0.2 g protein / g glucose by increasing concentrations of toluene.

The reduction of the yield in the presence of solvents is due to a maintenance higher by a factor three or four as well as to a decrease of the maximum growth yield by 33%. Therefore, energy-consuming adaptation processes as well as the uncoupling effect of the solvents reduce the yield of the tolerant cells.

INTRODUCTION

Many organic solvents are toxic for living organisms because of their devastating effects on biological membranes (Sikkema et al. 1995). This toxicity correlates with the hydrophobic character of a solvent expressed by the logarithm of its partition coefficient between octanol and water ($logP_{o/w}$ -value). Solvents with a $logP_{o/w}$-value between 1 and 5, like toluene, are highly toxic for whole cells (Sikkema et al. 1994). Due to these toxic effects, the choice of solvents for whole-cell biotransformations in two-phase solvent-water systems is limited. Only less toxic solvents with higher hydrophobicities can be applied (Salter and Kell 1995). In the last decade, however, more and more bacterial strains that can adapt to toxic organic solvents have been isolated and characterized (Inoue and Horikoshi 1989, Cruden et al. 1992, Weber et al. 1993, Ramos et al. 1995). These solvent-tolerant strains presumably will become a useful key in the performance of whole-cell biotransformations in the presence of these toxic, more polar solvents.

In recent years, many efforts have been made to uncover the mechanisms behind the solvent-tolerance of these strains belonging to the genus *Pseudomonas*. Up to now different adaptation mechanisms have been found. Alterations at the level of the cell-envelope structure, which suppress the effects of the solvents on the membrane stability or limit their rate of diffusion into the cell, have been described (Heipieper et al. 1992, Weber et al. 1994, Pinkart et al. 1996, Ramos et al. 1997). Furthermore, enhanced rates of phospholipid-biosynthesis, speeding up repairing processes, have been reported (Pinkart and White 1997). Last but not least, active export systems have been shown to exclude the solvent toluene from the cell (Isken and de Bont 1996, Ramos et al. 1997, Kieboom et al. 1998a, Fukumori et al. 1998). Active efflux of solvents is an energy-dependent process. Therefore, it should increase the maintenance-requirement of the cells in the presence of solvents. To what extent organic solvents enhance the energy requirement of the solvent-tolerant strains must still be determined.

However, although the adaptation mechanisms of some solvent-tolerant *Pseudomonas* strains have been studied in detail, no studies have been made of the effects of solvents on growth yields and maintenance requirements of these organisms. We now have determined the effect of toluene and other solvents on the growth parameters of *Pseudomonas putida* S12.

MATERIALS AND METHODS

Microorganism and media

Pseudomonas putida S12 was isolated as a styrene-degrading organism (Hartmans et al. 1990). This strain grows in the presence of a second phase of various organic solvents even if these solvents, like toluene, cannot be metabolized (Weber et al. 1993). *P. putida* JK1 is a solvent-sensitive transposon mutant of *P. putida* S12. This mutant has inactive *sprABC* genes which code for the energy-dependent solvent-efflux-system (Kieboom et al. 1998a). Both strains were cultivated in a minimal medium as described by Hartmans et al. (1989) with 1.8 g/l glucose as the sole source of carbon and energy. For cultivation on solidified medium 3.5 g/l yeast extract and 15 g/l agar were added. For the mutant strain 50 mg/l kanamycin was always added to the growth medium.

Batch cultivation

Batch culture cells were grown in 25-ml shaken cultures in 250 ml bottles sealed with Mininert valves (Phase Separations, Waddinxveen, The Netherlands) to prevent evaporation of the solvent. These valves possess movable teflon-rubber septa for both sealing with teflon and sampling. Different concentrations of toluene were added to the medium and equilibrated at 30°C for at least 12 h. The amount of solvent necessary to achieve a certain amount of solvent in the medium was calculated as follows:

$$V_{solvent,\ addition} = \frac{1}{\rho_{solvent}} * \left(\frac{1}{K_{water/air}} * \frac{m_{solvent,medium}}{V_{medium}} V_{air} + m_{solvent,medium} \right)$$

where $V_{solvent,\ addition}$ is the volume of solvent [µl] necessary to achieve the amount of solvent ($m_{solvent,medium}$ [mg]) in the medium, $\rho_{solvent}$ is the density of solvent [g/ml], $K_{water/air}$ is the partition coefficient of solvent between water and air as given by Amoore and Hautala (1983), V_{medium} is the volume of the medium and V_{air} is the volume of the air. Additionally, the concentration of a solvent was controlled by GC-analysis of the headspace.

Cells were precultivated in continuous culture (dilution rate = 0.2 h^{-1}) in medium saturated with toluene. After reaching the steady state these cells were used as inoculum (1 % (v/v)) for the batch cultures.

Cultivation of these batch cultures took place in a horizontally shaking water bath at 30°C. During growth we followed the optical density at 560 nm, the CO_2- and protein-production as well as the consumption of glucose. The maximal error occurred for the determination of protein and other parameters was 20% and 10%, respectively.

Continuous cultivation

Continuous-culture experiments were performed in a chemostat with 0.5 liter working volume at 30^{0}C, pH 7.0, 600 rpm and at the dilution rates mentioned. The amount of oxygen in the culture broth was determined with a Clark type oxygen electrode. Various solvents at different concentrations were supplied to the chemostat via the gas phase by passing a part of the air flow through a column filled with the solvent (at least 15 cm). The total air flow was kept constantly at 400 ml min^{-1}. Headspace samples of the air entering and leaving the chemostat were analyzed. The solvent concentrations in the medium were calculated from the concentration in headspace by using the partition coefficients given by Amoore and Hautala (1983). To achieve adaptation towards a certain solvent concentration the continuous culture was run first at a dilution rate of 0.05 hours^{-1} for at least 12 hours and then switched to the dilution rate of interest. The steady state was reached after five further exchanges of the volume. From the steady state we determined the concentration of protein in quintuplicate and the concentration of glucose remaining in the medium in duplicate. In this continuous approach the error of the protein and glucose determination was less than 15 % and 5 %, respectively.

Analytical methods

The amount of the metabolized carbon source, glucose, was determined by high-pressure liquid chromatography (HPLC) analysis of the culture supernatant. The supernatant was filtrated via a 0.2μm-pore-size filter prior to HPLC analysis performed at 70 ^{0}C on a ION-300 column (LC-Service, Emmen, The Netherlands) with 5mM H_2SO_4 as eluent and with refractive index detection (Grobben et al. 1995). Headspace analysis of organic solvents were performed by analyzing 100 μl of the gas phase on a model 437A gas chromatograph (Packard, Delft, The Netherlands) with a 10 % SE-30 Chromosorb WHP 80-100 mesh column (Chrompack, Middelburg, The Netherlands). The CO_2-concentration was measured via headspace analysis in a model 427 gas chromatograph (Packard) with Hayesep Q column (Chrompack). Dry weight was determined by drying washed cell suspensions at 105 ^{0}C for 24 hours prior to weighing. The method of Lowry et al. (1951) was used to determine the protein concentration with bovine serum albumin as standard.

Determination of yield and maintenance

We determined the yield by measuring the amount of protein produced per amount of glucose consumed. As protein constitutes to 60 % of the total cell dry weight in *Pseudomonas putida* S12 this value correlates with the amount of biomass produced per amount of glucose consumed.

The maximum growth yields and the maintenance coefficients were determined according the equation of Pirt (1975) from the data determined in the carbon-limited continuous culture as follows:

$$\frac{1}{Y_{obs}} = \frac{1}{Y_{max}} + \frac{m}{\mu_{obs}}$$

Y_{obs} is the observed growth yield, Y_{max} is the maximum growth yield, μ_{obs} is the observed specific growth rate as set by the dilution rate, and m is the maintenance metabolism rate.

Determination of solvent concentrations in the membrane

The amount of a solvent accumulating in the bacterial membrane was calculated from its concentration in the water phase and its log $P_{O/W}$, the logarithm of the partition coefficient of the solvent between octanol and water. For this calibration we made use of the equilibration found by Sikkema et al. (1994). This equilibration correlates the log $P_{O/W}$ with the log $P_{M/B}$, the logarithm of the partition coefficient of the solvent between membrane and buffer, as follows:

$$\log P_{M/B} = 0.97 \cdot \log P_{O/W} - 0.64$$

The values for the log $P_{O/W}$ were obtained from the list reported by Laane et al. (1987).

Chemicals

Toluene, benzene, ethylbenzene, propylbenzene, xylene, hexane, cyclohexane and hexadecane were obtained from Janssen Chimia (Tilburg, The Netherlands). All other chemicals were commercially available and used without any further purification.

RESULTS

Growth kinetics of P. putida S12 in batch cultures

P. putida S12 was precultivated in continuous culture in medium saturated with toluene. These adapted cells were used as inoculum for batch cultures and the growth was monitored. With this approach cells are already adapted in the beginning of a batch experiment. Therefore, the response of these cells to a new exposure of toluene demonstrates the effect of this solvent alone and not the induction of adaptation mechanisms. The batch growth rate was 0.8 h^{-1} and was not affected by the presence of toluene. However, variations in lag phase

occurred depending on the concentration of toluene present. Furthermore, the presence of toluene led to a lower yield. In the absence and presence of 6.2 mM toluene the yields observed at the end of the exponential phase were 0.34 and 0.20 g protein / g glucose, respectively. Growth kinetics are presented for cultures growing in either the absence or in the presence of 6.2 mM toluene (Figure 6.1). The yield and lag phase obtained for all concentrations of toluene tested are shown (Figure 6.4).

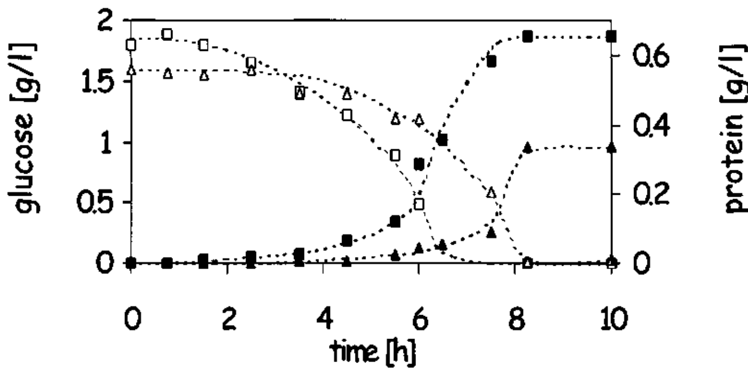

Figure 6.1 Growth kinetics of *Pseudomonas putida* S12 in batch cultures. Cells were adapted to toluene and transferred into minimal medium with glucose as sole source of carbon and energy. The concentrations of protein (filled symbols) and glucose (open symbols) were determined in the absence (square) or in the presence (triangle) of 6.2 mM toluene.

Effect of toluene on growth of P. putida S12 in carbon-limited continuous culture

P. putida S12 was cultivated in a carbon-limited chemostat at different dilution rates in the presence and absence of 6.2 mM toluene. After the cells had reached the steady states we determined the protein content and the concentration of glucose as the growth-limiting substrate (Figure 6.2). In the absence of toluene the wash out occurred at a dilution rate above 0.72 h^{-1}. In the presence of 6.2 mM toluene, however, this wash-out occurred at a lower growth rate and it was not possible to obtain a stable steady state at dilution rates above 0.4 h^{-1}. The presence of toluene also led to smaller amounts of biomass at all dilution rates. It was not possible to detect protein in the culture supernatant at all dilution rates tested.

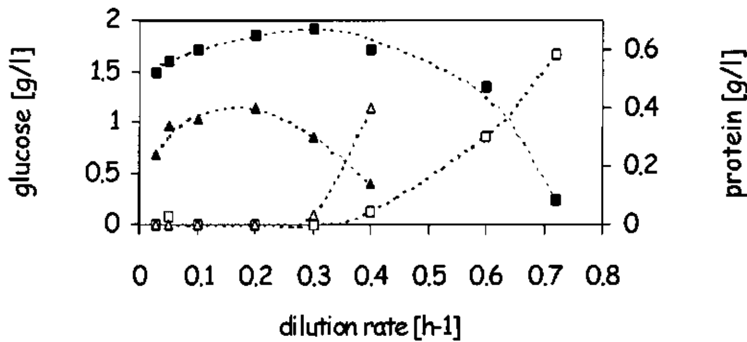

Figure 6.2 Growth of *Pseudomonas putida* S12 in a glucose-limited chemostat. Steady state values of the concentrations of protein (filled symbols) and glucose (open symbols) were determined at different dilution rates in the absence (square) and presence (triangle) of 6.2 mM toluene.

From the data in Figure 6.2 we calculated the growth parameters of *P. putida* S12 growing in the absence and presence of 6.2 mM toluene. The reciprocal values of yields were plotted against the reciprocal dilution rates (Figure 6.3). From the linear regressions of these plots the maintenance coefficients and the maximum growth yields were determined. In the absence and presence of toluene the maintenance coefficients were 0.023 and 0.076 g glucose / g protein • h^{-1}, respectively, and the maximum growth yields were 0.33 and 0.22 g protein / g glucose, respectively.

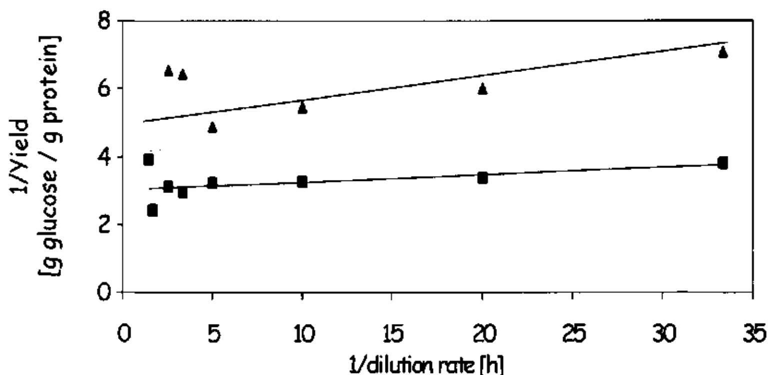

Figure 6.3 Graphic determination of maintenance coefficients and the maximum growth yields of *P. putida* S12 growing in a carbon-limited chemostat in the absence (■) and presence (▲) of 6.2 mM toluene. The linear regressions values (determined before wash out occurs) are as follows: 1/Y = 0.023/D + 3.00 (in the absence of toluene) and 1/Y = 0.076/D + 4.57 (in the presence of toluene).

Pseudomonas putida S12 was also cultivated in continuous culture at a dilution rate of 0.2 h^{-1} in the presence of various concentrations of toluene. Figure 6.4 shows the effect of toluene on the yield. Up to 3 mM concentration of toluene in the medium, the yield decreases linearly with increasing concentrations of toluene. Above this concentration of toluene, the yield remained nearly constant at 0.21 g protein / g glucose. This dose response is similar to results obtained in batch cultures.

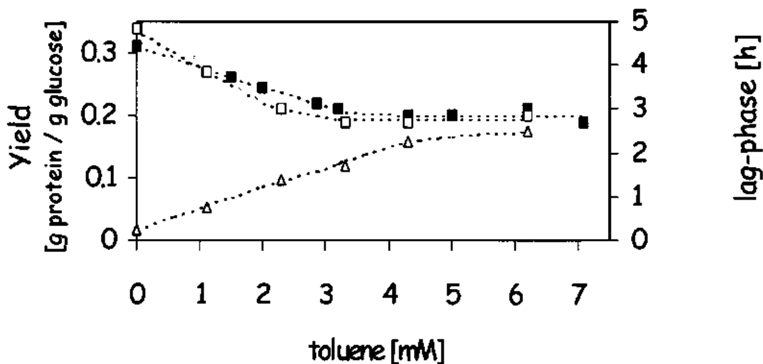

Figure 6.4 Effect of various concentrations of toluene on the lag-phase and yield of *Pseudomonas putida* S12. The lag-phase (△) is taken as the period of time from inoculation of batch cultures until an increase in the optical density was observed. Yields were determined in batch cultures at the end of the exponential phase (□) and from cells growing in a glucose-limited chemostat at a dilution rate of 0.2 h^{-1} (■).

Effect of different solvents on the yield of P. putida S12

P. putida S12 was grown in continuous culture at a dilution rate of 0.2 h^{-1}. Various aromatic and aliphatic organic solvents were added at different concentrations. We determined the concentration of the solvent in the medium and calculated the corresponding concentration in the bacterial membrane as described in Materials and Methods. We plotted the yield of *P. putida* S12 against these membrane concentrations (Figure 6.5). The plot shows a direct correlation between the yields observed and the concentration of solvents in the membrane, irrespective of the solvent tested.

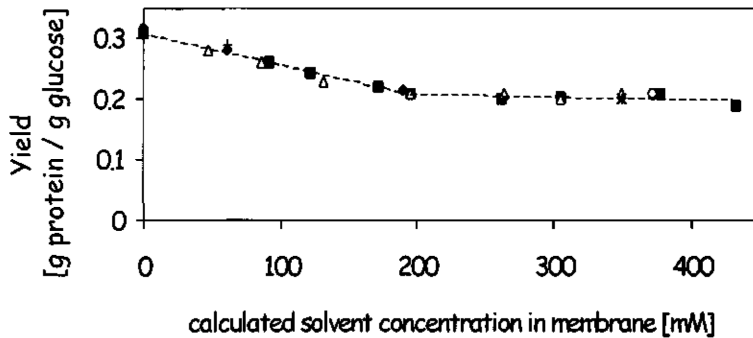

Figure 6.5 Effect of different solvents on the yield of *Pseudomonas putida* S12. Cells were cultivated in a glucose-limited chemostat at a dilution rate of $0.2h^{-1}$. Toluene (■), ethylbenzene (Δ), propylbenzene (*), xylene (O), hexane (+), cyclohexane (◆) and hexadecane (●) were added at different concentrations. The theoretical concentrations of the solvents in the bacterial membrane were calculated (see Materials and Methods) and the yields were plotted against these concentrations.

Effect of toluene on the yield of the solvent-sensitive mutant P. putida JK1

The effect of solvents was also studied in the solvent-sensitive mutant *P. putida* JK1. This mutant has an inactive operon for the solvent-efflux system. The cells were grown in batch cultures in the presence of various concentrations of toluene. The cells were transferred from lower toluene concentration to higher in small concentration steps of about 0.5 mM each at the end of the exponential phase. In this way growth of the mutant could be obtained up to toluene concentrations of 3.2 mM. Above this concentration no growth of the mutant strain was observed, while the wild type strain tolerated 6.2 mM of toluene. For both strains we monitored the protein production and glucose consumption in the presence of various concentrations of toluene. From these data yields were calculated and plotted against the concentration of toluene (Figure 6.6). Up to 2 mM concentration of toluene the yields of both strains were similar. Higher concentrations reduced the yield of the mutant to zero.

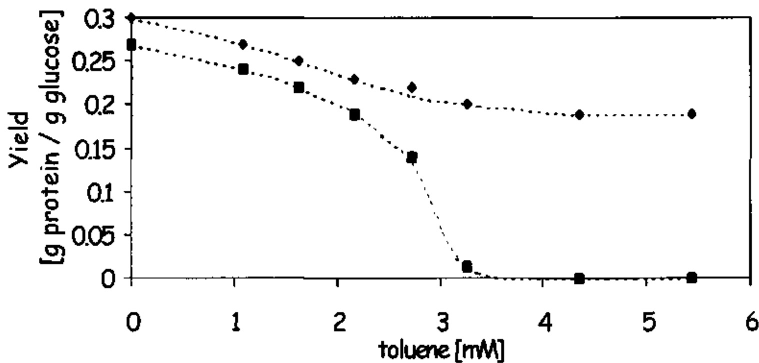

Figure 6.6 Effects of various concentrations of toluene on the yield of *P. putida* S12 (♦) and its solvent-sensitive mutant JK1 (■) growing in minimal medium in batch culture.

DISCUSSION

The growth-inhibiting effect of organic solvents on micro-organisms has been reported repeatedly (Salter and Kell 1995, Sikkema et al. 1995). The presence of solvents may lead to a reduction in the maximum growth rate, but cells which can adapt to solvents can achieve the same maximum growth rate in the presence of solvents (Inoue and Horikoshi 1989, Aono et al. 1992, Heipieper and de Bont 1994, Ramos et al. 1995). These results were obtained by employing batch systems, and we also observed that in batch cultivation no effect of toluene on the growth rate occurred. However, these results in the case of glucose as carbon source may be misleading as it was reported previously that gluconic acid transiently accumulates during the cultivation of *P. putida* on glucose (Hack et al. 1994). Under more defined conditions in continuous cultures we observed that the maximal growth rate is strongly affected by the presence of solvents. This reduction was not caused by oxygen limitation as we measured sufficient oxygen was present in all cases. We speculate the reduction of the maximum growth rate in the presence of toluene is caused by a reduction in the affinity of the cells for glucose. Such a change in the affinity for glucose may be caused by the adaptation mechanisms to toluene that reduce the permeability and change the structure of the cell envelope (Isken and de Bont 1998, Weber and de Bont 1996, Pinkart et al. 1996, Ramos et al. 1997).

The effects of solvents on the biomass yield was implied in previous reports (Aono et al. 1992, Abe et al. 1995, Ramos et al. 1997). From these reports it can be deduced that the presence of toluene leads to reduced yields. We observed similar results. In our batch cultures the yield as affected by toluene dropped by 30 %.

In continuous culture, yields and maintenance coefficients were obtained for *Pseudomonas putida* S12 grown under glucose limitation in either the presence or absence of toluene and other solvents. In the absence of solvents, both the maximal yield and the maintenance coefficient were similar to those reported for other *Pseudomonas* species growing on glucose under aerobic conditions (Pirt 1975, Verdoni et al. 1992, Hack et al. 1994, Wang et al. 1996).

The presence of toluene decreased the yield and increased the maintenance coefficient. These effects will be caused both by energy-consuming adaptation mechanisms and by a less effective energy metabolism in the presence of solvents. Specific energy-consuming processes include the active export of solvents in *P. putida* S12 (Isken and de Bont 1996, Kieboom et al. 1998a) and possibly an enhanced phospholipid biosynthesis rate as observed in *P. putida* Idaho (Pinkart and White 1997). Ineffective energy metabolism will occur due to the uncoupling character of organic solvents (Cartwright et al. 1986, Heipieper et al. 1991, Sikkema et al. 1992, Sikkema et al. 1994) and by disturbing effects of the solvents on the energy-transducing proteins (Sikkema et al. 1992, Sikkema et al. 1994) as observed in nontolerant microorganisms.

The dose-response effect of toluene on the overall yield of *P. putida* S12 was studied both in batch and chemostat culture. The yield decreased linearly with increasing concentrations (up to 3 mM) of toluene. Above this concentration, no further drop in the yield occurred. Adaptation of *P. putida* S12 to toluene has been observed at 3 mM or higher concentrations of toluene. Adaptation mechanisms triggered at this concentration include changes in the fatty acid profiles of membranes (Weber et al. 1994) and the active efflux of solvents from the membrane (Kieboom et al. 1998b). Cells precultured at this concentration or higher not only survived the presence of a second phase of toluene (Weber et al. 1993), but they also showed an enhanced resistance towards various antibiotics (Isken et al. 1997). Consequently, a toluene concentration of 3 mM is critical. Below 3 mM cells do not react to toluene and thus are slightly affected by the solvent. Above this concentration of toluene, various mechanisms come into operation to protect cells from excessive damage.

The constant yield values observed from 3 - 6 mM of toluene may indicate either that (i) the energy requirement of the adaptation mechanisms acting at these concentrations is very limited or (ii) the systems require substantial energy input but are compensated by the effective removal of toluene from the cell.

The results obtained for the wild type were confirmed in experiments with the solvent-sensitive mutant *P. putida* JK1. At low concentrations of toluene, the effects of solvents on the yield were similar in the wild type and the solvent-sensitive mutant *P. putida* JK1. The slightly lower yield of the mutant strain is caused by the presence of kanamycin as the selective marker. The mutant lacks the energy-consuming solvent-efflux system. Therefore, the reduction of the

yield observed at low concentrations of toluene cannot be caused by the energy requirement of the active efflux system.

Our results on yields of *P. putida* S12 as affected by various other solvents show that the dose-response relation of solvents is the same when the actual concentration in the bacterial membrane is taken as dose. Therefore, not the chemical structure but the amount of solvent accumulated in the bacterial membrane determines the effect of a solvent on the yield. Hence, the results found for toluene can be used for other solvents as well. It has been reported earlier that the concentration of solvents in bacterial membranes correlates directly with changes in the fatty acid profile and with the reduction of the maximal growth rate (Heipieper et al. 1995). In artificial membranes the same concentration of a solvent in the membrane results in the same expansion of the membrane. This membrane-concentration does also determine the release of ions and effect on the proton and electrical gradient (Sikkema et al. 1994).

We conclude that, at low concentrations, the effect of solvents on *P. putida* S12 is not different from their effects on any other cell. The difference between solvent-tolerant and -intolerant cells seems to be that the effect of solvents is counterbalanced at higher concentrations by specific mechanisms. We suggest that these mechanisms keep the actual concentration of solvents in the membrane constant. We think that this constant concentration of solvent in the membrane is approximate 200 mM, the concentration reached when adaptation starts.

7
CONCLUDING REMARKS

Although organic solvents are highly toxic for living organisms due to their ability to accumulate in and disrupt cell membranes, several bacterial strains have been isolated that can adapt to survive in the presence of these solvents (Inoue and Horikoshi 1989, Cruden et al. 1992, Weber et al. 1993, Ramos et al. 1995). In this thesis, the physiology of one such a microorganism, *Pseudomonas putida* S12, was studied with respect to its solvent-tolerance.

Adaptation mechanisms

Solvent-tolerant strains, like *Pseudomonas putida* S12, survive the presence of organic solvents because they induce or activate various defense mechanisms (chapter 2). The absence of one mechanism causes only a small decrease of the tolerance of an organism to solvents. Alone, even an important mechanism, like the active efflux pump for solvents discussed below, is not able to introduce the whole solvent-tolerant phenotype like that of *P. putida* S12 to a solvent-sensitive *Pseudomonas* strain (Kieboom et al. 1998a). Therefore, only the interplay of a number of mechanisms is sufficient to obtain solvent-tolerance. Although many mechanisms have been described up to now, it is still likely that additional mechanisms involved in solvent adaptation will be discovered in the future.

Changes in the membrane

The mechanisms described on the level of the bacterial cell envelope structure suppress the effect of solvents in the membrane. Most of these changes, like the *cis/trans* isomerization of unsaturated fatty acids, have been described also in solvent-sensitive microorganisms as a response to solvents or other membrane active compounds (Ingram 1986, Keweloh and Heipieper 1996). Indeed, almost all microorganisms change their membrane composition in response to various changes in the environment (Sinensky 1974, Shinitzky 1984, Grau and Mendoza 1993, Suutari and Laasko 1994). The *cis/trans* isomerization of unsaturated fatty acids in the membranes of solvent-tolerant bacteria is a short-term response (Keweloh and Heipieper 1996, Ramos et al. 1997). Therefore, such changes might be a fast general sign for a microorganism that it is exposed to stress (Heipieper et al. 1994, 1995, 1996).

Active efflux of solvents

The results presented in this thesis show, that changes in the structure of the cell envelope alone cannot explain the resistance to solvents like toluene: additional adaptation mechanisms are necessary. The active efflux system for toluene described in chapter 3 seems to be such an additional important adaptation mechanism. The active efflux system was the first defense mechanism described that does not only suppress the effect of solvents. It acts against the source of these effects, the solvents themselves. Recently, it was shown that strains of *Pseudomonas putida* can possess even more than one solvent efflux system (Mosqueda and Ramos 2000).

In the presence of an active efflux system the concentration of a solvent in the bacterial membrane can be below the theoretical equilibrium. However, such a system can only be effective if the permeability of the cell envelope is low. Otherwise the influx of the solvent would be too high even for the most effective pump. In general, the permeability of Gram-negative strains is lower than that of Gram-positive strains (Nikaido 1994b). This might be the explanation for the high tolerance of Gram-negative strains towards organic solvents.

Solvent-adaptation should decrease the permeability of the cell envelope of *P. putida* S12 for more hydrophobic compounds.

Permeability of the cell envelop

The permeability of the cell envelope of other *Pseudomonas* strains has been determined before (Plesiat et al. 1997). It was shown that a decrease of the permeability led to lower affinity of cells for substrates as expressed by the K_s values of the substrate oxidation. Therefore, we measured the affinity of adapted and non-adapted *P. putida* S12 for several substrates (Table 7.1).

Table 7.1 Effect of toluene-adaptation of *P. putida* S12 on the affinity for different substrates. Affinity constants (K_s) are given in μM.

substrate	non-adapted P. putida S12	adapted P. putida S12
ethanol	512	567
heptanol	0.17	0.6
octanol	0.5	7.5
glucose	10	420
acetate	2	2
succinate	50	151

The affinity for different aliphatic alcohols, that are carbon sources for this bacterium, changed hardly for the hydrophilic alcohol ethanol, but decreased for the more hydrophobic alcohols heptanol and octanol 3 and 15 fold,

respectively. The solvent adaptation seems to decrease the permeability of the cells for the more hydrophobic substrates.

However, the solvent adaptation will not only cause a general decrease of the permeability for hydrophobic compounds. In addition, the permeability for some compounds will change specifically. Adapted cells had a lower affinity for the hydrophilic substrates glucose and succinate. Polymyxins did not change the affinity of cells to these substrates when added to assays with toluene-adapted cells. Polymyxins are polycationic antibiotics with well-known unspecific outer membrane permeability - increasing properties (Vaara 1992). Therefore, the decrease of the affinity for glucose and succinate seems to be caused by a decrease of the permeability specific for these compounds.

The uptake of many substrates into *Pseudomonas aeruginosa* is facilitated by channels in the membrane (Nikaido 1992). Maybe the solvent adaptation of *P. putida* S12 changes the amount or specificity of such porines. As most compounds enter cells of *Pseudomonas* via such proteins, alternation on the levels of this porines may decrease the permeability of the cell envelope for some compounds.

Other compounds, however, will enter the cell via diffusion across the membrane. It has often been suggested that the diffusion of such compounds into Gram negative cells is limited by the hydrophilic layer on the upper side of the outer membrane (de Bont 1998). The outer membrane of adapted cells of *P. putida* S12 is more hydrophilic (Weber and de Bont 1996) and may, thereby, limit the entrance of hydrophobic compounds. However, other parts of the bacterial cell envelope are involved in limiting the entrance of hydrophobic compounds as well. In general, an interplay of the different parts of the cell envelope, the lipopolysaccarides, porines, periplasmatic compounds and the lipid bilayers with the embedded proteins determines the permeability of the cells.

Regulation of adaptation

Although the regulation of the various mechanisms is unknown, I speculate that they are connected to general stress response systems as described for heat shock (Mager and Kruijff 1995). Solvents are known to induce such a response. Otherwise, the tolerance to organic solvents would not correlate to the resistance to other harmful environmental factors (chapter 4). The fast-response adaptation mechanisms on the membrane level seem to be engaged in the first steps of a stress-response cascade. Their main function thereby is to close the membrane and thereby to hinder the cells to die in the presence of the solvents. After these fast-response mechanisms worked, the cells have time to induce long-term response mechanisms, which then allow the cells to adapt completely to the solvent. The regulation must be quite complex, as adaptation mechanisms must be active on various levels in the cell (Figure 7.1).

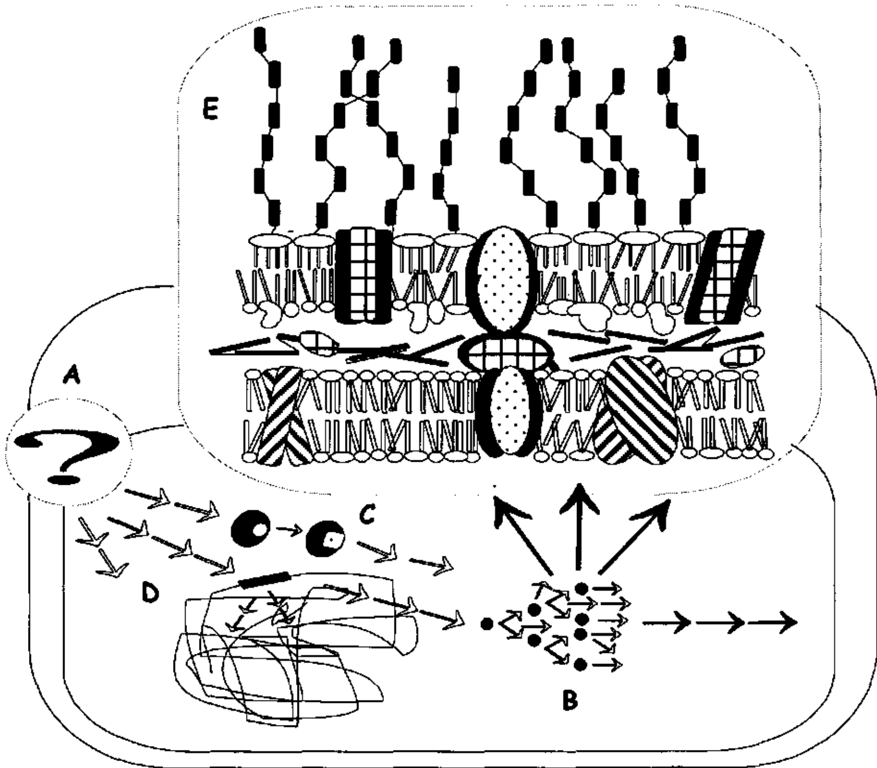

Figure 7.1 Speculation about the complex system that may mediate solvent tolerance in *Pseudomonas putida* S12. The diffusion of solvents into the membrane must activate a signal to start the first steps of a stress-response cascade (A). Within such a cascade (B) regulation may take place on both, protein or DNA level (C, D). Fast- and long-term response mechanisms, both mainly located on the cell envelope level (E), will be induced or activated in such a way.

Biotechnological applications

In the future, a number of applications for solvent-tolerant bacteria are possible. First, the application of solvent-tolerant strains in waste gas and wastewater treatment can introduce more stable systems. Although the mineralization should not be different in tolerant and non-tolerant strains, the solvent-tolerant strains are better adapted to survive peaks of higher concentrations of organic solvents. Second, the survival in the presence of higher concentrations may speed up bioremediation processes limited by toxicity effects. For these applications solvent tolerance should also be studied in mixed cultures and soil systems. The level of tolerance to a certain organic solvent can be different in such more complex systems (Huertas et al. 1998).

A third group of possible applications are biotransformations with whole cells in two-phase solvent-water systems. Such two-phase systems have been studied in order to cope with the toxicity of accumulating products (Nikolova and Ward 1993, Salter and Kell 1995, de Bont 1998). Extraction of toxic products into an organic phase can allow and enhance the production of various compounds, like e.g. epoxides, with whole-cell biotransformations. As long as solvent-sensitive strains are used these systems can only be applied for hydrophobic products. To remove less hydrophobic compounds a more hydrophilic solvent is necessary. However, normal cells cannot survive a solvent as a second phase if its log $P_{O/W}$ value is less than 4 (Sikkema et al. 1995). With solvent-tolerant stains such solvents can be applied. Therefore, more solvents can be used in such two-liquid water-solvent systems.

Medical aspects

Besides the possible biotechnological applications, solvent tolerance is of interest in relation to antibiotic resistance in pathogenic microorganisms. The solvent-tolerant strains used in this and other studies are not pathogenic. However, we were able cultivate a pathogenic *Pseudomonas aeruginosa* strain in the presence of a second phase of toluene. Thus, at least some pathogenic strains can adapt to solvents. The adaptation to hydrophobic organic solvents increases the resistance of bacteria to various antibiotics (chapter 4, Aono et al. 1995, Nakajima et al. 1995a,b, Li and Poole 1999). Antibiotic resistance is one of the most important challenges in the fight against infectious diseases (Neu 1992, Nikaido 1994a, Chopra et al. 1997, Levy 1998). In part because of the rise in resistance to antibiotics, the death rates for some communicable diseases such as tuberculosis have started to rise again, after having declined in the industrial nations. Many studies have focused on *Mycobacterium tuberculosis, Staphylococcus aureus* and *Streptococcus pneumoniae*. However, also strains of *Pseudomonas aeruginosa* already evade every antibiotic in the clinician's armamentarium, a stockpile of more than 100 drugs (Levy 1998).

Until now, it has not been explained how pathogenic strains can keep antibiotic resistance in the absence of antibiotics. Maybe the presence of hydrophobic compounds in the environment of such bacteria is responsible for their constant resistance. Interestingly, many hydrophobic solvents have been used in or as disinfectants. I suggest that these disinfectants play a role in antibiotic resistance.

Final remark

When solvent-tolerant strains are exposed to a shock of toluene the major part of the population will die (Weber et al. 1993). All solvent-tolerant strains described so far need an adaptation period to cope with the solvent stress (chapter 2). Even after such an adaptation period, the presence of

solvents enhances the energy requirement of the cells (chapter 6). Therefore, I prefer to speak about solvent tolerance rather than resistance.

REFERENCES

1. Abe A, Inoue A, Usami R, Moriya K, Horikoshi K (1995) Degradation of polyaromatic hydrocarbons by organic solvent-tolerant bacteria from deep sea. Biosci Biotech Biochem 59:1154-1156
2. Amoore JE, Hautala E (1983) Odor as an aid to chemical safety: odor thresholds compared with threshold limit values and volatilities for 214 industrial chemicals in air and water dilution. J Appl Tox 3:272-290
3. Angelova B, Schmauder HP (1999) Lipophilic compounds in biotechnology - interactions with cells and technological problems. J Biotechnol 67:13-32
4. Aono R, Aibe K, Inoue A, Horikoshi K (1991) Preparation of organic solvent-tolerant mutants from *Escherichia coli* K-12. Agric Biol Chem 55:1935-1938
5. Aono R, Ito M, Inoue A, Horikoshi K (1992) Isolation of novel toluene-tolerant strain *Pseudomonas aeruginosa*. Biosci Biotech Biochem 56:145-146
6. Aono R, Kobayashi H, Joblin KN, Horikoshi K (1994a) Effects of organic solvents on growth of *Escherichia coli* K12. Biosci Biotech Biochem 58:2009-2014
7. Aono R, Negishi T, Nakajima H (1994b) Cloning of organic solvent tolerance gene *ostA* that determines *n*-hexane tolerance level in *Escherichia coli*. Appl Environ Microbiol 60:4624-4626
8. Aono R, Kobayashi M, Nakjima H, Kobayashi H (1995) A close correlation between improvement of organic solvent tolerance levels and alteration of resistance towards low levels of multiple antibiotics in *Escherichia coli*. Biosci Biotech Biochem 59:213-218
9. Aono R, Kobayashi H (1997) Cell surface properties of organic solvent-tolerant mutants of *Escherichia coli* K12. Appl Environ Microbiol 63:3637-3642
10. Asako H, Nakajima H, Kobayashi K, Kobayashi M, Aono R (1997) Organic solvent tolerance and antibiotic resistance increased by overexpression of *marA* in *Escherichia coli*. Appl Environ Microbiol 63:1428-1433
11. Asako H, Kobayashi K, Aono R (1999) Organic solvent tolerance of *Escherichia coli* is independent of OmpF levels in the membrane. Appl Environ Microbiol 65:294-296
12. Barr DP, Aust SD (1994) Pollutant degradation by white rot fungi. Rev Environ Contam Toxicol 138:49-72
13. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911-917
14. Blom A, Harder W, Matin A (1992) Unique and overlapping pollutant stress proteins of *Escherichia coli*. Appl Environ Microbiol 58:331-334

15. Blum DJW, Speece RE (1991) A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. Res J WPCF 63:198-207
16. Bolhuis H, Molenaar D, Poelarends G, Veen HW van, Poolman B, Driessen AJM, Konings WN (1994) Proton motive force-driven and ATP-dependent drug extrusion systems in multidrug-resistant *Lactococcus lactis*. J Bacteriol 176:6957-6964
17. Bont JAM de (1998) Solvent-tolerant bacteria in biocatalysis. Trends in Biotechnol 16:493-499
18. Bowles LK, Ellefson WL (1985) Effects of butanol on *Clostridium acetobutylicum*. Appl Environ Microbiol 50:1165-1170
19. Bringer S, Härtner T, Poralla K, Sahm H (1985) Influence of ethanol on the hopanoid content and the fatty acid pattern in batch and continuous cultures of *Zymomonas mobilis*. Arch Microbiol 140:312-316
20. Carrea G, Ottolina G, Riva S (1995) Role of solvents in the control of enzyme selectivity in organic media. Trends Biotechnol 13:63-70
21. Cartwright CP, Juroszek J-R, Beavan MJ, Ruby FMS, Morais SMF de, Rose AH (1986) Ethanol dissipates the proton motive force across the plasma membrane of *Saccharomyces cereviseae*. J Gen Microbiol 132:369-377
22. Chamberlain NR, Mehrtens BG, Xiong Z, Kapral FA, Boardman JL, Rearick JI (1991) Correlation of carotenoid production, decrease in membrane fluidity and resistance to oleic acid killing in Staphylococcus aureus. Infect Immun 59:1332-4337
23. Chen Q, Janssen DB, Witholt B (1995a) Effects of octane on the fatty acid composition and transition temperature of *Pseudomonas oleovorans* membrane lipids during growth in two-liquid-phase continuous cultures. Enzyme Microb Technol 17:647-652
24. Chen Q, Janssen DB, Witholt B (1995b) Growth on octane alters the membrane lipid fatty acids of *Pseudomonas oleovorans* due to the induction of *alkB* and synthesis of octanol. J Bacteriol 177:6894-6901
25. Chen Q (1996) Growth of *Pseudomonas oleovorans* in two liquid phase media. Effects of organic solvents and *alk* gene expression on the membrane. pHD-thesis ETH Zürich Switzerland
26. Chopra I, Hodgonson J, Metcalf B, Poste G (1997) The search for antimicrobial agents effective against bacteria resistant to multiple antibiotics. Antimicrob Agents Chemother 41:497-503
27. Clark DP, Beard JP (1979) Altered phospholipid composition in mutants of *Escherichia coli* sensitive or resistant to organic solvents. J Gen Microbiol 113:267-274
28. Cruden DL, Wolfram JH, Rogers RD, Gibson DT (1992) Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. Appl Environ Microbiol 58:2723-2729
29. Dagley S (1986) Microbial metabolism of aromatic compounds In: Moo-Young M (ed) Comprehensive biotechnology, Bull AT, Dalton H (eds) vol. 1: The principles of biotechnology: scientific fundamentals. Pergamon press, Oxford

30. Davidson PM, Branden AL (1981) Antimicrobial activity of non-halogenated phenolic compounds. J Food Prot 44:623-632
31. Diefenbach R, Heipieper HJ, Keweloh H (1992) The conversion of *cis* into *trans* unsaturated fatty acids in *Pseudomonas putida* P8: evidence for a role in the regulation of membrane fluidity. Appl Microbiol Biotechnol 38:382-387
32. Diefenbach R, Keweloh H (1994) Synthesis of *trans* unsaturated fatty acids in *Pseudomonas putida* P8 by direct isomerization of the double bond of lipids. Arch Microbiol 162:120-125
33. Dombek KM, Ingram LO (1984) Effects of ethanol on the *Escherichia coli* plasma membrane. J Bacteriol 157:233-239
34. Fay JP, Farias RN (1977) Inhibitory action of a non-metabolizable fatty acid on growth of *Escherichia coli*: role of metabolism and outer membrane integrity. J Bacteriol 132:790-795
35. Ferrante AA, Augliera J, Lewis K, Klibanov AM (1995) Cloning of an organic solvent-resistance gene in *Escherichia coli*: the unexpected role of alkylhydroperoxide reductase. Proc Natl Acad Sci 92:7617-7621
36. Fukumori F, Hirayama H, Takami H, Inoue A, Horikoshi K (1998) Isolation and transposon mutagenesis of a *Pseudomonas putida* KT2442 toluene-resistant variant: involvement of an efflux system in solvent resistance. Extremophiles 2:395-400
37. Gage DJ, Neidhardt FC (1993) Adaptation of *Escherichia coli* to the uncoupler of oxidative phosphorylation 2,4-Dinitrophenol. J Bacteriol 175:7105-7108
38. George AM (1996) Multidrug resistance in enteric and other Gram-negative bacteria. FEMS Microbiol Letters 139:1-10
39. Gibson DT, Subramanian V (1984) Microbial degradation of aromatic hydrocarbons. In: Gibson DT (ed) Microbial degradation of organic compounds. Marcel Dekker, Inc., New York
40. Gottesman MM, Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 62:385-427
41. Grau R, Mendoza D (1993) Regulation of the synthesis of unsaturated fatty acids by growth temperature in *Bacillus subtilis*. Mol Microbiol 8:535-542
42. Grobben GJ, Sikkema J, Smith MR, Bont JAM de (1995) Production of extracellulair polysaccharides by *Lactobacillus delbrueckii* ssp. *bulgaricus* NCFB 2772 grown in a chemically defined medium. J Appl Bacteriol 79:103-107
43. Guckert JB, Hood MA, White DC (1986) Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increase in the *trans/cis* ratio and proportions of cyclopropyl fatty acids. Appl Environ Microbiol 52:794-801
44. Hack CJ, Woodley JM, Lilly MD, Liddell JM (1994) The production of *Pseudomonas putida* for the hydroxylation of toluene to its *cis*-glycol. Appl. Microb Biotechnol 41:495-499
45. Harrop AJ Hocknull MD, Lilly MD (1989) Biotransformation in organic solvents: a difference between Gram-positive and gram-negative bacteria. Biotechnol Letters 11:807-810
46. Hartmans S, Smits JP, Werf MJ van der, Volkering F, Bont JAM de (1989) Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. Appl Environ Microbiol 55:2850-2855

47. Hartmans S, Werf MJ van der, Bont JAM de (1990) Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. Appl Environ Microbiol 56:1347-1351
48. Heipieper HJ, Keweloh H, Rehm HJ (1991) Influence of phenols on growth and membrane permeability of free and immobilized *Escherichia coli* Appl Environ Microbiol 57:1213-1217
49. Heipieper HJ, Diefenbach R, Keweloh H (1992) A possible mechanism of the protection of the phenol degrading strain *Pseudomonas putida* P8 from the toxicity of the substrate: The conversion of *cis-* into *trans-* unsaturated fatty acids. Appl Environ Microbiol 58:1847-1852
50. Heipieper HJ, Bont JAM de (1994a) Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of the fatty acid composition of membranes. Appl Environ Microbiol 60:4440-4444
51. Heipieper HJ, Weber FJ, Sikkema J, Keweloh H, Bont JAM de (1994b) Mechanisms of resistance of whole cells to toxic organic solvents. Trends Biotechnol 12:409-415
52. Heipieper HJ, Loffeld B, Keweloh H, Bont JAM de (1995) The *cis/trans* isomerization of unsaturated fatty acids in *Pseudomonas putida* S12:an indicator for environmental stress due to organic compounds. Chemosphere 30:1041-1051
53. Heipieper HJ, Meulenbeld G, van Oirschot Q, Bont JAM de (1996) Effect of environmental factors on the *trans/cis* ratio of unsaturated fatty acids in *Pseudomonas putida* S12. Appl Environ Microbiol 62:2773-2777
54. Holtwick R, Meinhardt F, Keweloh H (1997) *cis-trans* isomerization of unsaturated fatty acids: cloning and sequencing of the *cti* gene from *Pseudomonas putida* P8. Appl Environ Microbiol 63:4292-4297
55. Holtwick R, Keweloh H, Meinhardt F (1999) *cis/trans* Isomerase of unsaturated fatty acids of *Pseudomonas putida* P8: Evidence for a heme protein of the cytochrome c type. Appl Environ Microbiol 65:2644-2649
56. Hugo WB (1978) Phenols: a review of their history and development as antimicrobial agents. Microbios 23:83-85
57. Huertas MJ, Duque E, Marques S, Ramos JL (1998) survival in soil of different toluene-degrading *Pseudomonas* strains after solvent shock. Appl Environ Microbiol 64:38-42
58. Ikura Y, Yoshida Y, Kudo T (1997) Physiological properties of two *Pseudomonas mendocina* strains which assimilate styrene in a two-phase (solvent-aqueous) system under static culture conditions. J Ferment Bioeng 83:604-607
59. Ingram LO (1976) Adaptation of membrane lipids to alkohols. J Bacteriol 125:670-678
60. Ingram LO (1977) Changes in lipid composition of *Escherichia coli* resulting from growth with organic solvents and with food additives. Appl Environ Microbiol 33:1233-1236
61. Ingram LO (1986) Microbial tolerance of alcohols: role of the cell membrane. Trends Biotechnol 4:40-44
62. Ingram LO (1990) Ethanol tolerance in bacteria. Crit Rev Biotechnol 9:305-319
63. Inoue A, Horikoshi K (1989) A *Pseudomonas* thrives in high concentrations of toluene. Nature 338:264-266

64. Inoue A, Yamamoto M, Horikoshi K (1991) *Pseudomonas putida* which can grow in the presence of toluene. Appl Environ Microbiol 57:1560-1562
65. Inoue A, Horikoshi K (1991) Estimation of solvent-tolerance of bacteria by the solvent parameter Log P. J Ferment Bioeng 71:194-196
66. Isken S, Bont JAM de (1996) Active efflux of toluene in a solvent-resistant bacterium. J Bacteriol 178:6056-6058
67. Isken S, Santos PMAC, Bont JAM de (1997) Effect of solvent adaptation on the antibiotic resistance in *Pseudomonas putida* S12. Appl Microbiol Biotechnol 48:642-647
68. Isken S, Bont JAM de (1998a) Isolation of several solvent tolerant Bacillus strains from bank-soil. In: Abstracts of Meeting on Extremophiles 1998 Yokohama Japan
69. Isken S, Bont JAM de (1998b) Solvent-tolerant bacteria. Extremophiles 2:229-238
70. Isken S, Derks A, Wolffs PFG, Bont JAM de (1999) Effect of organic solvents on the yield of solvent-tolerant *Pseudomonas putida* S12. Appl Environ Microbiol 65:2631-2635
71. Jackson RW, Moss JA de (1965) Effects of toluene on *Escherichia coli*. J Bacteriol 90:1420-1425
72. Jarlier V, Nikaido H (1994) Mycobacterial cell wall: structure and role in natural resistance to antibiotics. FEMS Microbiol Lett 123:11-18
73. Jenkins RO, Stephens GM, Dalton H (1987) Production of toluene *cis*-glycol by *Pseudomonas putida* in glucose feed-batch culture. Biotechnol Bioeng 29:873-883
74. Jüttner F, Henatsch JJ (1986) Anoxic hypolimnion is a significant source of biogenic toluene. Nature 323:797-798
75. Keweloh H, Weyrauch G, Rehm HJ (1990) Phenol-induced membrane changes in free and immobilized *Escherichia coli* Appl Microbiol Biotechnol 33:66-71
76. Keweloh H, Heipieper H-J (1996) *Trans* unsaturated fatty acids in bacteria. Lipids 31:129-137
77. Kieboom J, Dennis JJ, Bont JAM de, Zylstra GJ (1998a) Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J Biol Chem 273:85-91
78. Kieboom J, Dennis JJ, Bont JAM de, Zylstra GJ (1998b) Active Efflux of organic solvents in *Pseudomonas putida* S12 is induced by solvents. J Bacteriol 180:6769-6772
79. Kim K, Lee S, Lee K, Lim D (1998) Isolation and characterization of toluene-sensitive mutants from the toluene-resistant bacterium *Pseudomonas putida* GM73. J Bacteriol 180:3692-3696
80. Komatsu T, Moriya K, Horikoshi K (1994) Preparation of organic solvent-tolerant mutants from *Pseudomonas aeruginosa* strain PAO1161. Biosci Biotech Biochem 59:1754-1755
81. Laane C, Boeren S, Hilhorst R, Veeger C (1987) Optimization of biocatalysis in organic media. In: Laane C, Tramper J, Lilly MD (eds) Biocatalysis in Organic Media. Elsevier Science Publishers, Amsterdam

82. Leão C, Uden N van (1984) Effects of ethanol and other alcohols on passive proton influx in the yeast *Saccharomyces ceriviseae*. Biochim Biophys Acta 774:43-48
83. León R, Fernandes P, Pinheeiro HM, Cabral JMS (1998) Whole-cell biocatalysis in organic media. Enzyme Microb Technol 23:483-500
84. Levy SB (1998) The challenge of antibiotic resistance. Scientific American March 1998:32-39
85. Lewis K, Naroditskaya V, Ferrante A, Fokina I (1994) Bacterial resistance to uncouplers. J Bioenerg Biomembr 20:639-646
86. Li L, Komatsu T, Inoue A, Horikoshi K (1995) A toluene-tolerant mutant of *Pseudomonas aeruginosa* lacking the outer membrane protein F. Biosci Biotech Biochem 59:2358-2359
87. Li XZ, Zhang L, Poole K (1998) Role of the multidrug efflux systems of Pseudomonas aeruginosa in organic solvent resistance. J Bacteriol 180:2987-2991
88. Li XZ, Poole K (1999) Organic solvent-tolerant mutants of Pseudomonas aeruginosa display multiple antibiotic resistance. Can J Microbiol 45:18-22
89. Loosdrecht MCM van, Lyklema J, Norde W, Zehnder AJB (1990) Influence of interfaces on microbial activity. Microbiol Rev 54:75-87
90. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265-275
91. Lucchini JJ, Corre J, Cremieux A (1990) Antimicrobial activity of phenolic compounds and aromatic alkohols. Res Microbiol 141:499-510
92. Lupi CG, Colangelo T, Mason CA (1995) Two-dimensional gel electrophoresis analysis of the response of *Pseudomonas putida* KT2442 to 2-chlorophenol. Appl Environ Microbiol 61:2863-2872
93. Mager WH, Kruijff de AJJ (1995) Stress-induced transcriptional activation. Microbiol Rev 59:506-531
94. Mokrasch LC, McGilvery RW (1956) Purification and properties of fructose-1,6-diphosphatase. J Biol Chem 221:909-917
95. Monti JA, Christian ST, Schutzbach JS (1987) Effects of dolichol on membrane permeability. Biochim Biophys Acta 905:133-142
96. Morrison WR, Smith LM (1964) Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. J Lipid Res 5:600-608
97. Moriya K, Horikoshi K (1993a) Isolation of a benzene-tolerant bacterium and its hydrocarbon degradation. J Ferment Bioeng 76:168-173
98. Moriya K, Horikoshi K (1993b) A benzene-tolerant bacterium utilizing sulfur compounds isolated from deep sea. J Ferment Bioeng 76:397-399
99. Mosqueda G, Ramos JL (2000) A set of genes encoding a second toluene efflux system in *Pseudomonas putida* DOT-T1E is linked to the *tod* genes for toluene metabolism. J Bacteriol 182:937-943
100. Naglak TJ, Hettwer DJ, Wang HY (1990) Chemical permiabilization of cells for intracellular product release. In: Asenjo JA (ed) Separation Processes in Biotechnology. Marcel Dekker, New York
101. Nakajima H, Kobayashi H, Aono R, Horikoshi K (1992) Effective isolation and identification of toluene-tolerant *Pseudomonas* strains. Biosci Biotech Biochem 56:18721873

102. Nakajima H, Kobayashi K, Asako H, Aono R (1995a) Overexpression of the *robA* gene increases organic solvent tolerance and multiple antibiotic and heavy metal ion resistance in *Escherichia coli*. Appl Environ Microbiol 61:2302-2307
103. Nakajima H, Kobayashi M, Negishi T, Aono R (1995b) *SoxRS* gene increased the level of organic solvent tolerance in *Escherichia coli*. Biosci Biotech Biochem 59:1323-1325
104. Neu HC (1992) The crisis in antibiotic resistance. Science 257:1064-1073
105. Nikaido H (1992) Nonspecific and specific permeation channels of the *Pseudomonas aeruginosa* outer membrane. In: Galli E, Silver S, Witholt B (eds) *Pseudomonas* - molecular biology and biotechnology. American Society for Microbiology, Washington, D.C., pp 146-153
106. Nikaido H (1994a) Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science 264:382-388
107. Nikaido H (1994b) Porins and specific diffusion channels in bacterial outer membranes. J Biol Chem 269:3905-3908
108. Nikaido H (1996) Multidrug efflux pumps of Gram-negative bacteria. J Bacteriol 178:5853-5859
109. Nikolova P, Ward OP (1993) Whole cell biocatalysis in nonconventional media. J Ind Microbiol 12:76-86
110. Nonino EA (1997) Where is the citrus industry going? Perfumer Flavorist 22:53-58
111. Ogino H, Miyamoto K, Ishikawa H (1994) Organic solvent-tolerant bacterium which secretes an organic solvent-stable lipolytic enzyme. Appl Environ Microbiol 60:3884-3886
112. Ogino H, Yasui K, Shiotani T, Ishihara T, Ishikawa H (1995) Organic solvent-tolerant bacterium which secretes an organic solvent-stable proteolytic enzyme. Appl Environ Microbiol 61:4258-4262
113. Osborne SJ, Leaver J, Turner MK, Dunnill P (1990) Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. Enzyme Microb Technol 12:281-291
114. Paje MLF, Neilan BA, Couperwhite I (1997) A *Rhodococcus* species that thrives on medium saturated with liquid benzene. Microbiology 143:2975-2981
115. Paulsen IT, Brown MH, Skurray RA (1996) Proton-dependent multidrug efflux systems. Microbiol Rev 60:575-608
116. Pedrotta V, Witholt B (1999) Isolation and characterization of the *cis-trans*-unsaturated fatty acid isomerase of *Pseudomonas oleovorans* Gpo12. J Bacteriol 181:3256-3261
117. Pich A, Narberhaus F, Bahl H (1990) Induction of heat shock proteins during initiation of solvent formation in *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 33:697-704
118. Pinkart HC, Wolfram JW, Rogers R, White DC (1996) Cell envelope changes in solvent-tolerant and solvent-sensitive *Pseudomonas putida* strains following exposure to *o*-xylene. Appl Environ Microbiol 62:1129-1132
119. Pinkart HC, White DC (1997) Phospholipid biosynthesis and solvent tolerance in *Pseudomonas putida* strains. J Bacteriol 179:4219-4226

120. Pirt SJ (1975) Principles of microbe and cell cultivation. p. 63-70. Wiley, New York, NY

121. Plesiat P (1997) Use of steroids to monitor alterations in the outer membrane of *Pseudomonas aeruginosa*. J Bacteriol 179:7004-7010

122. Ramos JL, Duque E, Huertas M-J, Haïdour A (1995) Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. J Bacteriol 177:3911-3916

123. Ramos JL, Duque E, Rodriguez-Herva JJ, Godoy P, Haïdour A, Reyes F, Fernandez-Barrero A (1997) Mechanisms for solvent tolerance in bacteria. J Biol Chem 272:3887-3890

124. Ramos JL, Duque E, Godoy P, Segura A (1998) Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. J Bacteriol 180:3323-3329

125. Rezessy-Szabo JM, Huijberts GNM, Bont JAM de (1987) Potential of organic solvents in cultivation microorganisms on toxic water-insoluble compounds. In: Laane C, Tramper J, Lilly MD (eds) Biocatalysis in Organic Media. Elsevier Science Publishers, Amsterdam

126. Salter GJ, Kell DB (1995) Solvent selection for whole cell biotransformation in organic media. Crit Rev Biotechnol 15:139-177

127. Seeman P (1972) The membrane action of anesthetics and tranquilizers. Pharmacol Rev 24:583-655

128. Shima H, Kudo T, Horikoshi K (1991) Isolation of toluene-resistant mutants from *Pseudomonas putida* PpG1 (ATCC 17453). Agric Biol Chem 55(4):1197-1199

129. Shimooka T, Shibata A, Terada H (1992) The local anesthetic tetracaine destabilizes membrane structure by interaction with polar headgroups of phospholipids. Biochim Biophys Acta 1104:261-268

130. Shinitsky M (1984) Membrane fluidity and cellular functions. In: Shinitsky M (ed.) Physiology of membrane fluidity. vol II, CRC Press Inc., Boca Raton, Florida

131. Shirai K (1987) Catechol production from benzene through reaction with resting and immobilized cells of mutant strains of *Pseudomonas*. Agric Biol Chem 51:121-128

132. Sierra-Alvarez R, Lettinga G (1991) The effect of aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge. Appl Microbiol Biotechnol 34:544-550

133. Sikkema J, Bont JAM de (1991) Isolation and initial characterization of bacteria growing on tetralin. Biodegradation 2:15-23

134. Sikkema J, Poolman B, Konings WN, Bont JAM de (1992) Effects of the membrane action of tetraline on the function and structural properties of artificial and bacterial membranes. J Bacteriol 174:2986-2992

135. Sikkema J, Bont JAM de, Poolman B (1994) Interactions of cyclic hydrocarbons with biological membranes. J Biol Chem 269:8022-8028

136. Sikkema J, Bont JAM de, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59:201-222

137. Sinensky M (1974) Homeoviscous adaptation- a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proc Natl Acad Sci 71:522-525

138. Smet de MJ, Kingma J, Witholt B (1978) The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli* Biochim Biophys Acta 506:64-80
139. Smith MR (1990) The biodegradation of aromatic hydrocarbons by bacteria. Biodegradation 1:191-206
140. Smith MR (1994) The physiology of aromatic hydrocarbon degrading bacteria. In: Ratledge C (ed) Biochemistry of microbial degradation. Kluwer Academic Publishers, Dordrecht, The Netherlands
141. Suutari M, Laakso S (1994) Microbial fatty acids and thermal adaptation. Crit Rev Microbiol 20:285-328
142. Uribe S, Ramirez J, Peña A (1985) Effects of ß-pinene on yeast membrane functions. J Bacteriol 161:1195-1200
143. Uribe S, Rangel P, Espinola G, Aguirre G (1990) Effects of cyclohexane, an industrial solvent, on the yeast *Saccharomyces cerevisiae* and on isolated yeast mitochondria. Appl Environ Microbiol 56:2114-2119
144. Vaara M (1992) Agents that increase the permeability of the outer membrane. Microbiol Rev 56:395-411
145. Verdoni N, Aon MA, Lebeault JM (1992) Metabolic and energetic control of *Pseudomonas mendocina* growth during transition from aerobic to oxygen-limited conditions in chemostat cultures. Appl Environ Microbiol 58:3150-3156
146. Vermuë M, Sikkema J, Verheul A, Bakker R, Tramper J (1993) Toxicity of homologous series of organic solvents for the Gram-positive bacteria *Arthrobacter* and *Nocardia* sp. and the Gram-negative bacteria *Acinetobacter* and *Pseudomonas* sp. Biotechnol Bioeng 42:747-758
147. Volsky VT, Grady CPL, Tabak HH (1990) Effect of selected RCRA compounds on activated sludge activity. Res J WPCF 62:654-664
148. Wang K-W, Baltzis BC, Lewandowski GA (1996) Kinetics of phenol biodegradation in the presence of glucose. Biotechnol Bioeng 51:87-94
149. Weber FJ, Ooijkaas LP, Schemen RMW, Hartmans S, Bont JAM de (1993) Adaptation of *Pseudomonas putida* to high concentrations of styrene and other organic compounds. Appl Environ Microbiol 59:3502-3504
150. Weber FJ, Isken S, Bont JAM de (1994) *Cis/trans* isomerization of fatty acids as a defense mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. Microbiology 140:2013-2017
151. Weber FJ, Bont JAM de (1996) Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. Biochim Biophys Acta 1286:225-245
152. White DG, Goldman JD, Demple B, Levy SB (1997) Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA, soxS*, or *robA* in *Escherichia coli*. J Bacteriol 179:6122-6126
153. Woldringh CL (1973) Effects of toluene and phenethyl alcohol on the ultrastructure of *Escherichia coli*. J Bacteriol 114:1359-1361
154. Yoshida Y, Ikura Y, Kudo T (1997) Production of 3 vinylcatechol and physiological properties of *Pseudomonas* LF-3, which can assimilate styrene in a two-phase (solvent-aqueous) system. Biosci Biotech Biochem 61:46-50

155. Yuli I, Wilbrandt W, Shinitzky M (1981) Glucose transport through cell membranes of modified lipid fluidity. Biochemistry 20:4250-4256

156. Zeng T, Driessen AJM, Konings WN (1988) Effect of cholesterol on the branched-chain amino acid transport system of *Streptococcus cremoris*. J Bacteriol 170:3194-3198

SUMMARY

Hydrophobic organic solvents, like toluene, are toxic for living organisms. This toxicity is an important drawback in the environmental biotechnology as well as in the application of solvents in the production of fine chemicals by whole-cell biotransformations. The effects of organic solvents on micro-organisms have been studied extensively. It was shown that the toxicity of hydrophobic organic solvents is mainly caused by the ability of such solvents to intercalate and accumulate in biological membranes.

In the last decade, however, several strains that can survive the presence of toxic organic solvents have been isolated (chapter 2). One of the solvent-tolerant strains is *Pseudomonas putida* S12, studied in this thesis. This strain can grow in the presence of a second phase of organic solvents that have a log $P_{O/W}$ (logarithm of the partition coefficient between octanol and water) value equal to or higher than 2.3. *P. putida* S12 is able to survive the presence of solvents because of different adaptation mechanisms. It has been known that this strain can suppress the effect of organic solvents on the membranes by a *cis* to *trans* isomerization of the unsaturated fatty acids of the membrane.

In chapter 3, a further adaptation mechanism is presented. It is shown that cells adapted to toluene posses an active export system for toluene. Therefore, *P. putida* S12 compensates not only the toxic effects of organic solvents on the membrane, but decrease also actively the amount of the toxic solvent in the cell. In the presence of the efflux system the concentration of a solvent in the bacterial membrane can be below the theoretical equilibrium. This active efflux system depends on the proton motive force.

Since we reported the presence of an active export system for solvents, it has been suggested repeatedly, that this system is connected with the well-described efflux systems for antibiotics. Indeed, the adaptation of *P. putida* S12 to toluene enhances the resistance of this strain to various chemically and structurally unrelated antibiotics, with different targets in the cell (chapter 4).

However, we could demonstrate that efflux system for toluene in *P. putida* S12 does not export antibiotics. This efflux system is specific for solvents like toluene and *p*-xylene (chapter 5). Therefore, the adaptation to an organic solvent must activate other mechanisms responsible for the resistance towards these antibiotics. I suggest that this is connected to a general stress

response (chapter 4 and chapter 2). This general stress response may also cause the decrease of the cell-envelope permeability discussed in chapter 7.

The broad effects solvents have even on the solvent-tolerant strain *P. putida* S12 are demonstrated in chapter 6. The presence of toluene reduces the maximum growth yield and increases the maintenance requirement. Interestingly, other solvents had a similar effect as toluene as long as they reached the same concentration in the bacterial membrane. Not the chemical structure but the amount of solvent accumulated in the bacterial membrane determines the effect of a solvent on the cells. Therefore, results obtained with toluene can be extrapolated to other solvents as well.

SAMENVATTING

Hydrofobe organische oplosmiddelen, zoals tolueen, zijn toxisch voor levende organismen. Deze toxiciteit is een belangrijk probleem in zowel de milieubiotechnologie als in de toepassing van oplosmiddelen bij de productie van fijnchemicaliën door hele cellen. De invloed van organische oplosmiddelen op micro-organismen is al uitgebreid onderzocht. De toxiciteit van hydrofobe organische oplosmiddelen wordt hoofdzakelijk veroorzaakt door de eigenschap van deze oplosmiddelen om in biologische membranen binnen te dringen en zich daar op te hopen.

In de laatste tien jaar zijn echter verscheidene stammen geïsoleerd die bestand zijn tegen toxische organische oplossmiddelen (hoofdstuk 2). Een van deze oplosmiddel-tolerante stammen is *Pseudomonas putida* S12, die is bestudeerd in dit proefschrift. Deze bacterie kan groeien in de aanwezigheid van een tweede fase van organische oplosmiddelen die een log $P_{O/W}$ waarde (logaritme van de verdelingscoëfficient tussen octanol en water) hebben die gelijk is aan of groter dan 2.3. *P. putida* S12 is in staat de aanwezigheid van oplosmiddelen te overleven dankzij verschillende adaptatiemechanismen. Bekend is dat deze stam het effect van oplosmiddelen op de membranen kan onderdrukken door een isomerisatie van de onverzadigde vetzuren in de membraan van een *cis* naar een *trans* vorm.

In hoofdstuk 3 is een ander adaptatiemechanisme beschreven. Er is aangetoond dat cellen die geadapteerd zijn aan tolueen een actief exportsysteem bezitten voor dit oplosmiddel. *P. putida* S12 compenseert dus niet alleen de toxische uitwerking van tolueen op de membraan, maar verlaagt ook actief de hoeveelheid tolueen in de cel. In aanwezigheid van een exportsysteem kan de concentratie van een oplosmiddel in de bacteriële membraan onder de theoretische evenwichtwaarde blijven. Deze actieve efflux van tolueen is afhankelijk van energie in de vorm van een *proton motive force*.

Sinds we de aanwezigheid van een actief exportmechanisme hebben bekendgemaakt, is herhaaldelijk gesuggereerd dat dit systeem gekoppeld is aan de veelbeschreven exportsystemen voor antibiotica. Inderdaad verhoogt de adaptatie van *P. putida* S12 aan tolueen de resistentie van deze bacterie tegen diverse chemisch en structureel verschillende antibiotica die verschillende aangrijpingspunten in de cel hebben (hoofdstuk 4).

Niettemin pompt het actieve exportsysteem voor tolueen van *P. putida* S12 geen antibiotica naar buiten. Dit exportsysteem is specifiek voor oplosmiddelen zoals tolueen en *p*-xyleen (hoofdstuk 5). Daarom moet de adaptatie aan een organisch oplosmiddel andere mechanismen activeren die verantwoordelijk zijn voor de resistentie tegen deze antibiotica. Ik suggereer dat dit is gekoppeld aan een algemene reactie op stress (hoofdstuk 4 en hoofdstuk 2). Deze algemene reactie op stress zou ook de in hoofdstuk 7 bediscussieerde afname van de doorlaatbaarheid van de celenvelop kunnen veroorzaken.

De uitgebreide effecten die oplosmiddelen hebben, zelfs op de oplosmiddel-tolerante stam *P. putida* S12, zijn weergegeven in hoofdstuk 6. De aanwezigheid van tolueen verlaagt de maximale groeiopbrengst en verhoogt de energie nodig voor celonderhoudsprocessen (maintenance). Interessant is dat verschillende andere oplosmiddelen een vergelijkbare uitwerking als tolueen hadden zodra zij dezelfde concentratie bereikten in de membraan. Daarom bepaalt niet alleen de chemische structuur, maar de hoeveelheid oplosmiddel die zich in de membraan heeft opgehoopt de invloed van een oplosmiddel op de celopbrengst en maintenance. Dus kunnen de met tolueen verkregen resultaten ook naar andere oplosmiddelen toe worden geëxtrapoleerd.

ZUSAMMENFASSUNG

Hydrophobe, organische Lösungsmittel, wie z.B. Toluol, sind giftig für Lebewesen. Sowohl in der Umweltbiotechnologie als auch bei der biotechnologischen Feinchemikalienproduktion kann diese Toxizität Probleme hervorrufen. Bei ausführlichen Untersuchungen bezüglich der Wirkung organischer Lösungsmittel zeigte sich, daß die Toxizität dieser Lösungsmittel hauptsächlich auf ihrer Eigenschaft beruht, in biologischen Membranen zu akkumulieren.

Im vergangenen Jahrzehnt wurden mehrere bakterielle Stämme isoliert, die in Anwesenheit organischer Lösungsmittel überleben können (Kapitel 2). Einer dieser Stämme ist *Pseudomonas putida* S12, der in dieser Dissertation untersucht wurde. Dieser Stamm kann in einem Zweiphasensystem aus Lösungsmittel und Wasser wachsen, solange der log $P_{O/W}$ des Lösungsmittels (Logarithmus des Verteilungskoeffizienten des Lösungsmittels zwischen Oktanol und Wasser) größer oder gleich 2.3 ist. Das Überleben dieses Stammes in Anwesenheit der Lösungsmittel wird hierbei durch verschiedene Adaptionsmechanismen gewährleistet. Es ist bereits seit längerem bekannt, daß dieser Stamm den Lösungsmitteleffekt auf die Membranen durch eine *cis / trans* Isomerisierung der ungesättigten Membranfettsäuren kompensieren kann.

Im dritten Kapitel dieser Dissertation wird ein weiterer Anpassungsmechanismus vorgestellt. An Toluol adaptierte Zellen besitzen ein aktives Exportsystem für Toluol. Auf diese Weise kann *P. putida* S12 nicht nur den giftigen Membraneffekt der organischen Lösungsmittel kompensieren, sondern auch die Konzentration eines giftigen Lösungsmittels in der Zelle aktiv herabsetzen. In der Anwesenheit eines solchen Exportsystems kann die Lösungsmittel-konzentration in der bakteriellen Membran unterhalb des theoretischen Gleichgewichtswertes bleiben. Dieser aktive Export ist abhängig vom elektrochemischen Protonengradienten.

Seit unserer ersten Beschreibung des Lösungsmittelexportsystems wurde es immer wieder mit bekannten Antibiotikaexportsystemen in Verbindung gebracht. Tatsächlich erhöht die Anpassung des Stammes *P. putida* S12 an Toluol die Resistenz dieses Stammes für eine ganze Reihe verschiedener Antibiotika (Kapitel 4).

Trotzdem konnte kein Antibiotikaexport durch dieses Lösungsmittelexportsystem von *P. putida* S12 nachgewiesen werden. Dieses Exportsystem ist spezifisch für Lösungsmittel wie Toluol und *para*-Xylol (Kapitel 5). Darum müssen während der Lösungsmitteladaption der Zellen andere Mechanismen aktiviert werden, die die Antibiotikaresistenz verusachen. Vermutlich liegt diesem Phänomen eine allgemeine Streßantwort zu Grunde (Kapitel 2 und Kapitel 4). Diese kann auch Grund für die niedrige Durchlässigkeit der Zellhülle nach erfolgter Anpassung an Lösungsmittel, so wie sie in Kapitel 7 diskutiert wird, sein.

Die weitreichenden Effekte, die Lösungsmittel auf *P. putida* S12 haben, zeigen sich deutlich in Kapitel 6. Die Anwesenheit des Lösungsmittels Toluol reduziert den maximalen Biomasseertrag und erhöht den Erhaltungsstoffwechsel. Es ist bemerkenswert, daß andere organische Lösungsmittel bei gleicher Konzentration in der Membran ähnliche Effekte wie Toluol zeigen.

Für den Effekt eines Lösungsmittels ist daher nicht die chemische Struktur sondern die Konzentration in der bakteriellen Membran entscheidend. Somit können die mit Toluol erhaltenen Ergebnisse auch auf andere Lösungsmittel extrapoliert werden.

Nawoord

De takken voor mijn raam bewegen zachtjes in de zomerwind. De Vlaamse Gaai, zo vaak mijn gezel als ik achter de computer zit, is er vandaag niet. Voor deze mooie gekleurde vogel die zo lelijk kan krassen betekenen de floppy's op mijn bureau niets. Voor mij wel - mijn proefschrift is af. De tijd waarin ik hieraan heb gewerkt leek op de voor mij zo speciale kraai - heel afwisselend, gekleurd, bont en soms ook een rauwe schreeuw. Het was een leuke tijd en ik wil hier graag al die mensen bedanken die direct of indirect hebben geholpen, dat dit proefschrift hier nu ligt.

In eerste plaats natuurlijk mijn promotor, Jan de Bont. Jan, zonder jouw initiatief was ik niet eens naar Wageningen gekomen om met dit onderzoek te beginnen. Vanaf het begin heb je mij jouw vertrouwen gegeven, de vrijheid gelaten mijn eigen ideeën te verwezenlijken en toch zo nodig met raad en daad terzijde gestaan. Je noemde mij wel eens *taai*. Voor mij was dat toen een compliment, maar voor jou moet het niet altijd even makkelijk zijn geweest om mij te begeleiden. Ik ben blij dat je ondanks jouw persoonlijke situatie hebt geholpen mijn promotie af te ronden.

Hermann, ich weiß, Du willst keinen Dank. Aber einmal muß es sein. Ich habe eine Menge von Dir gelernt. Du hast mir immer geholfen, und unsere Diskussionen über Wissenschaft und den Rest des Lebens tun einfach gut. Es gibt nur noch wenige Universalwissenschaftler, Du hast einen in Dir. Schön, Dich Freund nennen zu dürfen.

De studenten, die mij tijdens mijn promotieonderzoek veel (meet)werk uit handen hebben genomen, wil ik ook met name noemen. Karin Reijenga, Antoine Derks, Petra Wolffs en Ineke Boeijen, bedankt voor jullie bijdrage en prettige samenwerking. Pedro Santos, thank you very much for the co-operation, help and the amount of plating that led to chapter 4.

Bij en rond IM hebben mij veel mensen geholpen, en al was het maar om voor een prettige werksfeer te zorgen. Lab-uitjes, Veluwelopen, kroegentochten, karten en zelfs voetbalwedstrijden herinner ik me met plezier. Alle (ex-)

collega's mijn dank hiervoor. Jan Wery, door jou ben ik vlug een deel van IM geworden en ik hoop dat je nog een tijdje mijn kamergenoot blijft. Mariët van der Werf, jouw enthousiasme is besmettelijk. Het was leuk om op ons oplosmiddelkamertje ook over ander soort onderzoek te kunnen discussiëren. Sybe Hartmans, jouw belangstelling en hulp hebben mij goed gedaan. Martin de Wit, bedankt dat je er altijd voor hebt gezorgd dat GC, HPLC en computers deden wat ik wilde. Hans Tramper, bedankt voor je steun in de rug tijdens de eindsprint naar mijn promotie.

Buiten het werk waren er verschillende mensen die ervoor hebben gezorgd, dat ik de nodige ontspanning kreeg. Vooral mijn kajak-maatjes van de WKV wil ik hiervoor danken. Niets spoelt mijn hoofd zo vrij als water. Gerard en Angeline, er komt een dag, dat ik rond om Runde vaar....

Ma' und Va', ohne Euch wäre diese Doktorarbeit niemals geschrieben worden. Kein Buch ist dick genug um das zu erklären.

Gert, fern der Heimat hast Du mir ein Zuhause gegeben. Danke, bedankt.

Curriculum vitae

Sonja Isken werd geboren op 22 maart 1969 te Kamen in Westfalen (Duitsland). In 1988 behaalde zij het *Zeugnis der allgemeinen Hochschulreife* aan het Städtische Gymnasium Bergkamen en begon vervolgens met de studie Biotechnologie aan de Technische Universität Carolo-Wilhelmina te Braunschweig. Tijdens deze studie werden twee 9 maands afstudeervakken voltooid, een bij het Insitut für Bioverfahrenstechnik, Abteilung Microbielle Physiologie van de Technische Universität Stuttgart en een bij de sectie Industriële Microbiologie van de Landbouwuniversiteit Wageningen. In December 1994 werd de studie met *sehr gut* afgerond. Van 1995 tot 1999 was zij Assistent in Opleiding bij de sectie Industriële Microbiologie van de Landbouwuniversiteit Wageningen, en verrichtte zij het onderzoek dat beschreven is in dit proefschrift. Sinds 1 januari 1999 is zij werkzaam als docent aan de sectie Industriële Microbiologie van Wageningen Universiteit.

BIBLIOGRAPHY

Weber FJ, Isken S, Bont JAM de (1994) *Cis/trans* isomerization of fatty acids as a defense mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. Microbiology 140:2013-2017

Isken S, Bont JAM de (1996) Active efflux of toluene in a solvent-resistant bacterium. J Bacteriol 178:6056-6058

Isken S, Santos PMAC, Bont JAM de (1997) Effect of solvent adaptation on the antibiotic resistance in *Pseudomonas putida* S12. Appl Microbiol Biotechnol 48:642-647

Isken S, Bont JAM de (1998) Bacteria tolerant to organic solvents. Extremophiles 2:229-238

Isken S, Derks A, Wolffs P, Bont JAM de (1999) Effect of organic solvents on the yield of solvent-tolerant *Pseudomonas putida* S12. Appl Environ Microbiol 65:2631-2635

Isken S, Bont JAM de (2000) Physiological characterization of the solvent efflux system of *Pseudomonas putida* S12. Accepted for publication

Saliola M, Isken S, Heipieper HJ (2000) The role of fatty acid modification on ethanol tolerance and adaptation in *ADH* multiple and null mutants of *Kluyveromyces lactis*. Accepted for publication

Matsumoto M, Bont JAM de, Isken S (2000) Isolation and characterization of solvent-tolerant *Bacillus* sp. Submitted for publication