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Adaptation of bacteria to toxic solvents: mechanisms and application

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Adaptation of bacteria to toxic solvents: mechanisms and application

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Für meine Familie

Whatever we know is only a drop.
What we don't know is an ocean. (Sir Isaac Newton)

CONTENTS

ABBREVIATIONS

% (v/v)	percent per volume
μ	growth rate
ζ	zeta potential
16S-rRNA	16S-subunit of the ribosomal RNA
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATPS	aqueous two-phase system
BIOLOG	company which delivers test kits for the characterisation and identification of microorganisms via phenotypical properties (Hayward, USA)
BLAST	Basic Local Alignment Search Tool
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
cti	gene of the *cis-trans* isomerase
Cti	enzyme *cis-trans* isomerase
EC50	effective concentration; EC50: concentration of a compound at which the test organisms show 50 % of a defined effect, e.g. growth
EDTA	ethylenediamine tetraacetic acid
e.g.	for example (abbreviation of Latin: *exempli gratia*)
EPS(s)	extracellular polymeric substance(s)
et al.	and others (Latin: *et alteri*)
ETH	Swiss Federal Institute of Technology *(Eidgenössische Technische Hochschule)*, Zürich, Switzerland
FAME	fatty acid methyl ester
FID	flame ionisation detector
Fig.	figure
GC	gas chromatography/gas chromatograph
GC/MS	gas chromatography/mass spectroscopy
GMO(s)	genetically modified organism(s)
HPLC	high performance liquid chromatography
KCN	potassium cyanide
kPa	kilopascal (SI-unit of pressure)
log P	logarithm of the partitioning coefficient of a compound in an octanol-water standard mixture (also value for hydrophobicity)
LPS	lipopolysaccharides
M	Molar (mol/l)
MATH	microbial adhesion to hydrocarbons-test
min	minutes
MFP	membrane fusion protein
n	"normal"; e.g. concerning the homologous line of alkanes
Na_2-succinate	di-sodium succinate
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
OD_x	optical density at x nm
OMP	outer membrane protein
P.	*Pseudomonas*

PAH	polycyclic aromatic hydrocarbon
pH	negative decade logarithm of the proton concentration
psi	pound per square inch (unit of pressure; equals about 6,89 kPa)
PUFA(s)	polyunsaturated fatty acid(s)
rpm	rotations per minute
cf.	compare (abbreviation for Latin "*confer*")
SEM	Scanning Electron Microscopy
sp.	species, Latin: *species*
Tab.	table
t_D	doubling time
TPPB	two phase partitioning bioreactor
t_R	retention time
UFZ	Centre for Environmental Research Leipzig-Halle (*Umweltforschungszentrum Leipzig-Halle GmbH*)

1 INTRODUCTION

1.1 Early steps in biotransformation

Already in the "cradle of civilisations" in Mesopotamia, people were able to produce alcohol. In many different places in the ancient world, our ancestors consumed food and beverages produced by microorganisms without being aware of the participation of "little assistants" during those processes. Very common examples are the brewing of beer and the making of bread, cheese, wine, and vinegar.

A prototype bioreactor with immobilized bacteria has been known in France already in the 17th century (Vasic-Racki 2006). Since the discoveries of Louis Pasteur in the 19th century, it is known that microorganisms can catalyze and perform chemical reactions. Pasteur was the first to document an enantiomeric selective production of a chemical compound, tartaric acid, mediated by a microorganism, the mold *Penicillium glaucum* (Pasteur 1858). From this time on, mankind deliberately started to use the biocatalytic abilities of bacteria, yeasts, and fungi. In 1880, lactic acid was probably the first optically active compound to be produced by fermentation at an industrial scale (Sheldon 1993). In the middle of the 20th century, discoveries concerning organisation and transformation of the genetic material of bacteria had a deep impact on genetic engineering during the 1970s (Tatum & Lederberg 1941, Cohen *et al.* 1973). Ensley *et al.* (1983) reported first applications of this technology for the production of small molecules: the authors described a recombinant *Escherichia coli* strain able to produce and excrete indigo, one of the oldest dyes known on earth.

The so-called "classical biotechnology" became a separate branch of science. It deals with biocatalytic features of microorganisms and enzymes as well as with their optimisation and application for industrial scales.

1.2 Biotransformations in industrial processes

Biological systems in general are interesting for industrial purposes since they are efficient and able to catalyse reactions selectively. They synthesize stereo- and regio-specific compounds without the need for chemical protective groups. The goal of biotechnology is to substitute as many chemical processes as possible by

biologically catalysed reactions (**Fig. 1.1**). This sustainability approach is energy saving, waste avoiding, and it serves preserving natural resources. Furthermore, the microorganisms or enzymes used in those so-called biotransformations as biocatalysts can often be produced at low costs (Sebek & Perlmann 1979), they are usually active under mild reaction conditions, and they are considered to be ecologically sound (León *et al.* 1998, Faber 2000, Schmid *et al.* 2001, Straathof *et al.* 2002, Schauer & Borriss 2004).

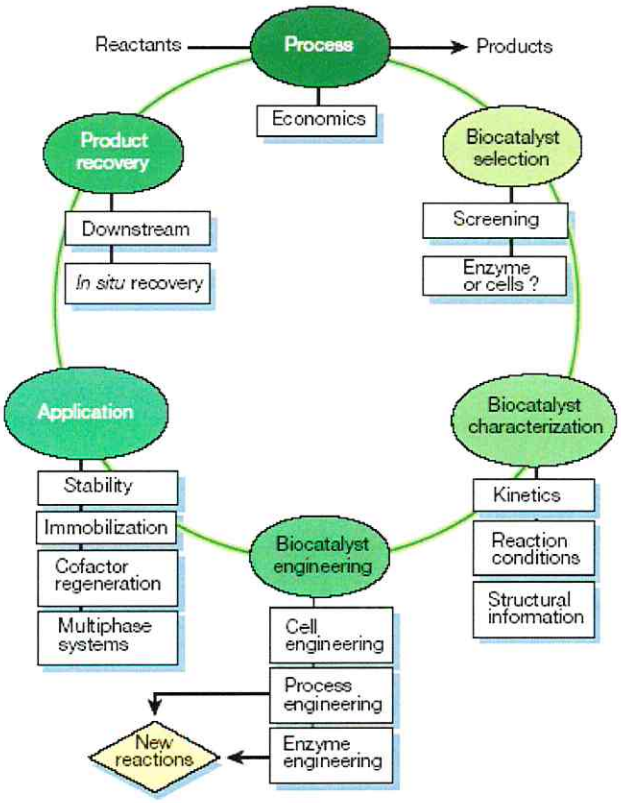

Fig. 1.1 The biocatalysis cycle (Schmid *et al.* 2001).

Those biotransformations can be divided into two types: on the one hand, isolated and purified enzymes can be chosen to catalyse a reaction – either directly in solution or immobilized in order to enhance process stability. On the other hand,

whole-cell biotransformations can be performed. The advantage of the latter is that all co-enzymes or co-factors, like NAD(P)H and ATP, are produced and regenerated by the biocatalyst itself. In addition, with whole-cell systems, complex conversions involving multiple enzymatic steps can be performed. Another disadvantage of enzymes as biocatalysts is that the product could be unstable in the artificial cell-free reaction environment, or the number of necessary reaction steps is so high that the yields are too low for feasible large-scale approaches. For complex processes, systems with whole cells are favoured over enzymes in order to ensure economic feasibility (Léon *et al.* 1998, Faber 2000, Schmid *et al.* 2001). A disadvantage of whole-cell transformations can be the formation and excretion of by-products and proteins into the medium, making downstream processing more complicated (Schmid *et al.* 2001).

Table 1.1 Some representative industrial biotransformations catalyzed by whole cells (cf. Vasic-Racki 2006)

Product	Biocatalyst	Operating since	Company
vinegar	Acetobacteria	1823	various
"Frings acetic acid generator"	*Acetobacter* sp.	ca. 1870	Heinrich Frings GmbH & Co. KG
lactic acid	*Lactobacillus*	1895	Boehringer Ingelheim, Germany
prednisolone	*Arthrobacter simplex*	1955	Schering AG, Germany
acrylamide	*Rhodococcus* sp.	1985	Nitto Chemical Ltd, Japan
L-carnitine	*Agrobacterium* sp.	1993	Lonza, Czech. Rep.
2-keto-L-gulonic acid	*Acetobacter* sp.	1999	BASF, Merck, Germany

Since the first applications of biotransformations by microorganisms at an industrial scale (**Table 1.1**), the amount of such processes introduced into industry has increased rapidly (**Fig. 1.2**). The complex stereo-, enantio- and regio-specificity of these processes cannot be reached by classical chemical syntheses and is an outstanding advantage of biotransformations. For this reason, the use of biocatalysts

has particularly become attractive for the production of fine chemicals as in pharmaceuticals, agrochemicals, and food additives. In the biotechnological industry, biocatalysis has become a standard technology (Schmid *et al.* 2001, 2002).

With molecular biological techniques, it is also possible to "manufacture" bacteria suitable for industrial production performing conversions that are otherwise difficult to accomplish chemically (Tatum & Lederberg 1947, Ensley *et al.* 1983, Léon *et al.* 1998, Panke *et al.* 1999, Held *et al.* 1999, Schmid *et al.* 2001). The same "engineering" approach is described for bacteria to be used in bioremediations for the treatment of environmental pollutions (Pieper & Reineke 2000). Yet, providing genetically modified organisms (GMOs) for *in situ* bioremediation remains an extremely demanding challenge. In comparison, delivering GMOs as biocatalysts under controlled conditions like in a reactor seems to be a relatively easy task (Cases & de Lorenzo 2005). Genetic engineering can help not only to optimize many biotransformations, but also to simplify downstream processing of the reaction products (Mathys *et al.* 1999).

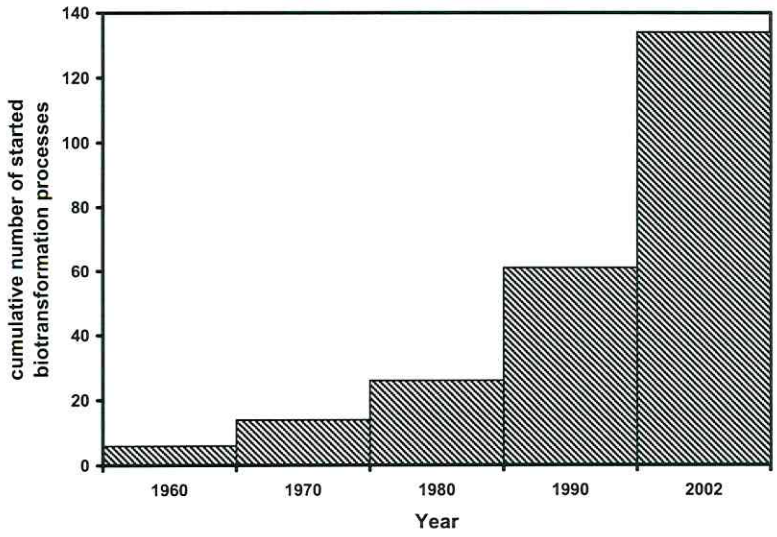

Fig. 1.2 The cumulative number of biotransformation processes that were started at an industrial scale (Straathof *et al.* 2002).

Although the advantages of biotransformations would suggest that biocatalytic systems are widely used, the number of processes already introduced in the

chemical industry is occasionally described as somewhat disappointing (Schmid *et al.* 2001, Schoemaker *et al.* 2003). One of the major drawbacks of replacing classical chemical processes by biotransformations is the toxic effect of many fine chemicals – of both substrates and products – on the required biocatalysts. Especially in whole-cell biocatalysis, the toxicity of chemicals plays a major role. In inverse correlation to that, another major difficulty with a variety of compounds is their poor water solubility (Léon *et al.* 1998, Wery *et al.* 2000) which impedes their bioavailability for the biocatalysts.

1.3 Whole-cell biocatalysis in organic-aqueous two-phase-systems

One major problem with biotechnological whole-cell processes – next to the substrate and product toxicity – is the product recovery from the aqueous medium. A promising solution for both problems is the implementation of a two-phase system with an organic solvent as the second liquid phase (**Fig. 1.3**). Two-phase biotransformation systems constituted of an aqueous phase containing the biocatalytic microorganisms (or even animal cells, Zijlstra *et al.* 1996a, b) and of an organic phase are often described and successfully implemented (Kaul & Mattiasson 1991, Witholt *et al.* 1992, Nikolova & Ward 1993, Van Sonsbeek *et al.* 1993, Salter & Kell 1995, Léon *et al.* 1998, Lye & Woodley 2001, cf. **Table 1.2**). This two-phase system also has the advantage of being self-regulatory in response to the metabolic activity of the cells. Aqueous phase biodegradation of the substrate results in a shift in the chemical equilibrium. The system enables renewed transfer from the organic phase and a long-term regeneration of the equilibrium (Van Sonsbeek *et al.* 1993, Munro & Daugulis 1997a, León *et al.* 1998, Cruz *et al.* 2004, Neumann *et al.* 2005a, cf. **chapter 4**).

Therefore, the major advantage of the addition of an extra organic phase is that it can act as a source and reservoir for the used substrates and simultaneously as a sink for the formed products. The concentration of toxic substrate in the aqueous phase can easily be maintained below inhibitory levels (Vermuë *et al.* 1993). The substrates are still bioavailable for the biocatalyst, but their toxicity is negligible. In this way, the overall concentration of the substrate in the system is very high, even if the compound is inhibitory, toxic or it dissolves very poorly into the water phase (Hack *et*

al. 2000, Park *et al.* 2000, Yeom & Daugulis 2001).

Similarly, the desired end-product will preferentially and continually partition into the solvent phase as well and therefore product toxicity and inhibition is prevented. Since the formed product accumulates in the organic solvent phase, it can be isolated by standard chemical purification methods. In this source-and-sink system, downstream processing is already partially performed during the fermentation itself by a simplified *in situ* product isolation and recovery – saving time, costs, and effort (Isken & de Bont 1997, 1998a, de Bont 1998, León *et al.* 1998, Lye & Woodley 1999, Malinowski 2001). This concept can be extrapolated to controlled delivery of a toxic substrate (Collins & Daugulis 1999a).

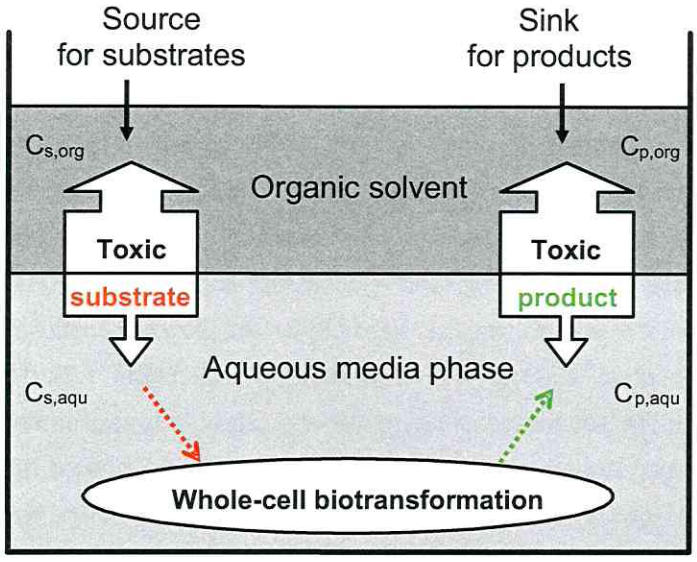

Fig. 1.3 Scheme of a two-phase biotransformation system. The bold arrows indicate the different equilibria of substrates and products between the organic solvent phase and the aqueous media phase. In the solvent, the concentrations of substrate ($C_{s,org}$) and product ($C_{p,org}$) are high. In the water phase, the concentrations of substrate ($C_{s,aqu}$) and product ($C_{p,aqu}$) are low enough to not cause any inhibitory or toxic effects. The toxic substrate partitions from the organic solvent phase into the water phase (red-dotted arrow), bioconversion by whole cells takes place, and the toxic product partitions into the solvent phase (green-dotted arrow) preventing product toxicity and product inhibition.

Diamond & Hsu (1992) promoted the use of two-phase bioreactor systems for the simultaneous production and extraction of biomolecules. They have previously been

Table 1.2 Selected biotransformations in organic-aqueous two-phase systems

Product	Biocatalyst	Organic phase	Reference
3-hydroxyoctanoate 3-hydroxyhexanoate	*Pseudomonas oleovorans*	*n*-octane	Kellerhals *et al.* 1998
3-methylcatechol	*Pseudomonas putida* MC2 (recombinant)	*n*-octanol	Hüsken *et al.* 2001, 2002, 2003
4-androstene-3,17-dione (AD)	*Rhodococcus equi*	cyclohexane, tetrachloroethane	Ahmad & Johri 1992
1,4-androstadiene-3,17-dione (ADD)	*Mycobacterium* sp. NRRL B-3805	different phthalate solvents	Cruz *et al.* 2004
6-pentyl-α-pyrone	*Trichoderma harzianum*	*n*-hexadecane	Serrano-Carreón *et al.* 2002
alkenes	*Pseudomonas oleovorans*	alkanes	Witholt *et al.* 1990
antibiotic WAP-8294A	*Lysobacter* sp. WAP-8294	*n*-butanol	Harada *et al.* 2001
cholestenone	*Arthrobacter simplex* (mutant)	tetrachloromethane	Liu *et al.* 1996
cholesterol derivatives	*Pseudomonas* sp.	xylene diphenylmethane	Aono *et al.* 1994
ethanol	*Saccharomyces cerevisiae*	dodecanol	Minier & Goma 1981
hexanal	*Pichia pastoris*	hexanol	Murray & Duff 1990
indigo	*Acinetobacter* sp. ST-550	diphenylmethane	Doukyu *et al.* 2002
lipase	*Pseudomonas* sp. LST-03	toluene, cyclohexane	Ogino *et al.* 1994
m-nitrotoluene reduction derivatives	*E. coli* JM101(pSPZ3) (recombinant)	*n*-octane	Meyer *et al.* 2005
naphthalene-*cis*-glycol	*Pseudomonas putida*	dodecane and others	Harrop *et al.* 1992
octanoic acid	*Escherichia coli* (recombinant)	*n*-octane	Favre-Bulle *et al.* 1991, 1993
phenol	*Pseudomonas putida* S12 (recombinant)	octanol	Wierckx *et al.* 2005
phenyl glycidyl ether	*Mycobacterium* M156	*n*-hexadecane	Prichanont *et al.* 1998
protease	*Pseudomonas aeruginosa* PST-01	e.g. chloroform, cyclohexane	Ogino *et al.* 1995
tryptophan	*Escherichia coli*	toluene and others	Bang *et al.* 2004
β-carotene	*Dunaliella salina*	dodecane	Hejazi & Wijffels 2003

used in extractive fermentations (Barton & Daugulis 1992, Jones *et al.* 1993, Daugulis *et al.* 1994). Even mathematical approaches were tried in order to calculate optimisation methods for aqueous two-phase extractions (Selber *et al.* 2000). Also for two-phase biotechnological approaches, the genetic "manufacturing" of bacterial strains was realized (Wubbolts *et al.* 1996, Meyer *et al.* 2005, **Table 1.2**).

The two-phase system bioreactor concept is also used as an approach for the biodegradation of xenobiotics (for review cf. Daugulis 2001, cf. **Table 1.3**). The potential applications are the biological treatment of industrial discharge streams as well as the *ex situ* remediation of contaminated soil and industrial air pollutions.

Table 1.3 Examples for biodegradation of xenobiotics with the help of two-phase bioreactors

Biodegraded xenobiotics	Biocatalyst	Reference
benzene	*Alcaligenes xylosoxidans* Y234	Davidson & Daugulis 2003
benzene, toluene, and phenol	*Pseudomonas putida* F1	Abu Hamed *et al.* 2004
BTX (benzene, toluene, and p-xylene	*Pseudomonas* sp. ATCC 55595	Collins & Daugulis 1999a, b, c
nitroaromatic explosive (hexahydro-1,3,5-trinitro-1,3,5-s-triazine, RDX)	*Enterobacter cloacae* ATCC 43560	Pudge *et al.* 2003
pentachlorophenol	*Arthrobacter* sp. ATCC 33790	Munro & Daugulis 1997a, b
phenanthrene	*Chlorella sorokiniana* and *Pseudomonas migulae*	Munoz *et al.* 2003
phenol	*Pseudomonas putida* ATCC 11172	Collins & Daugulis 1996, 1997a, b, Cruickshank *et al.* 2000, Vrionis *et al.* 2002a, b
polycyclic aromatic hydrocarbons	*Sphingomonas* sp.	Janikowski *et al.* 2002
styrene	*Pseudomonas aeruginosa*	El Aalam *et al.* 1993
toluene	*Alcaligenes xylosoxidans*	Daugulis & Boudreau 2003

In the literature, one can also find descriptions of comparable aqueous-organic phase systems. One is named "two-phase partitioning bioreactor" (TPPB, Daugulis 1987,

1988, 1994, 1997, Malinowski 2001, Janikowski *et al.* 2002, MacLeod & Daugulis 2003 etc.). Another similar system is the so-called "aqueous two-phase system" (ATPS). It is formed by the addition of aqueous solutions of two different polymers like polyethylenglycol and dextran or xanthan (Brumbauer *et al.* 1999, Bim & Franco 2000, Chethana *et al.* 2006). This system is often used for extractive fermentations (Zijlstra *et al.* 1998, for review see: Sinha *et al.* 2000).

The advantages and disadvantages of the use of organic solvents in whole-cell biotransformations were stated by Salter & Kell (1995) and are summarised in **Table 1.4**. Supportive to these rules, MacLeod & Daugulis (2005) reported the preferential active growth of a *Mycobacterium* strain in the organic phase of a two-phase bioreactor.

Table 1.4 Potential advantages and disadvantages of the use of organic solvents in whole-cell biotransformations (cf. Salter & Kell 1995)

Advantages	**Disadvantages**
concentration increase of poorly water-soluble substrates/products	cytotoxic/inhibitory effect on the biocatalyst (and on other life forms)
reduction of product and/or substrate inhibition/toxicity	non-toxic compounds tend to be highly apolar and have poor solvation properties and low reaction rates
prevention of substrate/product hydrolysis	clotting of biomass
organic solvents themselves as substrates	problem of waste disposal or recycling
reduction of mass transfer limitations	increase in system complexity
alteration of substrate/product partitioning	cost increase in order to guarantee safety within the reactor and downstream processing
improvement of biotransformation's stereoselectivity	precipitation of poorly water- and solvent-soluble reactant complexes or cells at the interphase
facilitation of product recovery	little experience, especially on large scales
better integration with chemical steps/processes	problem of product recovery, if surfactants are used and/or emulsions are formed

Furthermore, the extraction with the help of solvents has been reported as the cheapest in a number of cases. However, it is generally observed that the better the

solvent from the extraction point of view, the higher is its toxicity to the biocatalysts (Roffler *et al.* 1984, Mattiasson & Larsson 1985, Kaul & Mattiasson 1986, 1991).
The aqueous-organic two-phase bioreactor system with whole cells was found to efficiently increase the yield of many biotechnological processes of commercial interest (Van Sonsbeek *et al.* 1993, León *et al.* 1998, Angelova & Schmauder 1999, Panke *et al.* 2002, Stark & van Stockar 2003, Cruz *et al.* 2004).

1.4 Solvent properties and solvent selection

Solvents are liquids that dissolve solid, liquid, or gaseous compounds, without altering them chemically, resulting in a solution. In biotechnology, solvents enable and facilitate product recovery from aqueous reaction media (Schmid *et al.* 2001).
If a compound is suitable as a solvent for certain substrates and/or products is depending on its chemico-physical properties, which are also determined by the solvent's molecular structure (Léon *et al.* 1998, cf. paragraph **1.5**).
The use of an organic solvent phase in biocatalytic applications can provide both kinetic and thermodynamic advantages. When a product is continuously removed by a solvent phase, a biocatalyst that is subject to decreased productivity due to end-product inhibition will show an increase in its activity. For a successful implementation of this two-phase biotechnological approach in large scales, the proper solvent selection is absolutely crucial (Malinowski 2001).
An important parameter for the distribution of substrates and products in the organic phase is the hydrophobicity of the used solvent. The hydrophobicity can be defined as the common logarithm of the partition coefficient P of a particular solvent between a standardized 1:1 mixture of *n*-octanol and water (log P, Hansch *et al.* 1963, Hansch & Fujita 1964, Leo 1993, Sikkema *et al.* 1994a, 1995, Weber & de Bont 1996).
The ideal solvent for two-phase whole-cell biotransformations must not impede bacterial growth or interfere with any of the microorganism's biological and especially biocatalytic functions. The best suitable solvent for a two-phase biotransformation system will have a log P value similar to the one of the potential substrate (e.g. 4-chlorophenol, log P 2.4) and product (e.g. 4-chlorocatechol, log P 2.0). In this example, toluene (log P 2.5) would theoretically be an appropriate solvent for a two-phase system.

However, the organic solvents that fulfil the two-phase system requirements concerning extractive and reservoir properties usually belong to the group of compounds with log P values between 1 and 4 which are considered to be toxic for microorganisms. Therefore, organic solvents themselves are toxic to the biocatalysts (Sikkema *et al.* 1994a, 1995, Weber & de Bont 1996, Isken & de Bont 1998a, Sardessai & Bhosle 2002, Neumann *et al.* 2005a, **cf. chapter 4 and 5**).
The application of the best-suited organic solvent plays an essential role in delivering the designated properties of the biotransformation system. Daugulis summarized the desired characteristics a solvent should possess in such a process (Daugulis 1988, **Table 1.5**).

Table 1.5 Desirable solvent characteristics (Daugulis 1988)

- favourable distribution coefficient for product and substrate
- high selectivity
- low emulsion-forming tendency
- low aqueous solubility
- chemical and thermal stability
- favourable properties for product recovery
- non-biodegradability
- non-hazardous
- inexpensive
- available in bulk quantity
- biocompatibility

For effective aqueous-organic two-phase bioconversion systems, the proper solvent has to be biocatalyst-biocompatible, provide an adequate substrate reservoir and product sink to possess the necessary extraction, partition and mass transfer properties (Dias *et al.* 1994, De Bont 1998, León *et al.* 1998, Colins & Daugulis 1999, Aono *et al.* 2001, Hüsken *et al.* 2001, MacLeod & Daugulis 2003). The aqueous phase composition must also be defined in order to provide the requirements for prolonged maintenance of cell activity (Cruz *et al.* 2002).

The experimental determination of solvent biocompatibility and evaluating the solvent's extraction properties for each new biocatalytic system is time-consuming, thus making a general model to predict solvent toxicity a particularly useful tool. An empirical rule inversely correlating the toxicity of the solvent with its log P value (Laane *et al.* 1985, 1987b) is currently used (Inoue & Horikoshi 1991, Vermuë *et al.* 1993, Bassetti & Tramper 1994, Rajagopal 1996, Aono *et al.* 2001, MacLeod & Daugulis 2003). However, this model does not quantify solvent characteristics related to its molecular structure and the nature of its functional groups, which could be involved in specific interactions with microbial cells, ultimately leading to deviations from the predicted toxicity trend (Vermuë *et al.* 1993, Cruz *et al.* 2001, 2002). Thereby, the solubility parameter is helpful to assess the uptake-capacity of the organic solvent for the substrates and products involved in the bioconversion. The suitability of a water-immiscible organic solvent for the use in two-phase system biocatalysis can be predicted by evaluating the polarity and the molecular size of the organic solvent (Brink & Tramper 1985).

The distribution coefficient is a measure of the solvent's capacity for the product (and solvent) and is defined as the ratio of the product concentration in the solvent to the product concentration in the aqueous culture medium, at equilibrium (Bruce & Daugulis 1991). As the distribution coefficient increases, less solvent is required for effective extraction. Kollerup & Daugulis (1985) provided an empirical database for extractive fermentations with solvents based both on theoretical considerations as well as on experimental data allowing predictions concerning solvent properties and suitability.

With the correct choice of solvent, it is no longer necessary to work with diluted solutions, and product recovery and even wastewater treatment costs within bioremediational two-phase approaches can be reduced (Bruce & Daugulis 1991).

Again, fine chemicals have log P values ranging from 1 to 4, therefore the ideal solvents for those substances are within this log P range as well and are concomitantly toxic to the biocatalytic microorganisms. Due to the solvent toxicity, whole-cell biotransformations using a two-phase system with an organic solvent as the second phase were considered not being possible for a long time.

1.5 Toxicity of organic solvents

The toxicity of chemicals like organic solvents can be described in different ways.
The model of the so-called Quantitative Structure-Activity Relationship (QSAR) refers to the quantitative relation between the chemical structure of a molecule and its pharmacological, toxicological, ecological, chemical, biological, and physical effects. Sometimes the term QSPR (Quantitative Structure Property Relationship) can be found: that principle is limited to the relation between the molecule's structure and its physical and chemical properties. First works concerning this topic were already done in the middle of the 19th century. A linear correlation between the boiling points of alkanes and their chain length was proposed (Δt = 18°C, Kopp 1842). Both models are mainly used during the development of pharmaceuticals and allow the prediction of the so-called "drug efficacy" of a structurally related compound (Bochev & Rouvray 1991). The discovery of drugs often involves the QSAR approach in order to identify chemical structures with potentially good inhibitory effects on specific targets and low toxicities. Here, of special interest is the prediction of log P as a crucial mean in identifying "drug-likeness".
Leo *et al.* (1975) as well as Rekker & de Kort (1979) created models to calculate the log P values for a variety of compounds according to their atomic structure. Chemical compounds with log P values ranging between 1 and 4 are regarded as being highly toxic for microorganisms. Below log P 1, the hydrophobicity of a compound is too low to enable entering the membrane; above log P 4, the substances are too poorly water-soluble and concomitantly too poorly bioavailable to cause any toxic effect. The higher the hydrophobicity or log P within this range of 1 to 4, the higher the compound's toxic effect on bacteria, following a systematic, nearly linear correlation. The chemical toxicity of compounds to microorganisms is often expressed as the concentration that causes 50 % growth inhibition, the so-called effective concentration (EC50). It correlates negatively with the log P value (Sikkema *et al.* 1995, Isken & de Bont 1998a, Isken & Heipieper 2002, Ramos *et al.* 2002, Kabelitz *et al.* 2003). This correlation of hydrophobicity with toxicity is shown in **Fig. 1.4** and was proven for a variety of solvent classes, like alkanes, alkanols, alcohols, aromats, and phenols (Laane *et al.* 1987a, b, Rezessy-Szabo *et al.* 1987, Osborne *et al.* 1990, Sierra-Alvarez & Lettinga 1991, Sikkema *et al.* 1994a, Isken & de Bont 1998a, Isken & Heipieper 2002, Ramos *et al.* 2002).

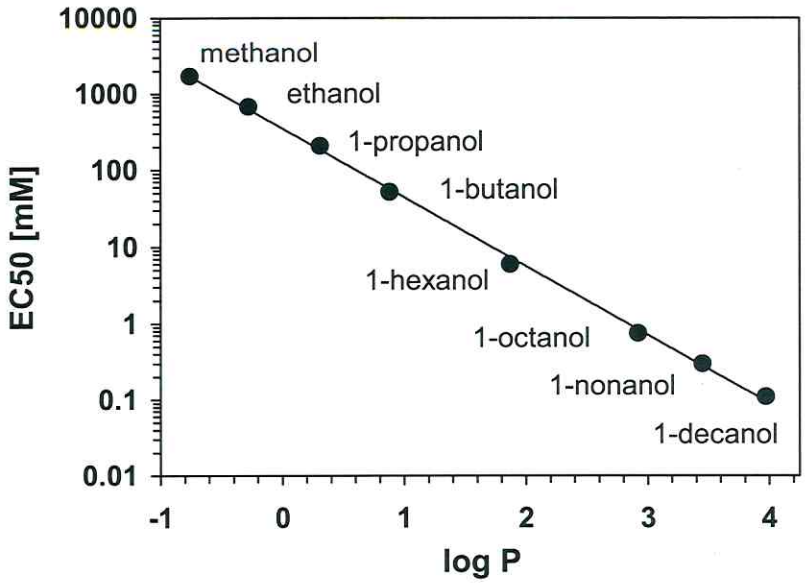

Fig. 1.4 Correlation between partitioning of different aliphatic alcohols in a standard octanol-water system (log P, hydrophobicity) and their chemical toxicity (EC50). Experimental results obtained with cells of *Acinetobacter calcoaceticus* (Kabelitz *et al.* 2003).

1.5.1 Effects of solvents on the membrane

Why are organic solvents toxic to cells? The major target for the toxic effects of solvents in bacteria is the membrane. As soon as solvents accumulate in the cell membrane, they disrupt the membrane's integrity and lead to a loss of the membrane's function as permeability barrier to the environment, as protein and reaction matrix, and as energy transducer what will concomitantly result in damage of cell metabolism, in growth inhibition or even in cell death (Isken & de Bont 1998a).

It is proven that a correlation is existing between the toxicity to microorganisms on the one hand, and the hydrophobicity of a compound and its partitioning into a lipid bilayer on the other hand (Osborne *et al.* 1990, Sikkema *et al.* 1992, 1994a). Compounds with a log P value below 1 possess a low hydrophobicity and hardly partition into the membranes, which prevents them from being toxic to cells. Most compounds with a log P higher than 4 are very poorly water-soluble, meaning their

bioavailability is too low to cause any toxic effect to the microorganisms (Heipieper *et al.* 1994, Weber & de Bont 1996, Isken & de Bont 1997, de Bont 1998).

Organic solvents with log P values ranging from 1 to 4 are able to accumulate within the hydrophobic layer of the cytoplasmic membrane, which leads to an increase in membrane fluidity and a decrease in membrane viscosity. The intercalation of solvents causes major disruptions in the membrane, leading to integrity loss and thereby influencing order, packing and permeability of the membrane (de Smet *et al.* 1978, Van der Meer 1984, Sikkema *et al.* 1992, 1994a, 1994b, 1995, Weber & de Bont 1996, Kim *et al.* 2002). The increased membrane permeability leads to inactivation and denaturation of membrane-embedded proteins like ion pumps and ATPases, to leakage of ions and intracellular macromolecules like RNA, phospholipids, and proteins (Jackson & de Moss 1965, Woldringh 1973, Isken & de Bont 1998a), and to a breakdown of the cell's energy status. The increased membrane permeability is considered the main reason for cell death because of exposure to toxic chemicals (Heipieper *et al.* 1992, 1994, Ramos *et al.* 1995, Sikkema *et al.* 1994a, 1995, Weber & de Bont 1996, Isken & de Bont 1998a, Isken & Heipieper 2002, Ramos *et al.* 2002). This disturbing effect of solvents on the cell membrane also inhibited active transport and glucose uptake in *Clostridium acetobutylicum* cells (Bowles & Ellefson 1985). It can partially or completely abolish membrane potential ($\Delta\Psi$), proton gradient (ΔpH), and decrease intracellular pH and energy charge due to lower ATP concentration (Bowles & Ellefson 1985, Huang *et al.* 1985, Terracciano & Kashket 1986, Sikkema *et al.* 1992). Heipieper *et al.* (1991) observed that potassium ions and ATP leak from the cell into the medium when the solvent phenol was applied in toxic concentrations (cf. **chapter 3**).

Due to the accumulation of solvents in the cell membrane, an increased permeability for small molecules and ions such as ATP, potassium ions, phosphate, and protons is caused (Lambert & Hammond 1973).

Already 40 years ago, it was determined that alkanols significantly increased the K^+ efflux (Bangham *et al.* 1965). Heipieper *et al.* showed that the potassium efflux (as well as the ATP efflux) from cells of *Escherichia coli* is induced by exposure of the cells to phenols and heavy metals (Heipieper *et al.* 1991, 1996). The same effect was also observed after exposure of non-adapted cells of *Pseudomonas putida* DOT-T1E to 1-decanol (Neumann *et al.* 2005a, cf. **chapter 4**). Solvent-adapted cells of

Pseudomonas putida DOT-T1E did not show an increased potassium efflux.
Cells have the adaptive ability to restore the lost potassium by active influx of potassium ions from their environment (Dinnbier *et al.* 1988).

Hydration and swelling caused by solvents affects the membrane surface (Shimooka *et al.* 1992) and the thickness of the membrane (Seeman 1972, McIntosh *et al.* 1980, Pope *et al.* 1984). This is important in relation to the relative surface and volume of the membrane since it is the major target for the toxic effects of solvents.
The effect that a lipophilic compound has on the membrane integrity depends on the position in the membrane where it accumulates. Short-chain alkanols like ethanol (C2) will preferentially interact with the phospholipid head-group area while long-chain alkanols (C8) will be incorporated in the centre, the fatty acid acyl chains.

1.5.2 Solvents' membrane concentration

There is a correlation existing between the toxicity of a solvent and its concentration inside the cell's membrane. Osborne *et al.* (1990) observed a correlation between biocatalytic activity and concentration of solvent in the membrane within an organic-aqueous two-phase system. Isken *et al.* (1999) suggested that the critical concentration of solvents in the membrane is to be 198 mM. The authors observed that the dose response concerning bacterial cell yield was linear up to approximately 200 mM solvent concentration in the bacterial membrane (cf. **chapter 4**).
It was established that the membrane concentration causing a comparable toxic effect is very similar for compounds with different log P values (Sikkema *et al.* 1994a, Heipieper *et al.* 1995, Neumann *et al.* 2005a, cf. **chapter 4**). Not the concentration of a compound within the medium, but its membrane concentration is crucial for any toxic effect it might have. The following equation describes the relation found by Sikkema *et al.* (1994a).

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

$P_{M/W}$ partitioning coefficient between membrane and water
$P_{O/W}$ partitioning coefficient between octanol and water (1:1)

The solvent's membrane concentration depends on the concentration of the solvent in the water phase, the partitioning of the solvent from the water phase into the membrane, and the ratio of the volumes of the two liquid phases. Thereby, a correlation has been found between the log P value of a solvent and its partitioning between membrane and buffer (Sikkema *et al.* 1994a). The maximum solvent membrane concentration in the cells can then be calculated if its water phase concentration is known, and it can be determined which solvents are too toxic (Bruce & Daugulis 1991, Neumann *et al.* 2005a, cf. **chapter 4**). Since the critical membrane concentration for the cell can be found, the corresponding critical aqueous concentration can be determined for each toxic or inhibitory solvent.

1.6 Solvent-tolerant bacteria

The toxic effect of suitable organic solvents on whole cells is a major drawback in their application in biotechnology and in the production of fine chemicals by whole-cell biotransformations (Salter & Kell 1995). Due to that, biotransformations with enzymes in the presence of organic solvents were established already in the 1970s (Buckland *et al.* 1975). Most enzymes were found to be able to function in organic solvents (Klibanov 1986, 1990, 2001, Blinkovski *et al.* 1992, Russell *et al.* 1994, Carrea *et al.* 1995). Nevertheless, two-phase biotransformations with whole cells using solvents with reasonable properties were still out of reach.

This situation changed in 1989, when Inoue & Horikoshi made a surprising discovery: they isolated a *Pseudomonas putida* strain, which was able to thrive in the presence of high concentrations of the toxic solvent toluene without being able to metabolize it (Inoue & Horikoshi 1989). Later, a number of research groups reported tolerance towards solvents like xylenes, styrene, and toluene for other species of *Pseudomonas* as well as for other genera, including *Flavobacterium*, *Bacillus,* and *Rhodococcus* (Cruden *et al.* 1992, Weber *et al.* 1993, Ramos *et al.* 1995, Isken & de Bont 1997, de Bont 1998, Isken & de Bont 1998b, Kim *et al.* 1998, Isken & Heipieper 2002). This solvent-tolerance of microorganisms opened new possibilities in the quest for whole-cell biocatalytic production processes for toxic fine chemicals and in overcoming limitations in industrial biotransformations. The usage of solvent-tolerant microorganisms now allows the application of otherwise toxic solvents with the

chemical properties needed for a successful implementation of whole-cell two-phase biotransformations (Sardessai & Bhosle 2004, Neumann *et al.* 2005a, cf. **chapter 4**). Several two-phase biotransformations have now been established using solvent tolerant bacteria, overcoming toxic effects of substrates and products (Al-Tahhan *et al.* 2000, Wery *et al.* 2000, Doukyu *et al.* 2002).

Table 1.6 Selected organic solvent-tolerant strains in chronological order (cf. Isken & de Bont 1998a)

Organism	Tolerated solvents	Reference
Pseudomonas putida IH-2000	heptanol, toluene	Inoue & Horikoshi 1989
P. putida PpG1 (mutant)	toluene	Shima *et al.* 1991
P. putida Idaho	dimethylphthalate, toluene	Cruden *et al.* 1992
P. aeruginosa ST-001	heptanol, toluene	Aono *et al.* 1992
P. putida S12	dimethylphthalate, toluene	Weber *et al.* 1993
Flavobacterium DS-711	benzene, toluene	Moriya & Horikoshi 1993a
Bacillus DS-994	benzene, toluene	Moriya & Horikoshi 1993b
P. aeruginosa PAO1161	xylene, hexane	Komatsu *et al.* 1994
P. aeruginosa LST-03	toluene	Ogino *et al.* 1994
P. putida DOT-T1	toluene	Ramos *et al.* 1995
Sphingomonas aromaticivorans B0695	toluene, naphthalene, xylenes, *p*-cresol, fluorene, biphenyl, dibenzothiophene	Frederickson *et al.* 1995, 1999
Arthrobacter ST-1	benzene	Kato *et al.* 1996
Pseudomonas LF-3	toluene	Yoshida *et al.* 1997
P. mendocina LF-1	dimethylphthalate	Ikura *et al.* 1997
P. mendocina K08-1	toluene	Ikura *et al.* 1997
Rhodococcus strain 33	benzene	Paje *et al.* 1997
Bacillus	toluene	Isken & de Bont 1998b
P. putida GM62, GM73	toluene	Kim *et al.* 1998
P. sp. strain GM80		
Bacillus cereus strain R1	toluene	Matsumoto *et al.* 2002
Bacillus sp. BC1	chloroform	Sardessai & Bhosle 2003
Chlorella vulgaris	isopropanol	McEvoy *et al.* 2004
Rhodococcus opacus	benzene, toluene, styrene, xylene, ethylbenzene, propylbenzene, octane, decane	Na *et al.* 2005
Staphylococcus sp. strain ZZ1	toluene	Zahir *et al.* 2006

From the bacteria listed in **Table 1.6** it is apparent that the solvent-tolerance potential is much higher in Gram-negative bacteria (Isken & de Bont 1998a).

One major disadvantage is that solvent-tolerant bacteria cannot perform all biotransformation reactions of interest, because they do not possess every enzymatic pathway necessary for fine chemicals production. There are two approaches to circumvent this problem: (1) genetic engineering of the solvent-tolerant bacterium to provide the appropriate set of genes required to perform the desired biocatalytic reactions (Ensley *et al.* 1983, Wery *et al.* 2000, Wierckx *et al.* 2005). And (2), the necessary set of genes providing solvent-tolerance could be introduced into more extensively studied and biocatalytic established bacteria, e.g. *Escherichia coli* (Schmid *et al.* 2001). Evidently, for both approaches and for the economic feasibility of two-phase biotransformations, broadening the knowledge on the mechanisms of solvent-tolerance is crucial.

1.7 Adaptive mechanisms of solvent tolerance

Since the first report on solvent-tolerant bacteria (Inoue & Horikoshi 1989), many research groups occupied themselves with the goal of investigating the mechanisms underlying the bacteria's ability to adapt to the presence of toxic solvents.

"Adaptation" itself can be defined as changes in cell physiology and/or composition in order to adapt to the environment, but without the means of genetic modifications (mutations).

The investigation of the discovered solvent-tolerant microorganisms revealed a broad variety of potentially possibilities used by those bacteria in order to survive the otherwise toxic concentrations of organic solvents:

- rigidification of the cell membrane (**1.7.1**)
- change in the membrane's protein content/composition (**1.7.1.4**)
- active excretion of the solvent (**1.7.2**)
- adaptation of the energetic status (**1.7.3**)
- changes in cell wall and outer membrane (**1.7.4**, **1.7.5**)
- modification of the cell surface properties (**1.7.6**)
- morphological changes (**1.7.7**)
- metabolization or transformation of the solvent (**1.7.8**)

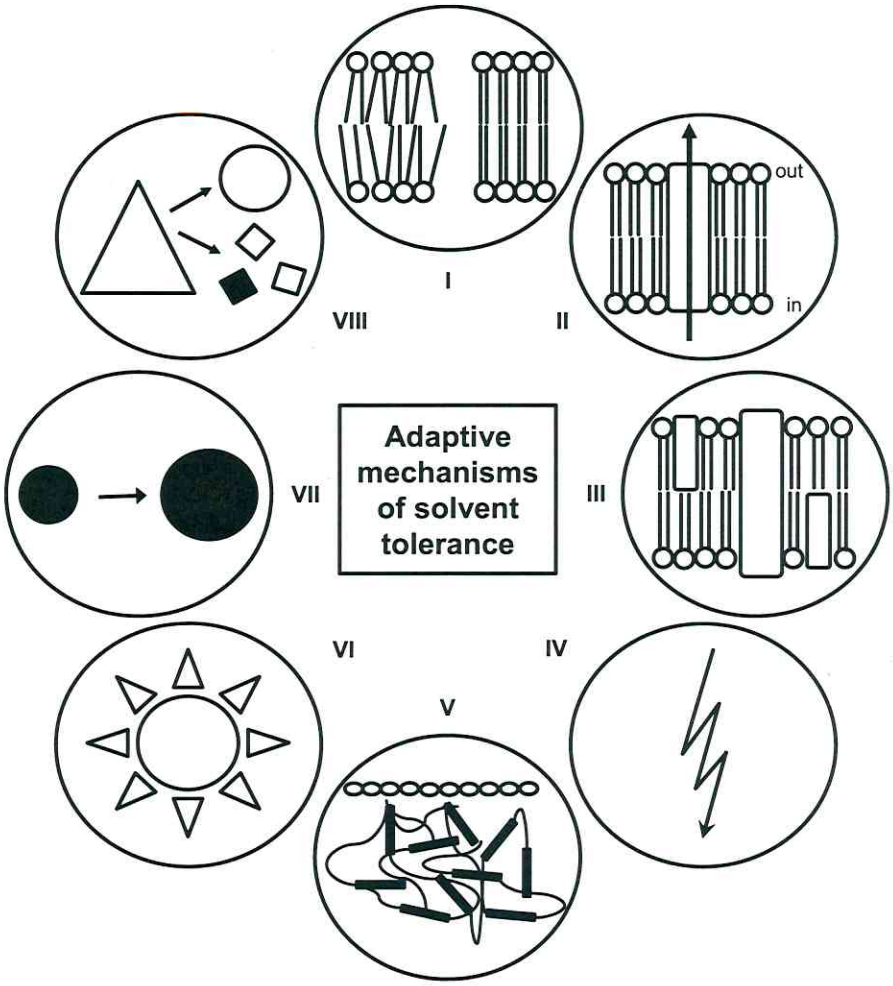

Fig. 1.5 Possible adaptation mechanisms that can protect cells against toxic effects of organic solvents/lipophilic compounds. (I) Modification of the membrane's fluidity by increased structuring of the lipid bilayer. (II) Active efflux of solvents from the membrane into the environment by energy-consuming transport systems (e.g. efflux pumps). (III) Modifications in the membrane proteins. (IV) Changes in the energetic status of the cell. (V) Changes in cell wall and outer membrane. (VI) Adaptation of the cell surface properties (surface charge and hydrophobicity). (VII) Morphological adaptation. (VIII) Transformation or degradation of the solvent. (cf. Sikkema *et al.* 1995, Isken & de Bont 1997, Isken & Heipieper 2002).

Overviews of the mechanisms possibly playing a role in solvent-tolerance are given in two reviews (Sikkema *et al.* 1995, Isken & de Bont 1997) and in **Fig. 1.5**. However, only a combination of those mechanisms enables tolerance towards solvents (Isken & de Bont 1998a, Kabelitz *et al.* 2003).

1.7.1 Adaptation of the cell membrane

Since the cell membrane is the main target for the toxic effects of solvents, it is not surprising that already since the first discovery of solvent-tolerant bacteria changes in the membrane composition were believed to play a crucial role in the mechanisms delivering solvent tolerance (Inoue & Horikoshi 1989). In the cytoplasmic membrane, changes upon solvent presence at the level of the lipids and proteins have been observed. Those adaptation mechanisms enable the reestablishment of fluidity and rigidity of the cell membrane after being subjected to a solvent (Weber & de Bont 1996). Four major adaptation targets on the membrane level were established (Isken & de Bont 1998a):

1. degree of saturation of the fatty acids;
2. *cis/trans* isomerisation of unsaturated fatty acids;
3. composition of phospholipid headgroups;
4. dynamics of phospholipid turnover.

These adaptation mechanisms prevent the influx of solvents by decreasing the membrane permeability and fluidity.

1.7.1.1 Membrane fluidity and transition temperatures

There are different possibilities for a bacterial cell to change its membrane, but all mechanisms are supposed to change the membrane fluidity in order to try to prevent the solvents from entering. The adaptive mechanisms are concerned with the maintenance of the bilayer gel phase of the membrane and therefore its proper functioning. Although there is a wide tolerance, there are upper and lower limits of growth-compatible membrane fluidity (McElhaney 1982, Melchior 1982). Most of the membrane alterations due to solvent presence were already reported earlier for adaptation to different growth temperatures. Generally, in eubacteria one can observe an increase in the lipid content at low temperatures (Russell & Fukunaga 1990). However, it is difficult to establish a direct comparison between eubacteria and archaebacteria, because of the unique ether lipids in the latter that make the archeael membrane more rigid, temperature resistant, and highly salt tolerant. That is why archeael membranes are better suited for extreme environments than the ester type lipids of eubacteria (for review see: van de Vossenberg *et al.* 1998).

Membranes can be modelled as a lipid double layer. The temperature required to induce a change in the lipid physical state from the ordered gel phase (where the fatty acid hydrocarbon chains are fully extended and closely packed) to the disordered liquid-crystalline phase (where the hydrocarbon chains are randomly oriented and "fluid") is called the phase transition temperature. There are several factors directly affecting the phase transition temperature of membrane phospholipids: hydrocarbon length, degree of unsaturation, charge, and headgroup species. As the hydrocarbon length is increased, van der Waals interactions become stronger requiring more energy to disrupt the ordered packing, thus the phase transition temperature increases. Likewise, introducing a double bond into the acyl groups puts a kink in the chain, which requires much lower temperatures to induce an ordered packing of the membrane. The membrane fluidity is therefore crucially connected with the composition of the membrane fatty acid chains. Depending on their length, degree of saturation, and configuration (*cis*, *trans, cyclo*, *iso*, *anteiso*), fatty acids exhibit different transition temperatures (melting points) at which they alter their gel phase (cf. **Table 1.7**). For example, the common unsaturated fatty acid palmitic acid shows a phase transition temperature of 63°C, palmitoleic acid with a *cis*-double bond "melts" at already 0°C, and palmitelaidic acid as the corresponding *trans*-configuration can only be altered into the liquid state at 33°C. Similar high differences in the melting points of fatty acids are observable in saturated compared to *anteiso*-branched fatty acids. For example, the phase transition temperature of C15:0 is more than twice as high as the one for the C15:*anteiso*-branched fatty acid. Nevertheless, *iso*-branched fatty acids possess similar melting points like the corresponding linear one (e.g. 53°C for C15:0 and 52°C for C15:*iso*).

The mechanism controlling fluidity and viscosity of the membrane is called "homeoviscous adaptation" (Sinensky 1974). By incorporating increasing proportions of saturated and long-chain fatty acids into membrane phospholipids, bacteria counteract the effect of solvents (and of rising growth temperatures), keep their membrane fluidity and viscosity constant, and ensure functioning of the membrane. All adaptive membrane changes upon solvent exposure lead to a decrease of the originally increased fluidity caused by the solvents.

Table 1.7 Transition temperatures of phospholipid fatty acids

Fatty acid	Transition temperature [°C]
C16:0 (palmitic acid)	63°C
C16:1*cis*-9 (palmitoleic acid)	0°C
C16:1*trans*-9 (palmitelaidic acid)	33°C
C15:0	53°C
C15:*iso*	52°C
C15:*anteiso*	23°C

The membrane lipid composition of *Pseudomonas oleovorans* has been studied during two-phase fermentations (Chen *et al.* 1995a, b). In the presence of octane, more unsaturated and *trans*-fatty acids are formed; the fatty acids show an increase in mean acyl chain length and in transition temperature leading to decreased membrane lipid fluidity as counteract adaptation to the alkane solvent.

1.7.1.2 Degree of saturation of membrane fatty acids

The most important and longest known membrane adaptive mechanism is the change in the saturation degree of the membrane lipid's fatty acids (Ingram 1976, Ingram & Buttke 1984). It is well known that the transition (melting) temperature of saturated fatty acids is higher than the one of unsaturated ones. Therefore, the fluidity of saturated fatty acids is lower.

Already in the 1970s, it was reported that *Escherichia coli* showed adaptations on the level of membrane lipids fatty acid's saturation upon alcohols, antibiotics, and food additives (Ingram 1976, 1977, Buttke & Ingram 1978a, b). Those fatty acid changes are similar to those induced by growth at changed temperatures (Marr & Ingraham 1962). A decrease in growth temperature means conversion of saturated to mono-unsaturated fatty acids in eubacteria (Russell 1997).

In many microorganisms, the degree of saturation is increased in order to counteract an increased fluidity (**Fig. 1.6**). Polar solvents as short-chained alcohols up to four carbon atoms are an exception. In their presence, the degree of saturation decreases (Ingram 1976, Kabelitz *et al.* 2003), they are also proposed to insert into the lipid

bilayer. The degree of saturation can only be altered by *de novo*-synthesis of membrane lipids. This is an energy-dependent process (Isken & de Bont 1998a). Under growth-inhibiting conditions, lipid biosynthesis is stopped due to the stringent-response regulation and that is why only growing cells can perform that kind of adaptation.

Prove came forward that alcohol-tolerance and fatty acid changes in *E. coli* are related (Ingram *et al.* 1980a). An increase in unsaturated fatty acid content during growth in the presence of short-chain alcohols (C1-C4, Ingram 1977, Ingram *et al.* 1980a, b) was observed. This alteration is identical with changes induced by a decrease in growth temperature (Marr & Ingraham 1962). The opposite effect occurred during growth in the presence of long-chain alcohols (C5-C8): an increased synthesis of phospholipids containing saturated fatty acids was observed (Ingram 1976, 1977), similar to the changes following an up-shift in growth temperature (Marr & Ingraham 1962). In *Acinetobacter calcoaceticus,* the same effect was reported: in the presence of long-chain alcohols, the degree of saturation is increased, whereas it decreased in the presence of short-chain alcohols (Kabelitz *et al.* 2003). The authors suggest that the adaptive response to alcohols in bacteria is connected with the physico-chemical properties of these compounds. It has been shown that also clostridial cells increase the content of saturated fatty acid chains (Vollherbst-Schneck *et al.* 1984, Baer *et al.* 1987, Lepage *et al.* 1987) and decrease the fluidity (Baer *et al.* 1989) of their lipid membranes in order to adapt to solvent stress. An increase in average acyl chain length of fatty acids has the same effect on membrane fluidity, but is outweighed by the greater fluidizing effect of increased unsaturation (Russell 1984a, b).

Reports of polyunsaturated fatty acids (PUFAs) in bacteria indicate that they might play a specific role in cold-temperature adaptation and in the homeoviscous adaptive response of bacteria in order to stabilize membrane fluidity (for review see: Russell & Nichols 1999).

Since many of the adaptive strategies are energy-dependent, they are carried out only in growing cells.

1.7.1.3 *Cis-trans* isomerisation of unsaturated membrane fatty acids

Another mechanism for adapting the membrane fluidity is the isomerisation of the *cis* bond of unsaturated esterified fatty acids into the corresponding *trans*-configuration, mainly of palmitoleic acid (C16:1Δ9) and *cis*-vaccenic acid (C18:1Δ11) (Heipieper *et al.* 1992). Remarkable is that the *cis-trans* isomerisation of unsaturated fatty acids was shown to be independent of energy, cell growth and *de novo*-biosynthesis of lipids. This allows the bacteria to adapt to toxic concentration of solvents, even when environmental situations are not suitable for growth and synthesis of new membrane fatty acids (Heipieper *et al.* 1992, Heipieper & de Bont 1994).

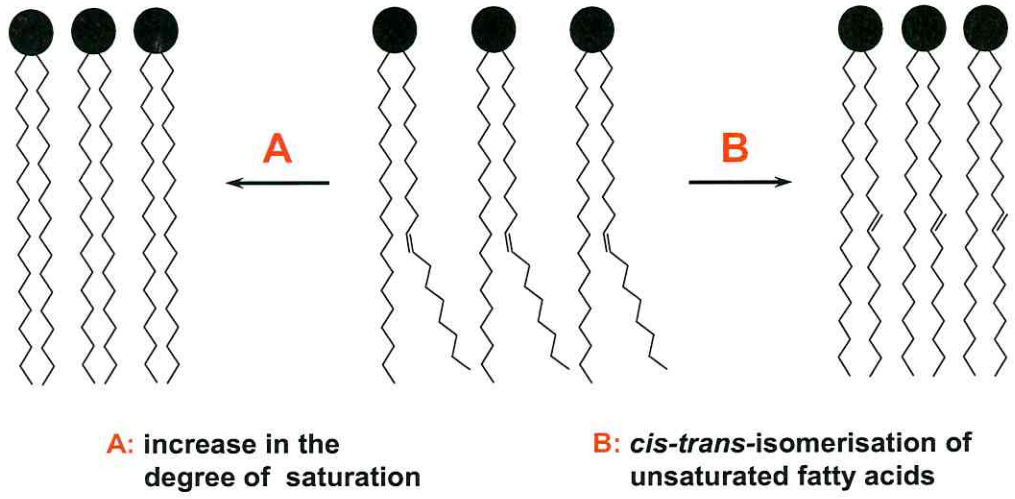

Fig. 1.6 Schematic illustration of the adaptation of membrane fatty acids to toxic solvents by increasing the degree of saturation (A, Heipieper & de Bont 1997) and by isomerisation of *cis*- (middle) into *trans*-fatty acids (right) (B, Heipieper *et al.* 1992, Ramos *et al.* 1997). Both mechanisms serve the increase of the cell membrane ordering and therefore the reduction of membrane fluidity, which counteracts the fluidizing effect of the toxic substances.

The *trans*-unsaturated membrane fatty acids are built by geometric isomerisation of the double bond – without shifting the position – directly from the complementary *cis*-fatty acid. Bacteria with the ability to perform this geometric isomerisation of fatty acid double bonds can alter and adapt their membrane fluidity and therefore adapt to environmental changes.

An increased *trans/cis* ratio enhances the ordering, density, and packing of the

membrane (**Fig. 1.6**) and consequently decreases the membrane fluidity (Diefenbach *et al.* 1992, Chen *et al.* 1995b, Keweloh & Heipieper 1996). This counteracts the fluidity increase resulting from the partitioning of the organic solvent into the lipid part of the membrane (Heipieper *et al.* 1996) and therefore prevents the solvent molecules from entering the membrane (Heipieper *et al.* 1996, Heipieper *et al.* 2003). Under normal growth conditions, the *cis*-isomers of unsaturated fatty acids are in the majority in bacterial membranes. Their acyl chain has an angle of 30° at the double bond. This is the reason for the steric hindrance of the closing of lipid molecules within the lipid bilayer. Therefore, the membrane lipids cannot be packed very tightly next to each other within the membrane. When isomerised to the *trans*-fatty acid, the angle of 30° is strongly reduced to about 6° (see **Fig. 1.6**). A closer proximity of the fatty acid hydrocarbon chains is now possible and therefore a rigidification of the membrane can be accomplished (Keweloh & Heipieper 1996, Heipieper *et al.* 2003, Härtig *et al.* 2005).

Cis-trans isomerisation was proven to be a reaction to the presence of solvents like toluene (Weber *et al.* 1994, Heipieper *et al.* 1995). A correlation was shown between the amount of unsaturated *trans*-fatty acids and the survival rate of solvent-tolerant bacteria in the presence of a second phase of toluene (Weber *et al.* 1994). The *cis-trans* isomerisation affects both phospholipids and free fatty acids (Chen *et al.* 1995b, Chen 1996).

The enzyme responsible for this conversion, the *cis-trans* isomerase (Cti), is located in the periplasmic space, is constitutively present and does not require any energy or co-factors like NAD(P)H (Diefenbach & Keweloh 1994, Holtwick *et al.* 1997, Junker & Ramos 1999, von Wallbrunn *et al.* 2003, Kiran *et al.* 2004). Several studies proved that Cti is a cytochrome *c* type protein with a heme-binding motif (Holtwick *et al.* 1999, von Wallbrunn *et al.* 2003, Heipieper *et al.* 2004). The biochemical mechanism of how the linkage in the double bonds of unsaturated fatty acids is broken, then changed by "rotating" from the *cis*- to the *trans*-configuration, and then again reconstituted, involves the electrophilic heme iron (Fe^{2+} and/or Fe^{3+}).

The *cti* gene that encodes for the Cti enzyme was cloned and sequenced. It was shown that the *cti* gene is constitutively expressed in several *Pseudomonas* and *Vibrio* species (Junker & Ramos 1999, Heipieper *et al.* 2003, von Wallbrunn *et al.* 2003, Kiran *et al.* 2005).

The reason for the Cti activation upon exposure to toxic organic compounds is not yet fully understood. It could be a result of the increase in membrane fluidity itself that heightens the availability of the enzyme's substrate: the double bonds of unsaturated fatty acids (Heipieper *et al.* 1996, 2001). An attractive theory concerning the regulation of the enzyme states that it works as long as it can reach the *cis*-fatty acids. By converting more and more *cis*-unsaturated fatty acids, the membrane becomes more rigid and it is more difficult for Cti to reach other *cis*-fatty acids. However, if the membrane becomes more fluid, for example due to the presence of solvent molecules, the enzyme is able to find substrate molecules again and can catalyse the reaction.

Changes in the fatty acid composition with the help of the enzyme Cti are considered short-term solvent adaptation mechanisms (de Bont 1998); the change in the degree of saturation is a long-term adaptation mechanism. Decrease of the *trans/cis* ratio of the membrane fatty acids is much slower because it can only take place via *de novo* synthesis of *cis*-fatty acids (Isken & Heipieper 2002). It was shown that *P. syringae* depends on *trans*-fatty acid synthesis during growth at higher temperatures due to the fluidity decrease and concomitant membrane stabilization caused by *trans*-fatty acids (Kiran *et al.* 2004).

Heipieper *et al.* (1995) observed a direct correlation between the hydrophobicity of organic compounds (log P), concentration dependent growth inhibition, and the *trans/cis* ratio of unsaturated membrane fatty acids. However, *cis-trans* isomerisation alone does not provide the necessary protection against organic solvents. There are bacterial strains able to perform the isomerisation, but they are still solvent-sensitive (Pinkart *et al.* 1996, Ramos *et al.* 1997). Starvation (Guckert *et al.* 1986) as well as the presence of antibiotics (Isken *et al.* 1997) or heavy metals (Heipieper *et al.* 1996) was also shown to evoke the activation of the *cis-trans* mechanism. Neumann *et al.* (2003, cf. **chapter 3**) proposed a correlation between the Cti-system and the activation of the potassium uptake system.

Stress for the bacterium due to the presence of an organic solvent (or other stress factors) can be measured by determination of the *trans/cis* ratio of unsaturated fatty acids in the membrane. Hence, this value can serve as a stress biomarker (Heipieper *et al.* 1996, de Bont 1998, Heipieper *et al.* 2003). There are several indications for the *cis-trans* isomerisation to be part of a general stress response of microorganisms.

1.7.1.4 Membrane proteins and phospholipid headgroups

Protein complexes embedded in the outer membrane of the cell are forming water-filled channels. The majority of them are so-called porins. Some studies have investigated their role in solvent tolerance since solvent molecules can pass through them. Therefore, mutants lacking porins or adapted bacteria having smaller porins than usual should be more resistant to the toxic effects of solvents (Ramos *et al.* 1996, Weber & de Bont 1996, Isken & de Bont 1998a).

While studying *E. coli*, an increase in membrane protein content was ascertained upon solvent exposure. In the presence of phenol and ethanol, respectively, growing cells showed a lower lipid-to-protein-ratio in the plasmic membrane (Dombeck & Ingram 1984, Keweloh *et al.* 1990). The fact emerged, that the increased protein content also serves the rigidification of the membrane and it counteracts the decreased saturation degree emerging upon ethanol presence/influence. The protein content of the membrane seems in fact to have a great influence on the fluidity of the membrane.

There are hints that flagellar proteins play a role in solvent tolerance as well (Kieboom *et al.* 2001, Segura *et al.* 2001).

It was ascertained that there is an increase of phospholipid biosynthesis in cells upon solvent stress. Compared to a solvent-sensitive mutant a significantly higher content of membrane phospholipids could be observed (Pinkart & White 1997).

As long-term response to the presence of the solvent toluene in *P. putida* DOT-T1, Ramos *et al.* (1996) observed changes in the phospholipid polar head groups: an enrichment of cardiolipin up to 22 % of the total phospholipids and a decrease of phosphatidylethanolamine. Such alterations lead to an increase of membrane viscosity counteracting the fluidizing effect of the solvent. It was shown by von Wallbrunn *et al.* (2002) that in mutants lacking the cardiolipin synthase, the *cis-trans* isomerisation was present on the same levels in wild type and mutant as response to 4-chlorophenol exposure, but this adaptive mechanism alone was not able to compensate for the lack of cardiolipin production.

In addition, alterations of the phospholipid headgroups have an effect on the physico-chemical properties of the membrane.

1.7.2 Active efflux of the solvent

In 1996, direct proof was found that solvents themselves could be excreted from the cell. The toluene concentration in the membrane can be reduced with the help of an ATP-dependent toluene-transporting system (Isken & de Bont 1996). Cells of *Pseudomonas putida* S12 that were adapted to toluene accumulate less solvent in their membranes compared to unadapted cells. It was shown that toluene-adapted cells accumulated more toluene in their membranes if treated with either the respiratory chain inhibitor potassium cyanide (KCN) or the proton conductor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Isken & de Bont 1996). Both compounds are structurally unrelated and are known to be energy-coupling inhibitors. The experiment suggested that energy is required for the cells to excrete solvent molecules actively (Isken & de Bont 1996).

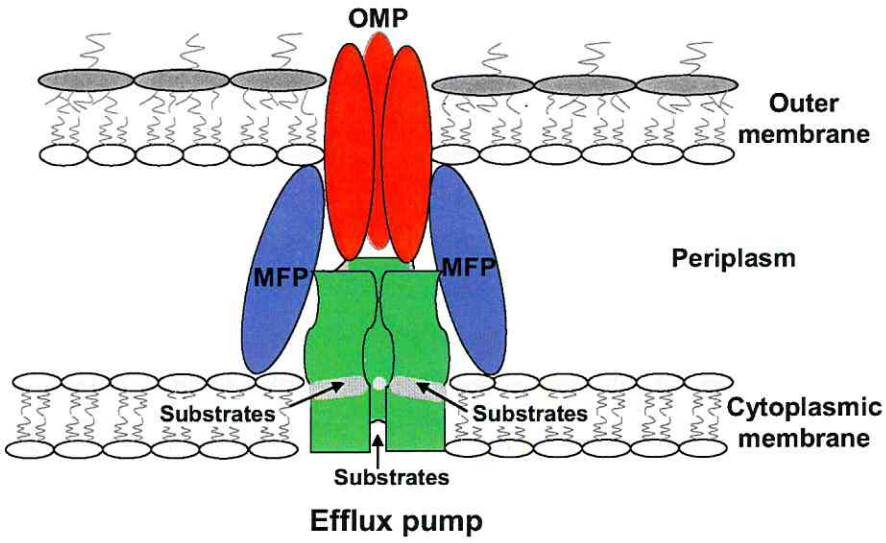

Fig. 1.7 Schematic model of the solvent efflux from the cytoplasmic membrane of a Gram-negative bacterium by active efflux pumps (Segura *et al.* 2004).

The solvent molecules are collected from the cytoplasmic membrane and are pumped out into the environment (de Bont 1998). **Fig. 1.7** shows a schematic representation of the functioning of the efflux pumps. The probably solvent-specific

efflux pumps take up the solvents in a manner that is described in the literature as "vacuum-cleaner effect" (Yu *et al.* 2003) and therefore they protect the bacteria against hydrophilic compounds that dissolve into the cytoplasmic membrane.
In the solvent-tolerant strain *Pseudomonas putida* DOT-T1E, three efflux-pumps located in the membrane and facilitating an efficient toluene tolerance were identified (Duque *et al.* 2001). The pumps named TtgABC and TtgGHI are constitutively expressed and next to toluene they "extrude" four different other solvents from the membrane. The third pump, TtgDEF, is induced by aromatic hydrocarbons such as toluene and styrene and the three encoding genes are linked to the chromosomal *tod* genes for toluene metabolism (Mosqueda & Ramos 2000). The pumps belong to the RND (resistance nodulation division) family, one of the four identified groups of the MDR (multi-drug resistant) families (Segura *et al.* 1999, Rojas 2001). Isken & de Bont (2000) showed that the solvent-efflux system in *Pseudomonas putida* S12 is specific for organic solvents and is not involved in antibiotic export or other compounds known to be substrates for pumps belonging to the MDR families, although it showed a striking resemblance to those (Kieboom *et al.* 1998a).
The membrane protein TolC from *Escherichia coli* exports solvents, heavy metals and antibiotics out of the cell providing a so-called multidrug efflux system (Koronakis *et al.* 2000). A completely different "extrusion system" is implemented by the toluene-tolerant strain *Pseudomonas putida* IH-2000 which uses membrane vesicles composed of phospholipids, lipopolysaccharides (LPS), and outer membrane proteins in order to extrude toluene molecules from its cells (Kobayashi *et al.* 2000).
The presence of this ATP-driven efflux system as well as other energy-consuming adaptive mechanisms is also reflected in the observation of lower growth yields of cells when grown in the presence of solvents (Isken *et al.* 1999, Neumann *et al.* 2006, cf. **chapter 5**).

1.7.3 Energetic status of the cell

For the energy transduction of the cell, the barrier properties of the cytoplasmic membrane are of special importance (Nicholls 1982). Due to the presence of solvents, it comes to an increase in the permeability of the membrane for ATP molecules, protons or other ions like potassium leading to a dissipation of the proton motive force, resulting in a less effective energy transduction. However, in the

literature one can find many adaptation mechanisms that are energy-consuming (Isken *et al.* 1996, de Bont 1998, Isken & de Bont 1998a, Kieboom *et al.* 1998a, b, Isken & Heipieper 2002, Ramos *et al.* 2002).

The energetic status of cells can be determined using a luciferin-luciferase bioluminescence reaction in which light is formed. The amount of light is proportional to the amount of ATP present in the sample. Typical ATP concentrations are within the range of 1-20 nmol/mg DW (Tran & Unden 1998). The overall energy charge of the cells can be calculated when the ADP and AMP concentrations in the cells are determined as well (Lundin *et al.* 1986). The energy charge (EC) is defined by the following equation:

$$EC = \frac{[ATP] + 1/2\,[ADP]}{[ATP] + [ADP] + [AMP]}$$ (Atkinson & Walton 1967).

Growth yield and maintenance coefficient are strongly connected. In general, cell growth and synthesis of proteins can only take place at high EC levels (> 0.75) and viability is maintained for EC values between 0.8 and 0.5; below that, the cells die (Chapman *et al.* 1971, Loffhagen & Babel 1985, Lundin *et al.* 1986, Barrette *et al.* 1988).

Nevertheless, the authors also present exceptions from that rule of thumb. Interestingly, in *Pseudomonas aeruginosa* biofilms, EC values of 0.2 at the base up to 0.6 at the top were observed, while AMP was the predominant nucleotide (Kinniment & Wimpenny 1992). In the presence of the solvent 1-decanol, Neumann *et al.* (2006) observed in *P. putida* cells a drop of the EC down to 0.3 - 0.45 at the end of the logarithmic growth phase (cf. **chapter 5, Fig. 5.1C**). That is comparable to values described in the literature at which viability of microorganisms still is maintained (Barrette *et al.* 1988, Lundin *et al.* 1986, Loffhagen & Babel 1985, Chapman *et al.* 1971). Very recently, it was observed that no significant differences exist between energy charge profiles during fermentations with and without a second phase of 1-decanol (Neumann *et al.* 2006, cf. **chapter 5, Fig. 5.1C**). That implies complete solvent adaptation of the cells on the energetic level. Although the bacteria needed additional energy for adaptation to the presence of the solvent, they were

able to maintain or activate electron transport phosphorylation, allowing homeostasis of the ATP level and energy charge in the presence of the solvent, at the price of a reduced growth yield. A similar behaviour was described for the membrane potential ($\Delta\psi$) of cells of *Acinetobacter calcoaceticus* during growth on acetate as carbon and energy source. The membrane potential also indicates the energetic state of living systems (Müller *et al.* 1996). Kayser *et al.* (2005) reported a decrease in the energy charge with increasing growth rates of *Escherichia coli* cells in continuous cultures. The maintenance energy is associated with the growth phase and it is an important factor at low growth rates (Pirt 1965). In the same study, it is stated that the yield is dependent on the growth rate. Actually, a reduction in energy yield leads to a higher maintenance coefficient. Isken *et al.* (1999) speculated that the difference in growth yield was caused by energy-consuming adaptation mechanisms and by a less effective energy metabolism in solvent-exposed cells.

1.7.4 The outer membrane: a protective barrier

In contrast to Gram-positive bacteria, Gram-negative bacteria additionally possess an outer membrane. Due to their relatively high impermeability, cell wall and outer membrane are important barriers against intrusive and toxic compounds like organic solvents (Beveridge & Graham 1991, Denyer & Maillard 2002).

A schematic picture of the Gram-negative cell wall and its main components is given in **Fig. 1.8**. The Gram-negative cell wall is surrounded by two membranes: an inner cytoplasmic and an outer membrane. The space between the inner and outer membrane is the periplasmic space. It contains negatively charged oligosaccharides as well as a large number of proteins for both nutrition supply and maintenance. Inside the outer membrane, hydrophilic channels called porins are present. They are divided in two classes: porins specific for a certain compound and non-specific porins. The latter porins facilitate the passive uptake into the periplasmic space of small (up to 600 Da), hydrophilic compounds. The outer membrane consists mainly of lipopolysaccharides (LPS, **Fig. 1.9**, cf. **1.7.5**), linked to the membrane by lipid A. LPS forms the outermost part of the cell wall and can extend up to 50 nm into the environment (Beveridge & Graham 1991, Denyer & Maillard 2002).

If one compares the number of solvent-tolerant Gram-negative bacteria with the number of solvent-tolerant Gram-positives, one could conclude that the former have

the higher potential for solvent-tolerance due to their outer membrane. In order to prevent the influx of solvent molecules into the cell membrane, it seems logical that microorganisms adapt themselves at the level of the first contact area with the toxin: the outer membrane. Nevertheless, not much is known yet about the changes in the outer membrane during exposure to organic solvents (Sikkema *et al.* 1995).

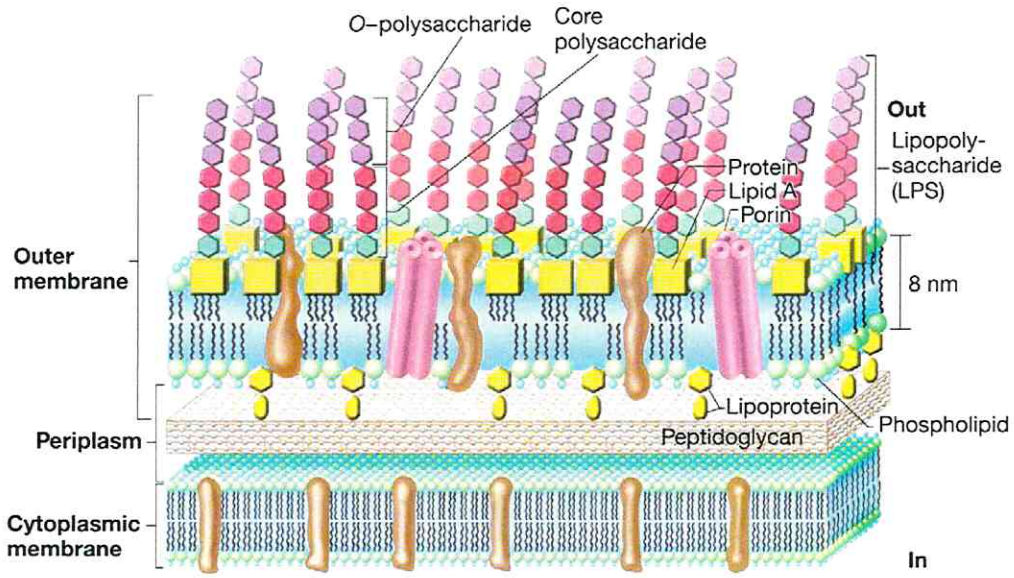

Fig. 1.8 Schematic picture of the Gram-negative cytoplasmic membrane, cell wall and outer membrane (Madigan & Martinko 2006).

A decrease in cell wall hydrophobicity had been postulated to repel the intrusive hydrophobic compounds effectively, therefore slowing the speed at which they diffuse through the cell wall and into the membranes (Sikkema *et al.* 1995, Isken & de Bont 1997, de Bont 1998). Studies have demonstrated that bacteria with an increased hydrophobicity of the cell wall show a higher affinity for hydrophobic compounds (van Loosdrecht *et al.* 1990, Jarlier & Nikaido 1994). This adaptation could increase the bioavailability of organic compounds that can be used as carbon and energy source and thereby be beneficial under those circumstances (Sikkema *et al.* 1994a, 1995) since Al-Tahhan *et al.* (2000) also showed an increased growth

rate on the hydrophobic substrate.

Pseudomonas putida Idaho is able to utilize the solvents toluene, *m*-xylene, *p*-xylene and other hydrocarbons. In this strain, it was observed that in the presence of *p*-xylene, the outer cell membrane became convoluted and membrane fragments were shed into the culture medium. At the same time, the cytoplasmic membrane invaginated, forming vesicles, and became disorganized (Cruden *et al.* 1992).

1.7.5 Lipopolysaccharide layer

Lipopolysaccharides (LPS) only exist as the principal component of the outmost part of the outer membrane of Gram-negative cell walls. This LPS layer has a large number of functions, including that as a physical and protective barrier for permeability in tolerance towards toxic compounds (Nikaido 1994). Indeed, LPS allows the traverse of hydrophobic molecules at about 1-2 % of the rates observed with typical phospholipid bilayers (Plesiat & Nikaido 1992, Allende & McIntosh 2003). Changes in LPS content and composition of *Pseudomonas aeruginosa* have been established for several stress factors such as heat shock, oxygen stress, and exposure to antibiotics like gentamicin (Russell & Furr 1986, Vaara 1992, 1993, Makin & Beveridge 1996b, Nguyen *et al.* 2003, Sabra *et al.* 2003).

LPS is a heteropolymeric layer of lipids and sugars and is composed of three regions: Lipid A, the core oligosaccharide, and the O-antigenic side chain (**Fig. 1.9**). The inner hydrophobic region, Lipid A, represents the pathogenic and endotoxin component of the LPS molecule. Linked to the core oligosaccharide, the outer region is an O-specific polysaccharide chain (also called O-antigen) which is responsible for the individual serotypes, meaning the antigenic properties of each bacterial strain.

Bacteria containing an LPS layer can be divided into two groups – based on the morphology of the colonies that are formed. So-called smooth or shiny colonies (S-form) contain all three LPS components. Mutants with a loss of or change in the O-antigen possess the rough-type LPS, form rough-surface colonies (R-forms), and are thus more susceptible towards antibiotics or hydrophobic compounds (Darveau & Hancock 1983, Nikaido & Vaara 1985, Russell 1995, El Hamidi *et al.* 2005). Norman *et al.* (2002) report that the loss of O-antigen in *P. aeruginosa* strains leads to shorter LPS molecules, increased cell surface hydrophobicity, and increased *n*-alkane degradation.

A second way of classifying LPS is based on size separation on SDS-PAGE gels. A distinction can be made between a shorter band (A-band, common antigen) and a longer band (B-band, serotype-specific) (Rivera *et al.* 1988, Lam *et al.* 1989, Rivera & McGroarty 1989, Arsenault *et al.* 1991, Norman *et al.* 2002). Mutants of *P. aeruginosa* possessing only A-band LPS demonstrated the highest surface hydrophobicity, followed by the strain lacking both A- and B-band LPS; adhesion to polystyrene mirrored the relative hydrophobicities of the strains (Makin & Beveridge 1996a).

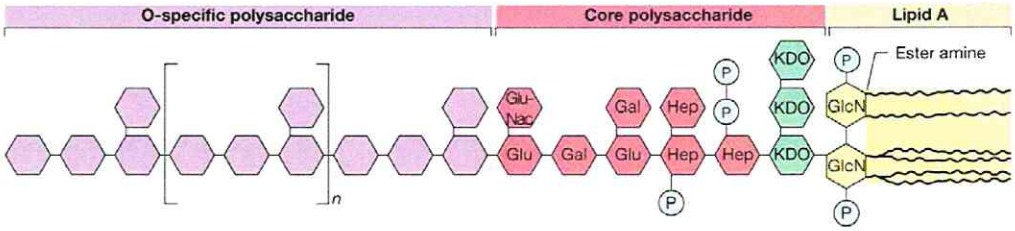

Fig. 1.9 Schematic picture of the lipopolysaccharide (LPS) structure of Gram-negative bacteria. LPS is divided into the highly variable O-specific polysaccharide, the core polysaccharide and lipid A. Glu: glucose. Glu-Nac: N-acetylglucosamine. Gal: galactose. Hep: heptose. KDO: 2-keto-3-deoxyoctonate. GlcN: glucosamine. P: phosphate. (Madigan & Martinko 2006).

Several *Pseudomonas* and *Salmonella* strains show antibiotic resistance due to changes of their LPS structure, which alter the cell surface hydrophobicity and permeability properties (Russell & Furr 1986, 1987, Arnoff 1988, Michéa-Hamzehpour *et al.* 1991, Piddock *et al.* 1992, Brözel & Cloete 1993, Sikkema *et al.* 1994a, Russell 1995). The loss of lipid A results in an increased susceptibility to hydrophobic antibiotics (Vuorio & Vaara 1992, Vaara 1993).

LPS are also partly responsible for the impermeability towards chemical compounds (Nikaido & Vaara 1985, Hancock 1991, Nikaido 1994, Wiese *et al.* 1999). Therefore, an alteration of LPS expression and structure is likely to have an effect on the cell membrane's permeability barrier function. The solvent-tolerant *P. putida* Idaho changes its LPS composition upon the presence of solvents (Pinkart *et al.* 1996).

The quantity and type of LPS found on the cell surface has a profound effect on cell

surface properties, such as the hydrophobicity, as well as on the interactions with solid surfaces (Darveau & Hancock 1983, Makin & Beveridge 1996a, Jucker *et al.* 1998, Denyer & Maillard 2002, Norman *et al.* 2002, El Hamidi *et al.* 2005).

1.7.6 Cell surface properties

Makin & Beveridge (1996a) suggested a connection between LPS profile and bacterial cell surface properties like surface charge, hydrophobicity, and adhesion. Strains lacking B-band LPS possessed more electronegative surfaces than those with B-band LPS; cells lacking both A- and B-band LPS had the highest surface electronegativity.

One parameter representing the surface charge is called zeta potential. This parameter strongly influences interactions with other charged particles, thereby affecting other parameters, like adhesive properties, by changing the electrostatic repulsion between the cells (van Loosdrecht *et al.* 1987a, Neumann *et al.* 2005a, 2006, cf. **chapter 4 and 5**). The adhesive properties of cells may influence the way bacteria interact with solid surfaces, such as the surface of a bioreactor or soil particles. It may be advantageous for the microorganisms to attach to a solid surface and form a biofilm. Furthermore, adhesion is influenced by the hydrophobicity of the cell surface. The most accurate method for the determination of the cell surface hydrophobicity is the so-called water contact angle.

The problem with physico-chemical properties of cells is more complicated since the cell surface is inhomogeneous and consequently contact angles and surface charges reflect only average properties (Vadillo-Rodríguez *et al.* 2003).

1.7.6.1 Cell surface charge

All bacterial surfaces possess an electronegative cell surface charge. From the literature, one exception is known: the clinical isolate *Stenotrophomonas maltophilia* (Jucker *et al.* 1996). Its positive charge is probably due to proteins located in the outer membrane lacking negatively charged groups. As a result, the adhesion to negatively charged surfaces is favoured compared with negatively charged bacteria like *Pseudomonas*. Phosphate plays a major role in the determination of the surface electrostatic charge since it is the most important charged component of the outer

cell surface. Furthermore, parts of the positive ions are loosely attached to the charged particle in form of a diffuse ion layer (van Loosdrecht *et al.* 1987b), which will move together with the particle as it moves through the liquid. This ion cloud increases the resistance against movement of the bacteria. These effects decrease the electrophoretic mobility of the bacterium and the obtained potential is therefore not the actual surface potential, but a value called zeta (ζ) potential (Ohshima & Kondo 1985, van Loosdrecht *et al.* 1987b, Sonohara *et al.* 1995, Tsuneda *et al.* 2004, for review on zeta potential method see: Wilson *et al.* 2001). The zeta potential can be calculated from the electrophoretic mobility using the Smoluchowski equation (von Smoluchowski 1918). This calculation assumes that all the charge is distributed in the form of a thin shell around a solid core particle. In bacteria, however, the charge is distributed in a relatively thick layer. Such a particle is called a "soft particle" according to the corresponding theory (Ohshima 1994, 2000, Ohshima & Kondo 1989, Tsuneda *et al.* 2004). In the case of bacteria, this "softness parameter" may also give information about the presence or absence of lipopolysaccharides.

Zeta potential measurements revealed that cells of *Pseudomonas putida* grown on phenanthrene (that has a low water solubility) were much more negatively charged than glucose-grown cells, suggesting that the polycyclic aromatic hydrocarbon (PAH) substrates induced modifications in the physical properties of bacterial surfaces (Rodrigues *et al.* 2005). Neumann *et al.* (2006, cf. **chapter 5**) observed a major increase in zeta potential of *P. putida* DOT-T1E cells upon exposure to the solvent decanol, meaning the cells became more negatively charged (**Fig. 5.2**). The very rapid changes in surface properties observed in those cells may indicate that occurring reactions are directed towards an energetically more favourable situation as seen before with *cis-trans* isomerisation (Heipieper *et al.* 1996, 2001). Another possible explanation is that components are simply lost from the cell wall. In *Pseudomonas putida* Idaho, a partial release of LPS upon the presence of organic solvents has been shown, supporting this theory (Kobayashi *et al.* 2000). Additionally, the release of membrane vesicles containing the solvent could be a protective mechanism against toxic organic solvents.

The zeta potential is also used within medical research, for example to determine the possible interaction or adhesion between tablets and the mucosa (Bogataj *et al.* 2003) or to assess the oligonucleotide-lipid-ratio of liposomes (Ciani *et al.* 2004).

Cationic liposomes are used in gene delivery because of their ability to form stable complexes with DNA fragments (lipoplexes).

The isoelectric point (IEP) is strongly connected with the cell's charge. Neumann *et al.* (2006, cf. **chapter 5**) determined the IEP of *Pseudomonas putida* DOT-T1E before and after exposure to 1-decanol. IEP-values of around 2.5 were found in all samples. An iso-electric point below 2.8 indicates the presence of cell surface lipopolysaccharides containing negatively charged phosphate and/or carboxyl groups (Lambert *et al.* 1975, Nikaido & Vaara 1985, Hancock *et al.* 1991, James 1991). These surface polymers inhibit adhesion onto both hydrophilic and hydrophobic surfaces. Bacteria with an IEP ≥ 3.2 appear to be free from polymer coatings that inhibit adhesion (Rijnaarts *et al.* 1995).

1.7.6.2 Cell surface hydrophobicity

The cell surface hydrophobicity is mainly analyzed by the method of the water contact angle (θ) measurement (van Loosdrecht *et al.* 1987a, **Fig. 1.10**). Contact angles for bacterial surfaces range from 15° (*E. coli*) over 38.5° (*P. putida*) up to 70° (*Mycobacter phlei*) (van Loosdrecht *et al.* 1987a). The measured contact angles correlated with the extent of the cells adhesion to a solid surface (linear regression coefficient 0.8). Alternatively, the hydrophobicity can be analyzed by the so-called microbial-adhesion-to-hydrocarbons test (MATH, Rosenberg *et al.* 1980).
Cells grown on glucose have a higher surface hydrophobicity (contact angle) than cells grown on phenol (Weber *et al.* 1993). The solvent-tolerant *Pseudomonas putida* S12 became less hydrophobic after adaptation to toluene (Weber & de Bont 1996). The observed reduction in the hydrophobicity of the outer membrane is expected to reduce the permeability of the membrane for the lipophilic compound toluene. Another possibility might be that the membrane itself does not become more hydrophobic, but rather the surface layer around it as it has been observed for surface layer proteins (Kotiranta *et al.* 1998, Vadillo-Rodríguez *et al.* 2004). A strain of *Mycobacterium* was reported to grow preferentially in the organic phase of a two-phase partitioning bioreactor while it was shown to have very high cell hydrophobicity (MacLeod & Daugulis 2005). Mozes *et al.* (1988) observed a direct correlation between the hydrophobicity of bacteria and the surface carbon

concentration in form of hydrocarbon – possibly due to the nature of cell membrane's phospholipid chains. For a *Pseudomonas putida* strain it was shown that the surfactant Triton X had no effect on the cell surface hydrophobicity (Tsubata *et al.* 1998).

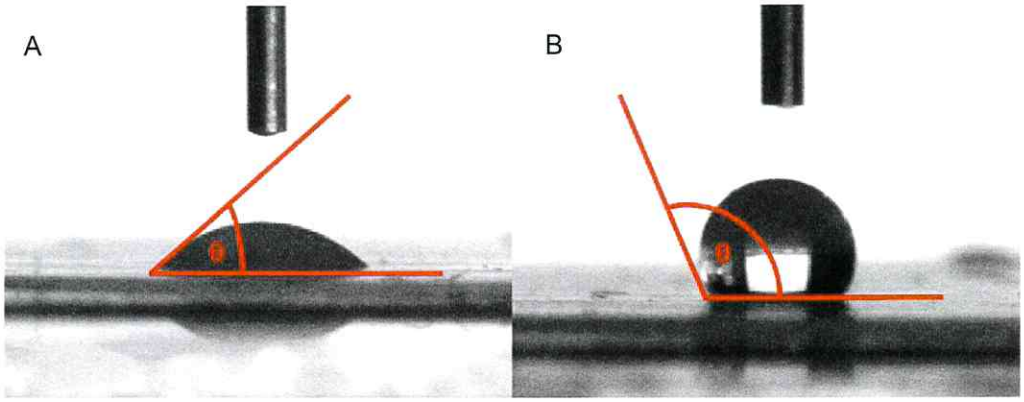

Fig. 1.10 Pictures of contact angle (θ) measurements. A) Hydrophilic bacterial layer causing a low contact angle. B) Hydrophobic bacterial cells causing a high contact angle.

Neumann *et al.* (2006, cf. **chapter 5**) observed an increase in the surface hydrophobicity of *Pseudomonas* cells in the presence of the solvent decanol. This is in contrast with previous findings (Weber *et al.* 1993, Weber & de Bont 1996) and assumptions by de Bont (1999) who speculated that solvent adaptation of *P. putida* at the level of the outer membrane should lead to a more hydrophilic surface in order to "repel" the solvent molecules and decrease the cell's affinity for them (Sikkema *et al.* 1995). However, the surface hydrophobicity of *Mycobacterium frederiksbergense* LB501T was studied by Wick *et al.* (2002, 2003). This bacterium showed an increased hydrophobicity and a reduced zeta potential when grown on the hydrophobic compound anthracene as sole carbon source – in comparison with glucose-grown cells. The increased hydrophobicity caused a higher affinity of the cells towards anthracene, which optimised the uptake of this compound in soils with low anthracene bioavailability (Wick *et al.* 2002, 2003). In a similar way, the observed increase in surface hydrophobicity could be beneficial for cells of *Pseudomonas putida* DOT-T1E (Neumann *et al.* 2006, cf. **chapter 5**), taken into account that

1-decanol can act as carbon and energy source for this bacterium (Neumann *et al.* unpublished results).

In connection with medical research, a decreased hydrophobicity was observed in antibiotic resistant *Salmonella enterica* strains (Braoudaki & Hilton 2005). Masuoka & Hazen (1997, 2004) suggest a relation between cell wall protein mannosylation and cell surface hydrophobicity in the pathogenic yeast *Candida albicans*. This is in support of earlier findings showing that rough-type mutants of *C. albicans* (that missed LPS parts) possessed increased cell surface hydrophobicities while lacking three quarters of the wild-type mannan content (Shimokawa & Nakayama 1986).

Cell surface hydrophobicity is an important factor in the ability of pathogenic opportunistic microorganisms to adhere to surfaces. The more hydrophobic the cells, the more easily they adhere to host cells.

1.7.6.3 Adhesive properties

Both the cell surface hydrophobicity and the cell surface charge have been shown to influence the adhesion of bacteria to solid surfaces (Busscher *et al.* 1984, van Loosdrecht *et al.* 1987a). There are examples for bacterial adhesion found with respect to the infection of various tissues (Woods *et al.* 1980), biomedical implants (Harkes *et al.* 1991), surfaces in the oral cavity (Marsh & Martin 1992), food-processing equipment (Visser & Jeurnink 1997), ship hulls (Cooksey & Wigglesworth-Cooksey 1995), in connections with waste treatment (Characklis 1973), and fermentations (Atkinson & Fowler 1974). Bacterial adhesion will be favoured if the process itself causes decrease of thermodynamic or free energy function (Absolom *et al.* 1983). Investigations concerning the interfacial adsorption of cells via dynamic surface tension measurements have been done in aqueous two-phase systems; it appears that increasing the overall polymer composition in the system causes the cells to adsorb at the interface (Alam *et al.* 1989). However, an increased surface charge may facilitate changes in adhesive properties to solid surfaces (van Loosdrecht *et al.* 1987a).

Van Loosdrecht *et al.* (1987a) have shown a relation between a combination of the electrophoretic mobility and hydrophobicity and the adhesion to a negatively charged surface. In general, a combination of low hydrophobicity and a high electrophoretic mobility seems to have a negative influence on adhesion, whereas both a decrease

in electrophoretic mobility and an increase in hydrophobicity positively affect adhesion. Thus, bacterial adhesion is controlled by the hydrophobicity (i.e. cell-water contact angle θ) as well as the negative electrokinetic potential of the cell surface and substrate (Dahlbäck *et al.* 1981, van Loosdrecht *et al.* 1987b, Stenström 1989). Chen & Zhu (2005) proposed that the bacterial adhesion could be explained in terms of the interaction free energies with the substrate, which are determined by the surface properties of bacteria, substrate and the surrounding medium. Van Loosdrecht *et al.* (1987b) confirmed that cell surface characteristics determining adhesion are influenced by growth conditions.

Several extracellular structures, such as LPS, flagella, and membrane proteins may affect the adhesion of bacteria – for example to the substrate (Cammarota & Sant'Anna 1998, Jucker *et al.* 1998, Briandet *et al.* 2001, Gómez-Suárez *et al.* 2002). Tsuneda *et al.* (2004) used a variety of heterotrophic bacterial strains for their investigations, e.g. *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Paracoccus denitrificans,* etc. Extracellular polymeric substances (EPSs) such as proteins, ketoses, pentoses, hexosamines, and hexoses were quantitatively analysed for each strain. The results indicated that EPSs covering the cell surface generally contribute to an increase in softness based on the soft-particle theory.

Neumann *et al.* (2006, cf. **chapter 5**) observed relatively little attachment of *Pseudomonas putida* DOT-T1E to glass. Interestingly, upon decanol exposure and the corresponding increase in hydrophobicity, the degree of attachment decreased by about 40 % (Neumann *et al.* unpublished results). This contrasts to the general finding that hydrophobic cells generally adhere better to solid surfaces than hydrophilic cells (van Loosdrecht *et al.* 1987b). Apparently, hydrophobicity and zeta potential are not the only factors influencing adhesion. This suggestion is supported by the observation that dead *P. putida* cells show very little adhesion, regardless whether they had been exposed to 1-decanol or not (Neumann *et al.* 2006, cf. **chapter 5**).

1.7.7 Morphological adaptation

Very little is known about the morphological changes in bacteria as response to solvents. Nevertheless, it has always been observed that cells increase their size upon toluene exposure. Park *et al.* (2001) reported that *Pseudomonas* cells showed

destructive openings on the cell envelopes upon treatment with aromatic hydrocarbons; the cells treated with ethanol displayed irregular rod shapes with wrinkled surfaces and crumpled shapes. Results obtained for mammalian cells showed that short-chain fatty acids (up to hexanoate) and parabens induce, at partially inhibitory concentrations, a jagged cell shape (Ginsburg *et al.* 1973).

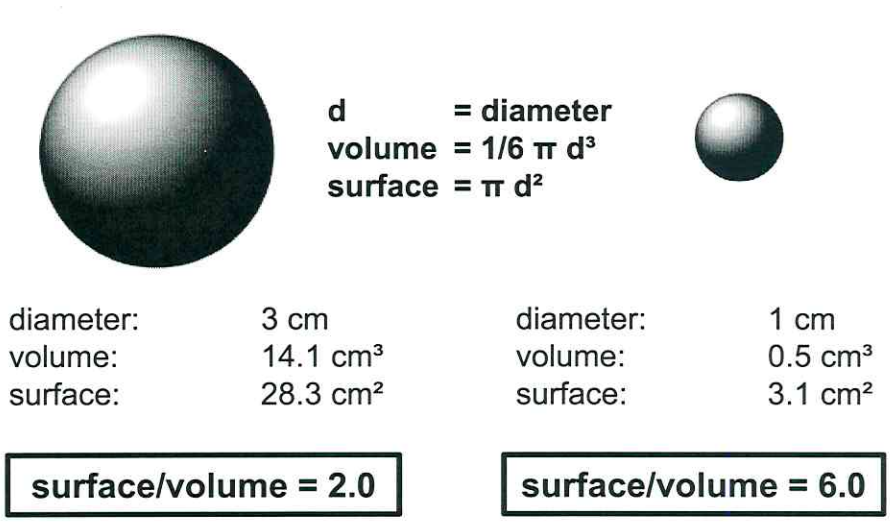

Fig. 1.11 Visualisation of the relation between volume and surface of a body. The bigger the corpus, the lower the surface/volume ratio, meaning the lower the relative surface.

In the 19th century, the physiologist Carl Bergmann stated a simple law for homoeothermic animals: the colder the climate, the bigger the organism (Bergmann 1847). Since the volume of a body increases with the cubic (of the diameter), but the surface only with the square (cf. **Fig. 1.11**), Bergmann's law refers to the biological selection advantage of the decreased temperature loss of a relatively smaller surface. Astonishingly, comparable adaptive results were obtained with bacteria as response to the presence of solvents. Neumann *et al.* (2005b) present findings obtained with *P. putida* and *Enterobacter* sp. cells showing an increased cell size upon exposure to phenol, 4-chlorophenol, and butanol (cf. **chapter 2**). It could be concluded that it is favourable for the bacterium to minimize the surface-to-volume ratio as this strategy facilitates a smaller target for the toxic effects of solvents with

respect to the cell membrane. On the other hand, a decrease in cell size has been shown in cells of *Enterobacter* sp. as an adaptive response to the solvent 1-butanol when grown on it as sole carbon and energy source (Veeranagouda *et al.* 2006). In this case, a bigger cell surface might provide an enhanced possibility for the uptake of potential growth substrates. For glucose-grown cells, which were not solvent-adapted, the addition of butanol led to the opposite effect: a decrease in the surface-volume ratio.

A recent work by de Carvalho *et al.* (2004) studied the morphological changes of a *Mycobacterium* species in aqueous, two-phase aqueous-organic and organic media. It was shown that cells in biphasic systems tended to shrink and decrease their surface roughness, meaning they decreased their surface area. A similar study with regard to biphasic systems containing different organic solvents (de Carvalho & da Fonseca 2004) observed morphological changes in very different bacterial species upon solvent exposure and concomitant incorporation of solvent molecules into the cellular membrane result in a loss of cell viability.

1.7.8 Transformation or degradation of solvents

A microbial resistance towards antibiotics is often caused by their degradation or by their transformation to less toxic compounds. Many toxic organic solvents can also be metabolised or transformed by microorganisms. Similar to antibiotic-resistance, the solvent-tolerance of bacteria could also be caused by those two mechanisms. This, for example, was postulated for a benzene-tolerant *Rhodococcus* strain (Paje *et al.* 1997) and for *E. coli* (Ferrante *et al.* 1995). Both bacteria can metabolise the solvent. Obviously, biotransformation of the solvent only plays a role if the organisms possess the relevant biodegradation pathway. In contrast to that, many solvent-tolerant bacterial strains can thrive in the presence of different solvents without being able to degrade or to modify them. For the toluene-tolerant and toluene-degrading strain *Pseudomonas putida* DOT-T1E, it was proven that the toluene degradation has no influence on the solvent-tolerance. Tol-negative mutants are on the same level solvent-tolerant like those who are able to degrade toluene via the tol-pathway (Ramos *et al.* 2002). Although it is useful in decreasing the toxic solvent concentration, the presence of a catabolic pathway for solvent degradation is not necessary for completing adaptation to the presence of a solvent. In fact, several

studies showed that solvent tolerance also occurs without any metabolism of the organic solvent (Inoue *et al.* 1991, Cruden *et al.* 1992, Pinkart *et al.* 1996). Therefore, it can be concluded that biodegradation is only a part of the adaptation mechanisms, but not necessarily the main mechanism of solvent tolerance (Isken & de Bont 1998a). Moreover, biodegradation is generally highly specific and does not play a major role in a broad solvent tolerance (Weber & de Bont 1996, Isken & de Bont 1997, 1998a, Isken & Heipieper 2002).

1.7.9 General stress response by the cell

Until now, many mechanisms facilitating solvent adaptation have been described. It is obvious that only the combination of different mechanisms leads to solvent tolerance (Isken & de Bont 1998a). A cascade of short-term and long-term mechanisms acts jointly in order to reach a complete adaptation of the cell. This suggests the presence of a general stress response, which is probably induced directly or indirectly by solvents since no molecular signal responsible for those mechanisms has been identified yet.

The induction of a great number of proteins in the presence of solvents, pollutants or xenobiotics has been described several times. Recently, a proteomic essay has revealed that many of the proteins involved in solvent tolerance of *Pseudomonas putida* DOT-T1E are stress related proteins (Segura *et al.* 2005). The authors reported that 35 proteins were induced by at least twofold in the presence of the solvent toluene. Lupi and colleagues documented an extensive change of the proteome of *Pseudomonas putida* KT2442 in the presence of 2-chlorophenol (Lupi *et al.* 1995). Earlier in this chapter, it was described that antibiotic-resistance is connected with solvent-tolerance (**1.7.1.2**, **1.7.1.3**, **1.7.2**, **1.7.8**). Furthermore, the adaptation to solvents raises tolerance towards other solvents (Heipieper & de Bont 1994) and additionally it increases tolerance towards heavy metals (Heipieper 1996) and antibiotics (Isken 1996). These observations give further hints that the regulation of the diverse solvent-adaptation response system is connected with a general stress response towards the observed stresses (Ramos *et al.* 2001). Alsaker *et al.* (2004) reported a connection between overexpression of the regulator *spo0A* and an increased tolerance and prolonged metabolism in response to butanol stress in *Clostridium acetobutylicum*. As response to primary alcohols (ethanol, butanol,

hexanol) in *Acinetobacter calcoaceticus*, the heat shock proteins GroEl, HtpG and DnaK were induced (Benndorf *et al.* 1999). For *Pseudomonas putida* KT2440 it was shown that when grown in the presence of a potential solvent nutrient (toluene), the strain rather expresses the general stress transcriptional machinery than the degradative one (Domínguez-Cuevas *et al.* 2006). Investigations concerning the functional genomics in *P. putida* KT2440 revealed that also adaptations of the membrane barrier, like adhesion properties and outer membrane proteins, are a subject of the general stress response (Reva *et al.* 2006).

Obviously, the variety of presented solvent adaptation mechanisms implies that bacterial solvent tolerance cannot possibly be provided by one single mechanism. The cell's goal of solvent adaptation can only be reached by a combination of a variety of different processes including an extensive stress response machinery activated by the solvent.

1.8 Outline of this thesis

The scientific and the industrial community regard biocatalysis as a promising research field. Particularly the sustainability approach regarding the development of new technologies for the production of fine chemicals for a broad variety of precursors and ingredients for the pharmaceutical, food, and agrochemical industry validates future biotechnological research (Schmid *et al.* 2001, 2002, Schoemaker *et al.* 2003, Shaw *et al.* 2003).

The establishment of a cost-efficient two-phase aqueous-organic biotransformation system is a promising approach in overcoming the limitations of the substrate and product spectrum within fine chemicals production.

Until recently, the two-phase system was not applicable because the suitable solvents with the necessary substrate reservoir and product extraction properties were toxic themselves to the used biocatalysts. The discovery of solvent-tolerant microorganisms able to adapt to otherwise toxic solvents offers new possibilities for the classical biotechnology.

In order for this concept to come into reach, an extensive knowledge about the adaptive mechanism of solvent-tolerant biocatalysts is crucial (**chapter 1**). Therefore, the goal of this thesis was to investigate how microorganisms manage to obtain solvent tolerance in that way that they grow in the presence of an otherwise toxic solvent, but are nevertheless able to maintain their stability and all their potential biocatalytic activities.

In particular, the work was focussed on investigations concerning the adaptation mechanisms on the level of morphology (**chapter 2**), cell membrane and permeability (**chapter 3**), energetic status as well as surface properties (**chapter 5**).

For our goal, the applicability of an economically sound biotransformation system with an organic solvent as second phase (**chapter 4**), all adaptive mechanisms investigated during this thesis and presented in **chapter 1** are essential.

2 CELLS OF *PSEUDOMONAS PUTIDA* AND *ENTEROBACTER* SP. ADAPT TO TOXIC ORGANIC COMPOUNDS BY INCREASING THEIR SIZE

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ABSTRACT

The phenol-degrading solvent-tolerant bacterium *Pseudomonas putida* P8 changed its cell shape when grown in the presence of aromatic compounds such as phenol and 4-chlorophenol. The sizes of cells that had been growing after addition of different concentrations of the toxic compounds were measured using a Coulter Counter that calculates the sizes of the rod-shaped bacteria to diameters of virtual spheres. The cells showed an increase in the diameter depending on the toxic effects of the applied concentrations of both solvents. The same effect was measured for an alkanol degrading bacterium, *Enterobacter* sp. VKGH12, in the presence of *n*-butanol. The reaction of the cells to different concentrations of *n*-butanol was examined by scanning electron microscopy. With this technique, it could be shown that the size of the bacteria increased with increasing concentrations of *n*-butanol. These changes in cell size were dependent on the cellular activity and occurred only after addition of non-lethal concentrations. In the presence of lethal concentrations that completely inhibited cell growth, the cell sizes were similar to those of cells without intoxication. Taking into account the mathematical formula for spherical and cylindrical diameter and surface, respectively, the cells reacted to the presence of

organic solvents by decreasing the ratio between surface and volume of the cells and therefore reducing their relative surfaces. As the cell surface and especially the cytoplasmic membrane are the major targets for the toxic effects of membrane-active compounds this reduction of the relative surface represents an adaptive response to the presence of such compounds.

INTRODUCTION

Organic solvents are known to be extremely toxic to microbial cells. The toxicity of these compounds to organisms is due to their preferential partitioning into membranes causing an increase in the fluidity of the membrane that leads to its non-specific permeabilization (Heipieper *et al.* 1994, Sikkema *et al.* 1995). The dose-dependent growth inhibition caused by organic solvents is very similar for a variety of aerobic microorganisms (Isken & Heipieper 2002).

However, microorganisms can adapt to different organic substances and other forms of environmental stress by several adaptive mechanisms (for review see: Ramos *et al.* 1997, Isken & de Bont 1998a, Segura *et al.* 1999, Isken & Heipieper 2002, Ramos *et al.* 2002). Here, especially bacteria of the species *Pseudomonas putida* have been shown to be the most solvent-tolerant strains. Among them, strain *P. putida* P8, a solvent-tolerant phenol degrading bacterium has been intensely investigated (Diefenbach *et al.* 1992, Heipieper *et al.* 1992, Diefenbach & Keweloh 1994). Thereby, changes in the membrane composition are known to play a crucial role in the mechanism contributing to solvent tolerance (Inoue & Horikoshi 1989, Ramos *et al.* 2001, Heipieper *et al.* 2003). Among them, the major mechanisms involved in solvent tolerance are changes in the degree of saturation of fatty acids (Pinkart *et al.* 1996), *cis-trans* isomerisation of unsaturated fatty acids (Heipieper *et al.* 1992, Heipieper *et al.* 1995), composition of phospholipid head groups (Ramos *et al.* 1997) and modification of lipopolysaccharides (Pinkart *et al.* 1996). Thus, the major adaptive responses of microorganisms to externally occurring changes in the environment are modifications of the cell envelopes. As an open question remains, whether these changes in the cell envelope are also connected with a change in the overall morphology of the cells.

In this contribution, we investigated the effect of toxic concentrations of solvents such as phenols and butanol on cell size and shape of two different bacteria, *Pseudomonas putida* and *Enterobacter* sp.

MATERIALS AND METHODS

Strains and chemicals

Pseudomonas putida P8 was isolated as a phenol-degrading bacterium and has previously been described (Bettmann & Rehm 1984). It was one of the first strains of *P. putida* in which solvent-tolerance was investigated (Diefenbach *et al.* 1992, Heipieper *et al.* 1992). *Enterobacter* sp. VKGH12 was recently isolated as an alkanol-degrading organism tolerating high alkanol concentrations and was identified using the culture system BIOLOG. Additionally, the strain was identified as *Enterobacter* sp. by sequencing of 16S-rRNA and alignment using the BLAST algorithm with BLAST server of the National Centre for Biotechnology (homology 99%, AY297785.1).

Culture conditions

Pseudomonas putida P8 and *Enterobacter* sp. VKGH12 were cultivated in a mineral medium as described by Hartmans *et al.* (1989) with 20 mM Na_2-succinate or glucose as sole carbon source. Cells were grown in 50 ml shake cultures in a horizontally shaking water bath at 30°C. Growth was monitored by measuring the turbidity (optical density) at 560 nm (OD_{560}).

Incubation with toxic compounds

For the measurements of the toxic effects of the investigated compounds, they were added to exponentially growing cultures as described by Heipieper *et al.* (1995). Cultures were incubated in the presence of the compounds for three hours in a shaking water bath at 30°C. Cells were then harvested and immediately used for the measurement of cell size. Growth inhibition caused by *n*-butanol was measured by comparing the differences in growth rate μ (h^{-1}) between intoxicated cultures ($\mu_{1,\ toxin}$) with that of control cultures ($\mu_{0,\ control}$). The growth inhibition of different concentrations

of *n*-butanol was defined as the percentage of the growth rates of cultures grown with *n*-butanol and that of control cultures without toxin addition.

$$\text{Inhibition growth (\%)} = \frac{\mu_1, \text{toxin}}{\mu_0, \text{control}} \times 100$$

Coulter Counter

The cell size of *P. putida* cells was measured using a Coulter Counter Multisizer 3 (Beckman Coulter, High Wycombe, UK). Before being measured, samples were diluted by factor 500 with an Isoton 2 standard solution (Beckman, High Wycombe, UK) using a Coulter Diluter DD3 (Beckman Coulter, High Wycombe, UK). Cell sizes, given as sphere diameters, were calculated using an MS-Multisizer 3 software (Beckman Coulter, High Wycombe, UK).

As the Coulter Counter calculates the measured cells to spheres, the volumes and surface areas of the cells were calculated as follows:

$$\text{Volume V } (\mu m^3) = 4/3\pi r^3$$

$$\text{Surface a } (\mu m^2) = 4\pi r^2$$

Scanning Electron Microscopy (SEM)

The effect of different *n*-butanol concentrations on morphology of *Enterobacter* sp. VKGH12 was studied using SEM. After three hours of incubation in the presence of different concentrations of *n*-butanol, bacterial cells were harvested and washed twice with 50 mM potassium phosphate buffer (pH 7.0). Bacteria were then fixed by immersion in 2.5 % glutaraldehyde (prepared in 50 mM potassium phosphate buffer) over night at 4°C. Then the specimens were washed twice with phosphate buffer, dehydrated by an ethanol series 30, 40, 50, 60, 70, 80, 90, and 100 % (v/v) ethanol, and stored in 100 % ethanol. Specimens in 100 % ethanol were gold coated for 15 min using an IB-3 (Giko E. Co., Japan) sputter cotter and examined under an S-2500C (Hitachi Co., Japan) SEM. Cell dimensions of cylindrical bodies were directly measured from the SEM photographs to calculate cell volume and surface

area by following equations:

$$\text{Volume } V\ (\mu m^3) = r^2\pi h$$

$$\text{Surface a}\ (\mu m^2) = 2r^2\pi + 2\pi rh$$

where r is the radius and h the length of the cylindrical cells. Average cellular volumes and surface area were calculated by using 30 individual bacteria per population. Cells showing deformations/depressions were not considered.

Statistics

All experiments were carried out 5-fold. For Coulter Counter experiments, the mean values as well as the standard deviations were taken as calculated by the MS-Multisizer 3 software (Beckman Coulter, High Wycombe, U.K.) of the Coulter Counter equipment. For the SEM-method, the mean values calculated from SEM-photographs by taking 30 bacteria per population. For the SEM-method, the mean values of the measures examined for 30 cells for each SEM-photograph were calculated. Statistics were calculated using the Parametric Statistic programme, version 1.01 (Lundon Software, Inc., Chagrin Falls, Ohio, USA).

RESULTS

Changes in the cell size of *Pseudomonas putida* in the presence of phenol and 4-chlorophenol

Cells of *P. putida* were grown in a mineral medium with Na_2-succinate as energy and carbon source. The growth rate μ of the cells was about 0.65 h^{-1}, which corresponds to a doubling time (t_D) of about 1.08 h. Phenol and 4-chlorophenol were added in different concentrations during the exponential growth phase. The organisms continued to grow exponentially, but at reduced growth rates. After three hours in the presence of the toxins, samples were taken to measure cell sizes using the coulter counter technique. **Fig. 2.1**shows the curves of the coulter counter measurements for *P. putida* cells for phenol (A) and 4-chlorophenol (B). In the presence of both solvents, a dose-dependent

increase in the diameter of the cells can be observed. *P. putida* P8 can use phenol as carbon and energy source. This explains the slight increase in the cell number in the presence of 100 mg/l whereas all other concentrations of phenol and 4-chlorophenol lead to a reduction of the cell number due to the toxic, growth reductive effect of the solvents.

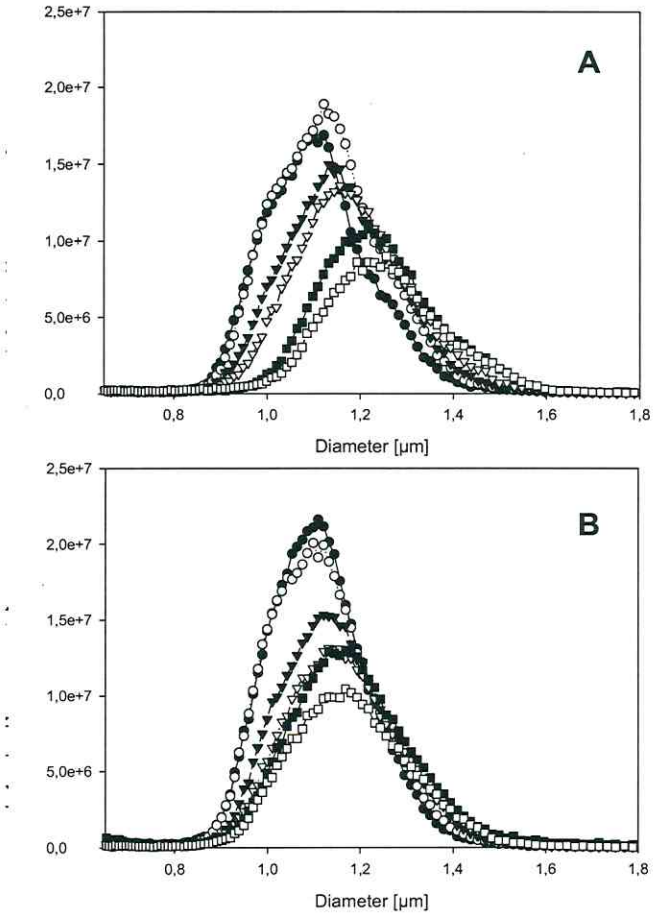

Fig. 2.1 Effect of toxic concentrations of phenol (**A**) and 4-chlorophenol (**B**) on the diameter of cells as well as cell number of *P. putida* P8 grown in mineral medium with Na_2-succinate as carbon source. Samples were taken after three hours in the presence of the toxins and measured by Coulter Counter. The concentrations added to the cultures were for (**A**) phenol (mg/l) 0 (●), 250 (❍), 500 (▼), 750 (▽), 1000 (■), 1250 (□); and for (**B**) 4-chlorophenol (mg/l) 0 (●), 100 (❍), 200 (▼), 300 (▽), 400 (■), 500 (□).

In the presence of both toxic aromatic compounds, the cells increased their size in relation to the added concentration of the toxin but regardless of the fact that this compound was added as carbon and energy source (phenol) or as a non-degradable compound (4-chlorophenol). This effect was depending on the growth inhibitory effect of the added non-lethal concentration of the specific solvent. **Fig. 2.2** shows the relation between the concentration dependent growth inhibition caused by phenol (A) and 4-chlorophenol (B) and the change in the ratio between surface and volume of the cells. Depending on the concentration, a significant reduction of the surface-to-volume-ratio can be observed.

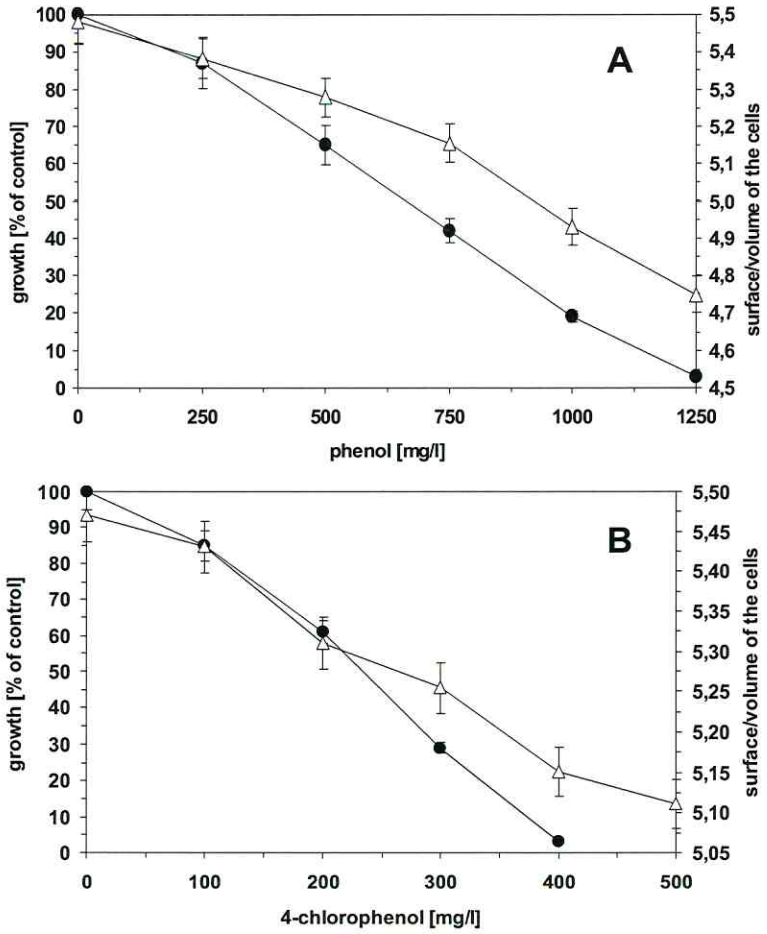

Fig. 2.2 Effect of phenol (**A**) and 4-chlorophenol (**B**) on growth (●) and the ratio between cell surface and volume (Δ) of cells of *P. putida* P8 measured by Coulter Counter.

Changes in the cell size of *Enterobacter* sp. VKGH12 in the presence of *n*-butanol

Using coulter counter technique, we could clearly show a dose-dependent effect of toxins on the cell size of *P. putida.* These coulter counter results were carried out with other bacteria and other toxins and always gave similar results (data not shown). To control these results and the applicability of the coulter counter, respectively, we used a second technique to measure the development of the cell size of a solvent-tolerant *Enterobacter* sp. VKGH12 grown in the presence of *n*-butanol. Therefore, the cell size was measured directly from SEM photographs that had been taken from cells growing in the presence of different concentrations of the toxin. This SEM technique has already been applied for studying the deleterious effects of stressful environment on the bacterial morphology (Shi & Xia 2003). The objects are clearly visualized and a three-dimensional view of the intact cells and their surface structure can be clearly observed.

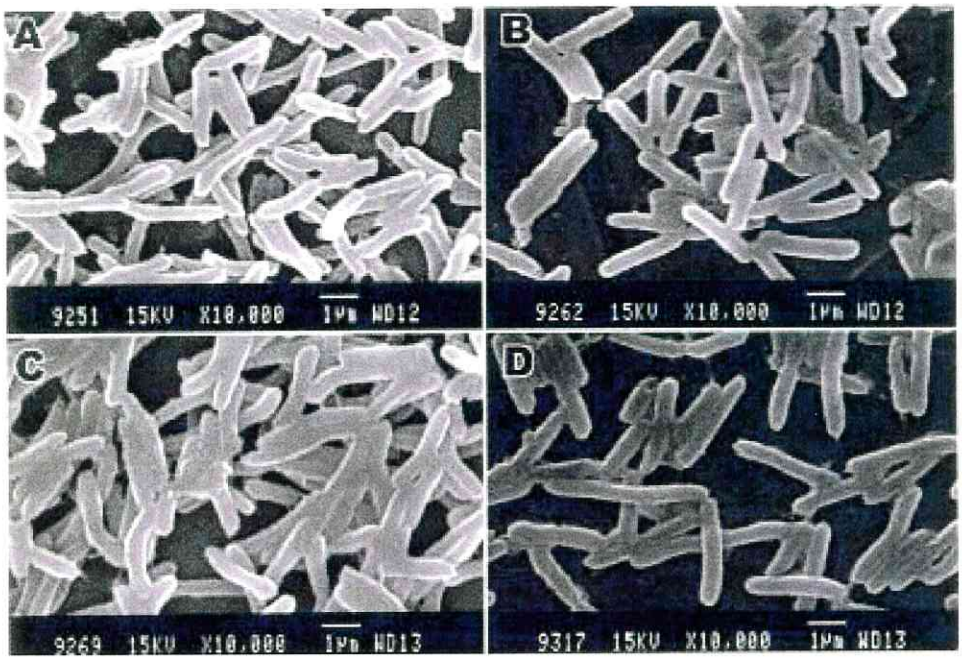

Fig. 2.3 SEM photographs of cells of *Enterobacter* sp. VKGH12 incubated for three hours in the presence of 0 % (**A**), 0.25 % (**B**), 1.0 % (**C**), and 1.5 % (**D**) (v/v) *n*-butanol.

Cells of *Enterobacter* sp. VKGH12 were grown in a mineral medium with Na_2-succinate as energy and carbon source. The growth rate μ of the cells was about 0.55 h^{-1}, which corresponds to a doubling time (t_D) of about 1.33 h. *n*-butanol was added in different concentrations during the exponential growth phase. These organisms continued to grow exponentially as well, but at reduced growth rates. **Fig. 2.3** shows SEM photographs of *Enterobacter* sp. VKGH12 cells that had been grown for three hours in the presence of different concentrations of *n*-butanol. The results are summarised in **Table 2.1**.

Table 2.1 Effect of *n*-butanol on growth, cell shape and the ratio between cell surface and volume of exponentially growing cells of *Enterobacter* sp. VKGH12. Cell length and radius were estimated by measuring out SEM photographs. For calculations, cells shape was assumed to be cylindrical.

n-butanol (%, v/v)	Growth (%)	Length (μm)	Radius (μm)	Surface (μm^2)	Volume (μm^3)	Surface/ volume
0	100	1.29	0.199	1.862	0.160	11.60
0.1	93	1.40	0.211	2.136	0.196	10.91
0.25	87	1.43	0.219	2.269	0.215	10.53
0.5	75	1.44	0.221	2.306	0.221	10.44
0.75	45	1.45	0.222	2.332	0.224	10.39
1	20	1.69	0.225	2.707	0.269	10.07
1.25	2	1.85	0.234	3.064	0.318	9.63
1.5		1.93	0.242	3.302	0.355	9.30
1.75		1.55	0.205	2.260	0.205	11.05
2		1.35	0.201	1.959	0.171	11.43

In the presence of *n*-butanol, the cells increased their size in relation to the added non-lethal concentrations of the toxin. However, in the presence of lethal concentrations of each toxin (i.e. 2 % v/v butanol) the cells show nearly the size of control cells without intoxication. Thus, the observed effect was strongly dependent on the fitness of the cells and is not only a chemico-physical effect of solvent

addition. By increasing their size, the cells reduce the relative area of their cell envelope, which is known to be the major target for the toxic actions of aromatic compounds such as phenols that are also defined as membrane-active toxins. **Fig. 2.4** shows the relation between the growth inhibition caused by *n*-butanol and the change in the ratio between surface and volume of the cells.

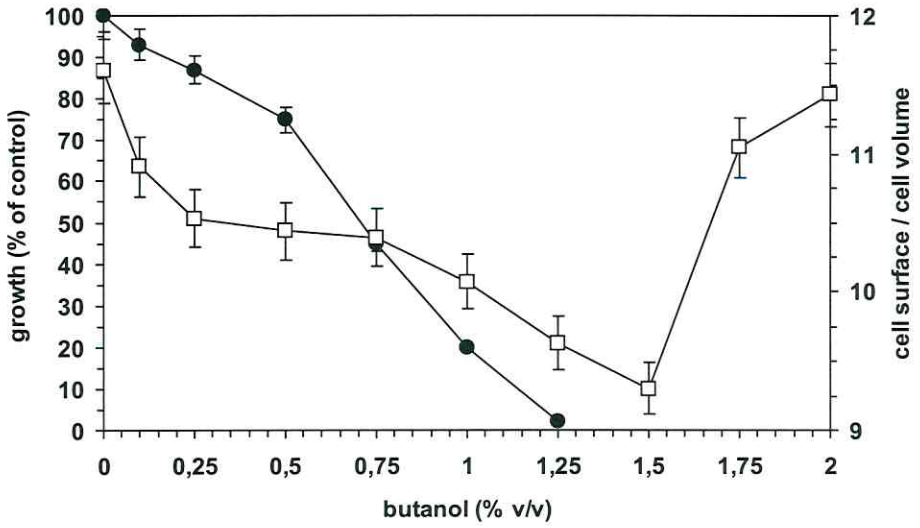

Fig. 2.4 Effect of toxic concentrations of n-butanol on growth (●) and the ratio between cell surface and volume (□) of cells of *Enterobacter* sp. VKGH12 measured from SEM photographs.

DISCUSSION

In eubacteria an external shell of peptidoglycan opposes internal hydrostatic pressure and prevents membrane rupture and death, as well as it gives each cell a certain size and shape that is also heritable allowing the bacteria to adapt to changes in environmental conditions (Young 2003). Indeed, changes in the morphology as an adaptive response have already been described for several bacteria and environmental conditions (stresses) (Chen *et al.* 1996, Rasanen *et al.* 2001, Ritz *et al.* 2001, Daniel & Errington 2003, Shi & Xia 2003). The changes in cell morphology as a response to toxic organic solvents observed in our experiments can

be explained by a relative reduction of cell surface with respect to its volume. By increasing the size, the volume of an organism increases with the cubic (for spheres, the volume $V = 4/3\pi r^3$), whereas the surface only increases with the quadrate (for spheres, the surface $a = 4\pi r^2$). This phenomenon is also known as Bergmann's law. Carl Bergmann, a 19th century's biologist, pointed out that amongst birds and mammals, individuals of the same species tend to be larger and heavier when they live in colder climates. Thus, the bigger a body is the smaller is its surface with respect to its volume. For homoeothermic organisms such as birds and mammals, a smaller surface/volume ratio causes a relatively smaller loss of body heat in colder climates. This gives bigger animals an advantage in the competition with smaller conspecifics.

Transferring this law to microorganisms, this means that a bigger size reduces the relative surface and consequently reduces the attachable surface for toxic organic compounds. Therefore, bigger cells are better protected against toxic organic compounds than smaller cells of the same species. This relative reduction of the cell surface represents an effective mechanism of the cells to reduce the toxic effect of environmental stress factors just by reducing the attachable surface in relation to the whole cell volume. This reaction makes even more sense by taking into account that one of the major adaptive mechanisms to various environmental factors, including toxic organic solvents, is the active efflux of those compounds by ATP-driven efflux pumps (Lewis 1994, Nikaido 1994, Isken & de Bont 1996, Rojas *et al.* 2001). It is obvious that the functioning of such solvent efflux pumps is more effective if the overall membrane surface is reduced. This leads to a reduction of the area that allows diffusion and partitioning of solvents into the membrane where they are recognised and excluded by the efflux pump proteins (Rojas *et al.* 2001).

For starving cells, the opposite effect has been described. When entering the stationary growth phase, bacteria are known to reduce their size and shape to smaller, nearly coccoidal structures (Givskov *et al.* 1994, Moller *et al.* 1996). This observation can also be explained by the surface/volume ratio already used for solvent tolerance: under starvation the cells increase their relative surface, their membrane area, to allow a better uptake and consumption of nutrients under these limiting environmental conditions.

Acknowledgments

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3 THE REGULATION OF THE *CIS-TRANS* ISOMERASE (CTI) OF UNSATURATED FATTY ACIDS IN *PSEUDOMONAS PUTIDA*: CORRELATION BETWEEN CTI ACTIVITY AND K^+-UPTAKE SYSTEMS

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ABSTRACT

The regulation of an urgent stress adaptive mechanism of *Pseudomonas putida*, the isomerisation of *cis* to *trans* unsaturated fatty acids, was investigated. By comparing the responses of the cells to a series of stress factors, a direct correlation between the activation of this unique mechanism and the well-investigated K^+-uptake pumps was observed. Only those stress conditions (osmotic stress caused by glycerol, cold shock, high pH) that are well known not to activate cellular K^+-uptake, showed no effect on the *cis-trans* isomerase (Cti). On the other hand, organic solvents, osmotic stress caused by NaCl and sucrose, heavy metals, heat shock and membrane-active antibiotics activated both K^+-uptake and the Cti system. This seems to be another indication for an activation of the constitutively present Cti by increasing membrane fluidity. The enzyme, located in the periplasm, can only reach its substrate, the double bond in the hydrophobic zone of the membrane bilayer, when the membrane fluidity is disturbed by an environmental factor.

INTRODUCTION

One of the solvent adaptation mechanisms enabling several *Pseudomonas* strains to grow in the presence of membrane disrupting compounds is the *cis-trans* isomerisation of unsaturated fatty acids (Heipieper *et al.* 1994, Keweloh & Heipieper 1996). The extent of the isomerisation apparently correlates with the toxicity and the concentration of such organic compounds in the membrane (Heipieper *et al.* 1992, Heipieper & de Bont 1994, Heipieper *et al.* 1995). A mutual dependence was found between the activation of this system and the induction/activation of other stress response mechanisms (Heipieper *et al.* 1996).

The *cis-trans* isomerase activity is constitutively present, does not require ATP or other cofactors like NAD(P)H or glutathione, and works in the absence of *de novo* synthesis of lipids (Heipieper *et al.* 1992, Morita *et al.* 1993, Heipieper & de Bont 1994, Diefenbach & Keweloh 1994).

The *cis-trans* isomerase represents the first description of an enzyme that is able to modify the configuration of the double bond of unsaturated fatty acids without changing the position of the double bond. Even non-enzymatic catalysis by using metals leads to a shift in the position of the double bond.

Therefore, the cti offers many interesting applications for the industrial modification of lipids that very often contain high percentages of *cis*-unsaturated fatty acids. Their isomerisation into the corresponding *trans*-isomer causes a drastic change in the physico-chemical properties of such fats and oils. For this reason, the *cti*-gene was already patented (Cognis Gesellschaft für Biotechnologie mbH 1997).

The enzyme has been purified from the periplasmic fraction of *Pseudomonas oleovorans* (Pedrotta & Witholt 1999) and *Pseudomonas* sp. strain E-3 (Okuyama *et al.* 1998). The *cis-trans* isomerase gene cloned and sequenced from *P. putida* P8 (Holtwick *et al.* 1997) and *P. putida* DOT-T1E (Junker & Ramos 1999) made clear that the isomerase has an N-terminal hydrophobic signal sequence, which is cleaved off after targeting the enzyme to the periplasmic space. Holtwick *et al.* (1999) provided evidence that the enzyme is a cytochrome *c*-type protein. For an enzyme preparation from *Pseudomonas* sp. strain E-3 it was suggested that iron (probably Fe^{3+}) plays a crucial role in the catalytic reaction (Okuyama *et al.* 1998).

Despite the wealth of available information, the biochemical mechanism of the solvent-

induced regulation of isomerase activity, in relation to membrane homeostasis (Ramos *et al.* 1997), still remains obscure.

As the copy number of the enzyme is estimated to be only about 2-3 copies per cell, a successful and economically interesting application must be initiated by the large scale synthesis of the protein in a recombinant bacterium. To get a successful transfer of the enzyme system into *E. coli*, major investigations must be done in order to understand the regulation of the protein and to describe the system leading to an export of the enzyme into the periplasmic space. While the latter must be performed mainly using molecular biological research and technology, the regulation will be investigated by classical physiological techniques.

In the present paper, we describe a strong correlation between the activity of *cis-trans* isomerisation and that of the well-known activation of potassium uptake systems, giving indication for the regulation of the isomerase by increasing membrane fluidity.

MATERIALS AND METHODS

Strain and materials

Pseudomonas putida S12 was isolated as a styrene-degrading organism and has previously been described (Hartmanns *et al.* 1990). All chemicals were reagent grade and obtained from commercial sources.

Culture conditions

Pseudomonas putida S12 was cultivated in a mineral medium as described by Hartmans *et al.* (1989) with 15 mM glucose as sole carbon source. Cells were grown in 100 ml shake cultures in a horizontally shaking water bath at 30°C. Growth was monitored by measuring the turbidity (optical density) at 560 nm (OD_{560}).

Measurement of growth and growth inhibition

An inoculum from an overnight culture was transferred into 40 ml fresh medium leading to a starting OD of 0.05. After 4 hours of exponential growth, the stress factors were added or the environmental conditions were modified, respectively. Cell growth was measured by monitoring the turbidity (OD560nm) of the cell suspensions

with a spectrophotometer. Growth inhibition caused by stress factors was measured by comparing the differences in growth rates μ (h^{-1}) between the test cultures and the control culture, as described by Heipieper *et al.* (1995). The different concentrations of growth inhibiting toxins were defined as the percentages of the growth rates μ (h^{-1}) of cultures grown in the presence of stress factors and that of a control culture. For the data presented in **Table 3.1**, all stress factors were applied to exponentially growing cells.

Table 3.1 Correlation between the activation of *cis-trans* isomerization of unsaturated fatty acids in *P. putida* and the activation of K^+-uptake systems in bacteria caused by different forms of stress

Stress	*Cis-trans* isomerisation	K^+-Uptake	References
Phenol	+	+	Heipieper *et al.* 1992, Heipiper *et al.* 1991
4-Chlorophenol	+	+	Heipieper *et al.* 1992, Heipiper *et al.* 1991
Toluene	+	+	Heipieper & de Bont 1994
Ethanol	+	+	Heipieper *et al.* 1995, Heipieper & de Bont 1994
Alkanols	+	+	Heipieper *et al.* 1995
NaCl	+	+	Heipieper *et al.* 1996, Csonka 1989
Sucrose	+	+	Heipieper *et al.* 1996, Csonka 1989
Glycerol	-	-	Heipieper *et al.* 1996, Csonka 1989
Zn^{2+}	+	+	Heipieper *et al.* 1996
Cd^{2+}	+	+	Heipieper *et al.* 1996
Heat shock	+	+	Heipieper *et al.* 1996
Cold shock	-	-	Heipieper *et al.* 1996
Low pH	+	+	Heipieper *et al.* 1996, Booth 1985
High pH	-	-	Heipieper *et al.* 1996, Booth 1985
Polymyxin	+	+	Isken *et al.* 1997
Nigericin	+	+	Isken *et al.* 1997

For all stress factors (several organic solvents, several osmotic stresses, heavy metals, temperature shocks, pH stress, membrane-active antibiotics), conditions were chosen which reduced cell growth up to 50 % and caused the greatest effects in adaptive responses.

Measurement of cellular K^+-content

One-millilitre samples were withdrawn before and after addition of toxins. Separation of cells from the supernatant was carried out by centrifugation (Eppendorf Centrifuge 5415 D, 13,000 rpm, 5 min). The cell pellets were disrupted in 5 % (wt/vol) trichloroacetic acid, and the debris was removed by centrifugation. The released K^+ in the supernatant was measured by flame photometry in a Unicam Atomic Absorption Spectrometer (Unicam). The conditions where as follows: wavelength 766,5 nm, split size 0.2 nm, burner angle 200°, flame acetylene/air.

All experiments were repeated three times. The average data of these results are shown. The standard deviation was less than 5 %.

Lipid extraction, transesterification, and fatty acid analysis

The lipids were extracted with chloroform/methanol/water as described by Bligh & Dyer (1959). Fatty acid methyl esters (FAME) were prepared by incubation for 15 min at 95°C in boron trifluoride/methanol applying the method of Morrison & Smith (1964). FAME were extracted with hexane.

Determination of fatty acid composition

FAME analysis was performed using gas chromatography (GC) (capillary column: CP-Sil 88; 50 m; temperature program from 160 to 220°C; FID, flame ionisation detector). The instrument used was a CP-9000 gas chromatograph (Chrompack-Packard). The fatty acids 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 (Shimadzu, 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 amounts of the C16 *trans* unsaturated fatty acids (16:1*trans*Δ9) and the C16 *cis* unsaturated fatty acids (16:1*cis*Δ9) of this bacterium.

RESULTS AND DISCUSSION

Effect of organic compounds on the permeability of the cell membrane

Organic solvents are toxic to cells, mainly due to their action on the membranes as so-called "membrane-active compounds" (Heipieper *et al.* 1994, Isken & Heipieper 2002). One of the first indications of membrane damage in bacteria caused by organic solvents is the efflux of potassium ions (Heipieper *et al.* 1991). **Fig. 3.1** shows the cellular potassium concentrations of exponentially growing bacteria in the presence of 4-chlorophenol. Immediately after addition of the toxin, a dose-dependent loss of cellular K^+-content was observed. In the presence of non-lethal concentrations, this preliminary loss was compensated by an enhanced activity of K^+-uptake pumps as they are present in every bacterial membrane (Booth 1985, Csonka 1989).

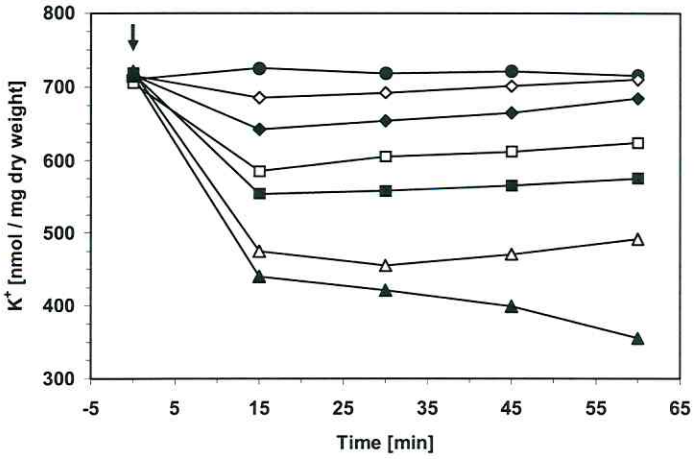

Fig. 3.1 Effect of 4-chlorophenol on the K^+-content of cells of *P. putida* S12. 4-chlorophenol was added to exponentially growing cells, as indicated by the arrow, in concentrations of 0.2 (◇), 0.4 (◆), 0.8 (□), 1.2 mM (■), 1.6 (△), or 2.0 (▲), respectively. Control cultures without 4-chlorophenol: (●).

Effects of organic compounds on *cis-trans* isomerisation of unsaturated membrane fatty acids

Fig. 3.2 shows the effect of 4-chlorophenol on growth, the increase in the *trans*/*cis* ratio and the cellular K^+-content one hour after addition of the toxin. After this time, all

possible cellular reactions had taken place in order to compensate the loss of K^+, by an enhanced uptake of potassium ions. A clear correlation was observed between the growth inhibition caused by the investigated compound, an activation of the *cis-trans* isomerase and the loss of cellular K^+-content. This had also been observed for several other organic compounds as well as for other forms of stress (Heipieper & de Bont 1994, Heipieper *et al.* 1996).

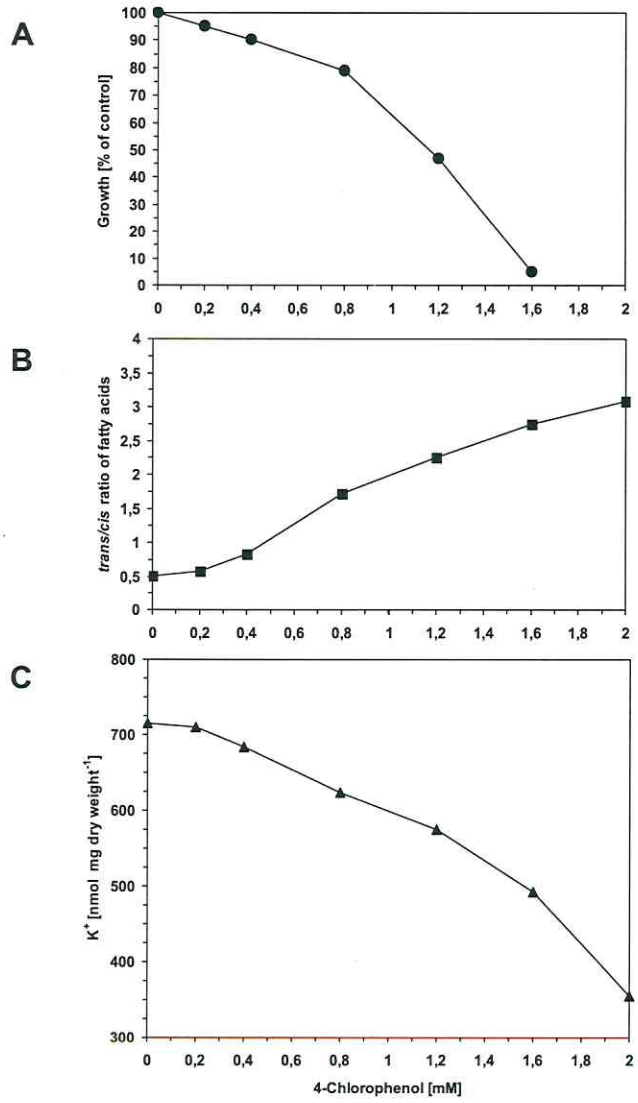

Fig. 3.2 Effect of 4-chlorophenol on (**A**) growth (●); (**B**) *trans/cis* ratio of unsaturated fatty acids (■); and (**C**) K^+-content one hour after addition of the stimulants (▲) of *P. putida*.

Correlation between the activation of the *cis-trans* isomerisation and that of K^+-uptake systems in *P. putida*

One of the main open questions regarding the *cis-trans* isomerase of unsaturated fatty acids in *Pseudomonas* (and several *Vibrio*) strains is how the activity of this constitutively expressed periplasmic enzyme is regulated. Regulation of enzyme activity may be brought about by giving the active centre of the enzyme the ability to reach its substrate, the double bond, which in turn depends on the fluidity of the membrane. An increase in the membrane microviscosity caused by changes of the acyl chain order in the presence of solvents was reported for many membrane systems and solvents, respectively (Killian *et al.* 1992). Accordingly, the observed regio-specificity reflects penetration of the active site of the isomerase to a specific depth in the membrane (Heipieper *et al.* 2001, von Wallbrunn *et al.* 2003). The hydrophilic structure and periplasmic location, respectively, of cti supports the assumption that the enzyme can only reach its target, the double bonds of unsaturated fatty acids that are located at a certain depth of the membrane, when the membrane is "opened" in the presence of environmental conditions that lead to a disintegration of the membrane (Heipieper *et al.* 2001). This is also known to be the signal for the activation of K^+-pumps. Table 1 shows the correlation of both adaptive mechanisms in the presence of several known environmental stress factors. All stress factors were applied to exponentially growing cells and under conditions that reduced cell growth up to 50 % and caused the greatest effects in adaptive responses.

Obviously, there is a very strong correlation between the activation of the well investigated K^+-uptake pumps and that of the *cis-trans* isomerase: only stress conditions (osmotic stress caused by glycerol, cold shock, high pH) that do not activate K^+-uptake also have no effect on the activation of the *cis-trans* isomerase. On the other hand, stresses (organic solvents, osmotic stress caused by NaCl and sucrose, heavy metals, heat shock, low pH, membrane-active antibiotics) caused an activation of both systems.

These results represent further indications for the regulation of the constitutively present cti by increasing membrane fluidity as it was suggested earlier (Heipieper *et al.* 2001). The enzyme that is located in the periplasm can only reach its substrate, the double bond in the hydrophobic zone of the membrane bilayer, when the

membrane fluidity is disturbed by an environmental factor. As the result, the reaction catalysed by the enzyme also causes reduction in membrane fluidity. This explains the relation between toxicity caused by a certain concentration of an environmental stress factor and cti activity that was already observed for many solvents and other forms of stress (Heipieper *et al.* 1994, Heipieper *et al.* 1995, Isken & Heipieper 2002). For a possible technical application of cti in hardening processes of plant oils that contain high percentages of *cis* unsaturated fatty acids this means that the enzyme can be applied in media containing high osmotic stress and organic solvents without being affected in its catalytic potential.

Further research will therefore be focussed on the cloning of the *cti*-gene in overexpression vectors to produce economically interesting amounts of the enzyme.

Acknowledgements

This work was partially supported by Contracts No. QLK3-CT-1999-00041 and QLRT-2001-00435 of the European Commission within its Fifth Framework Programme.

4 PREDICTION OF THE ADAPTABILITY OF *PSEUDOMONAS PUTIDA* DOT-T1E TO A SECOND PHASE OF A SOLVENT FOR ECONOMICALLY SOUND TWO-PHASE BIOTRANSFORMATIONS

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ABSTRACT

The strain *Pseudomonas putida* DOT-T1E was tested for its ability to tolerate second phases of different alkanols for their use as solvents in two-liquid-phase biotransformations. Although 1-decanol showed an about 10-fold higher toxicity to the cells than 1-octanol, the cells were able to adapt completely to 1-decanol only and could not be adapted in order to grow stable in the presence of a second phase of 1-octanol. The main explanation for this observation can be seen in the higher water and membrane solubility of 1-octanol. The hydrophobicity (log P) of a substance correlates with a certain partitioning of that compound into the membrane. Combining the log P value with the water solubility, the maximum membrane concentration of a compound can be calculated. With this simple calculation, it is possible to predict the property of an organic chemical for its potential applicability as a solvent for two-liquid-phase biotransformations with solvent tolerant *P. putida* strains. Only compounds that show a maximum membrane concentration of less than 400 mM – such as 1-decanol – seem to be tolerated by these bacterial strains when applied in super-saturating concentrations to the medium. Taking into consideration

that a solvent for a two-liquid-phase system should possess partitioning properties for potential substrates and products of a fine chemical synthesis, it can be seen that 1-decanol is a suitable solvent for such biotransformation processes. This was also demonstrated in shake cultures, where increasing amounts of a second phase of 1-decanol led to bacteria tolerating higher concentrations of the model substrate 3-nitrotoluene. Transferring this example to a 5-liter-scale bioreactor with 10 % (v/v) of 1-decanol, the amount of 3-nitrotoluene tolerated by the cells is up to 200-fold higher than in pure aqueous medium. The system demonstrates the usefulness of two-phase biotransformations utilising solvent-tolerant bacteria.

INTRODUCTION

Biocatalysis using whole cells promised to play an important role in the industrial synthesis of fine chemicals, pharmaceuticals, and precursors for chemical syntheses. However, the number of successful processes using whole-cell biotransformations is very small so far, because several factors limit the number of applications (Schmid *et al.* 2001, Schoemaker *et al.* 2003). One important limitation of a successful application of whole cell biotechnological processes towards classical chemical synthesis is that many reactions of interest involve substrates or products that are extremely toxic for the bacteria (Van Sonsbeek *et al.* 1993). This problem can be solved by the application of an organic solvent phase that functions as source and sink for toxic organic substrates and products, respectively (Vrionis *et al.* 2002a). For biotransformations with whole cells, the major advantage of an addition of a second organic phase lies within its ability to act as a sink for the substrates as well as in the continuous removal of products (Laane *et al.* 1987b, Leon *et al.* 1998, Vrionis *et al.* 2002a). In both cases, the presence of a solvent phase keeps the concentrations of substrates and toxins at a level that does not lead to toxic effects on the cells, which would decrease the activity of the biocatalyst. Additionally, the higher solubility of the product in the organic phase allows much higher product yield and volume productivities. That will lead to a massive reduction of the costs of downstream processing and product recovery (Bruce & Daugulis 1991, Leon *et al.* 1998).

To fulfil the needs for a certain process, the choice of an applicable organic solvent

for a two-liquid-phase system depends on the substrates, products, and the biocatalyst. The ideal solvent should be completely tolerated by the microorganism carrying out the biotransformation and should show high partitioning properties for the chosen substrates and resulting products. To allow an economically sound biotransformation process, both the substrates and the products should preferentially dissolve into the organic solvent phase. Each biotransformation process requires a series of experiments to test the behaviour of the biocatalyst in the system.

Organic solvents with a log P (the logarithm of the partition coefficient of a solvent in a two-phase water-octanol standard system) between 1.5 and 4 are extremely toxic for most microorganisms (Sikkema *et al.* 1995). Therefore, the ideal solvents for two-liquid-phase fermentations were not applicable because they themselves were toxic to the biocatalysts. In 1989, Inoue & Horikoshi discovered a bacterial strain that was able to thrive in the presence of the toxic organic solvent toluene (Inoue & Horikoshi 1989). The isolation and identification of several solvent-tolerant bacteria able to grow in the presence of organic solvents with toxic log P values like toluene (2.5) followed (Heipieper *et al.* 1994). Those discoveries were scientific breakthroughs that may help in overcoming limitations in industrial biotransformations and in expanding the applications of biocatalysts. The usage of solvent-tolerant bacteria as whole-cell biocatalysts in two-phase systems now allows the application of solvents with chemical properties needed for a successful implementation of whole-cell two-phase biotransformations.

One of the highly solvent-tolerant bacteria isolated is *Pseudomonas putida* DOT-T1E. This bacterium has been the subject for many investigations concerning the mechanisms responsible for these solvent tolerance properties (Segura *et al.* 1999, Ramos-Gonzalez *et al.* 2001). The bacterium is well described regarding its degradation properties. Molecular biological tools for its genetic transformation are also developed (Mosqueda *et al.* 1999, Ramos *et al.* 2002). Thus, *P. putida* DOT-T1E represents an ideal biocatalyst for biotransformations.

The aim of this study was to investigate the tolerance and growth of *P. putida* DOT-T1E in a two-liquid-phase system of aliphatic alcohols such as 1-octanol (log P 2.9), or 1-decanol (log P 4). These solvents were chosen because they are not harmful to humans like toluene and are thus better applicable for industrial processes. Additionally, they both are suitable as second phase in a two-liquid-phase

system in order to realise the extraction of fine chemicals of interest. This process is an example for the use of desirable agents in biotechnological productions of fine chemicals in a bioreactor with two liquid phases.

MATERIALS AND METHODS

Strain and chemicals

Pseudomonas putida DOT-T1E has previously been described (Ramos *et al.* 1995). All chemicals were reagent grade and obtained from commercial sources.

Culture conditions

Pseudomonas putida DOT-T1E was cultivated in a mineral medium as described by Hartmans *et al.* (1989), with Na_2-succinate as carbon and energy source. Adaptation of the cells to solvents was achieved by growing the cells semi-continuously in batch cultures with increasing concentrations of the solvents up to concentrations above saturation. Cells were grown in 50 ml shake cultures at 30°C in a horizontally shaking water bath. Growth was monitored by measuring the turbidity (optical density) at 560 nm (OD_{560}). A 1 ml inoculum from an overnight culture was transferred to 50 ml fresh medium and cells were grown exponentially for 3 to 4 hours (until an OD_{560} of 0.6).

Incubation with toxins

For the measurements of the toxic effects of the investigated compounds, they were added to exponentially growing, non-adapted cultures as described by Heipieper *et al.* (1995). Cultures were incubated in the presence of the toxins for 2 hours in a shaking water bath at 30°C. Then the cells were harvested, washed two times with potassium phosphate buffer (50 mM, pH 7.0) and stored at -20°C prior to their use for the lipid extraction.

Lipid extraction and transesterification

The lipids were extracted with chloroform/methanol/water as described by Bligh & Dyer (1959). Fatty acid methyl esters (FAME) were prepared by incubation for 15 min

at 95°C in boron trifluoride/methanol applying the method of Morrison & Smith (1964). FAME were extracted with hexane.

Analysis of fatty acid composition by GC-FID

Analysis of FAME in hexane was performed using a quadruple GC System (HP5890, Hewlett & Packard, Palo Alto, USA) equipped with a split/splitless injector. A CP-Sil 88 capillary column (Chrompack, Middelburg, The Netherlands; length, 50 m; inner diameter, 0.25mm; 0.25 µm film) was used for the separation of the FAME. GC conditions were: Injector temperature was held at 240°C, detector temperature was held at 270°C. The injection was splitless, carrier gas was He at a flow of 2 ml/min. The temperature programme was: 40°C, 2 min isothermal; 8°C/min up to 220°C; 5 min isothermal at 220°C. The pressure programme was: 27.7 psi (= 186.15 kPa), 2 min isobaric; 0.82 psi/min (5.65 kPa/min) to the final pressure 45.7 psi; 5.55 min isobaric at 45.7 psi (310.26 kPa). The peak areas of the carboxylic acids in total ion chromatograms (TIC) were used to determine their relative amounts. The fatty acids were identified by GC and co-injection of authentic reference compounds obtained from Supelco (Bellefonte, USA). The mean data of three independent experiments are shown. The standard deviation was less than 5 %.

Preparation of resting cells

50 ml of exponentially growing cells were harvested by centrifugation and suspended in the same volume of sodium phosphate buffer (50 mM, pH 7.0). Experiments were started 45 min after suspension of cells, by which time growth had stopped completely.

Cellular K^+ content

One-millilitre samples from cells suspended in sodium phosphate buffer were harvested at regular intervals before and after addition of solutes. Separation of cells from the supernatant was carried out by rapid centrifugation (Eppendorf table centrifuge, 13,000 rpm; 5 min). The cell pellet was disrupted by boiling in 5 % trichloroacetic acid, and debris was removed by centrifugation. The K^+ of the supernatant was measured by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) using a Spectroflame P/M (SPECTRO Analytical

Instruments, Kleve, Germany). All experiments were carried out three times; the average data of these results are shown. The standard deviation was less than 10 %.

Analysis of 3-nitrotoluene

The quantification of 3-nitrotoluene was done using HPLC. A C-18 column (CC250/4 Nucleosil 100-5 C18 HD) from Macherey-Nagel (Düren, Germany) with following attributes was used: pore size, 100 Å; particle size, 5 µm; inner diameter, 4 mm; length, 25 cm.

Fermentations

The fermentations were carried out in a 5 l fermenter (ISF-205, INFORS GmbH, Einsbach, Germany). Parameters such as temperature (°C), pH, CO_2-output (%), O_2-output (%), pO_2 (%), air flow (l/min), stirrer (rpm) and total weight (kg) of the fermenter were monitored online. The used IRIS software (INFORS CONTROL AG, Bottmingen, Switzerland) allowed the control of the measured parameters. Data were obtained and recorded with the Servomex Analyzer Series 1400 (East Sussex, England). Standard parameters for fermentations were as follows: 30°C, pH 7.2, and 1500 rpm. The fermenter was inoculated with an overnight culture in that way that a starting OD_{560} of 0.08 was reached. Due to difficulties measuring the optical density in a dispersed two-phase system, we obtained growth data via measuring the protein content of the cells (Bradford 1976). In the computer controlled fermentation unit, growth was continuously determined as CO_2 production. For the fermentations carried out in this study, a direct correlation between CO_2 production and protein content was observed.

RESULTS AND DISCUSSION

Adaptation of Pseudomonas putida DOT-T1E to the presence of 1-octanol and 1-decanol

In a set of first experiments, the effect of the four chosen alkanols, 1-hexanol, 1-octanol and 1-decanol and 1-dodecanol, on the growth of *Pseudomonas putida* DOT-T1E was investigated. From all investigated compounds, 1-hexanol featured the

lowest toxicity with a minimal inhibitory concentration (MIC, concentration leading to a complete inhibition of cell growth) of 16 mM (data not shown). This concentration was comparable to previous data with other bacteria (Kabelitz *et al.* 2003). In contrast, 1-dodecanol was not toxic to the cells at all (data not shown). As it was impossible to adapt cells to super-saturating concentrations of 1-hexanol and as the solvent properties of a very hydrophobic compound such as 1-dodecanol were not favourable for our purposes, further experiments were focussed on 1-octanol and 1-decanol as solvents. For this reason, adapted and non-adapted cells of *P. putida* were grown in a mineral medium with Na_2-succinate as energy and carbon source. The solvents were added in different concentrations during the exponential growth phase. The organisms continued to grow exponentially, but at reduced growth rates allowing a quantification of growth inhibition by comparing the growth rates as described by Heipieper *et al.* (1995). The results are summarised in **Fig. 4.1**. For non-adapted cells of *P. putida* DOT-T1E, 1-octanol showed a MIC of 2.2 mM. The MIC for 1-decanol was 0.23 mM und thus about 10-fold lower than for 1-octanol. Albeit this higher toxicity of 1-decanol, it was very easy to adapt the cells to a second phase of this solvent whereas this was not possible for 1-octanol. Microorganisms are able to adapt to the presence of toxic organic compounds using a whole cascade of adaptive mechanisms (for review: see Segura *et al.* 1999, Ramos-Gonzalez *et al.* 2001). One adaptive mechanism enabling several *Vibrio* and *Pseudomonas* strains to grow in the presence of membrane disrupting compounds is the isomerisation of *cis*- to *trans*-unsaturated fatty acids (Heipieper *et al.* 1992, Heipieper *et al.* 2003, Härtig *et al.* 2005). This mechanism could also be found in *Pseudomonas putida* DOT-T1E (Junker & Ramos 1999). The extent of the isomerisation, usually expressed as the *trans/cis* ratio of unsaturated fatty acids, apparently correlates with the toxicity of organic compounds (Heipieper *et al.* 1996, Heipieper *et al.* 2001). Therefore, we used the *trans/cis* ratio of unsaturated fatty acids as an indicator for stress and stress adaptation. In non-adapted cells, both solvents caused a strong dose-dependent increase in the *trans/cis* ratio of unsaturated fatty acids (**Fig. 4.1**).

The *cis-trans* isomerase is thought to be a kind of urgent response mechanism for the bacteria to adapt to stress (Heipiper *et al.* 2003) which is then substituted by other long-term adaptive mechanisms (Ramos-Gonzalez *et al.* 2001). When these adaptive steps have taken place, the *trans/cis* ratio is reduced to nearly zero (Härtig

et al. 2005). Therefore, low *trans/cis* ratios are an indicator for a complete adaptation of the bacteria to the environmental condition. Adapted cells showed a very low *trans/cis* ratio in the presence of 1-decanol, which proofs that the cells were completely adjusted and thus no longer stressed by the solvent. Contrary to that, 1-octanol also caused a reaction of the adapted cells in form of a slight increase in the *trans/cis* ratio of their unsaturated fatty acids. This indicated an incomplete adaptation to 1-octanol, which was confirmed by a reduced and unstable growth in the presence of a second phase of this solvent (data not shown).

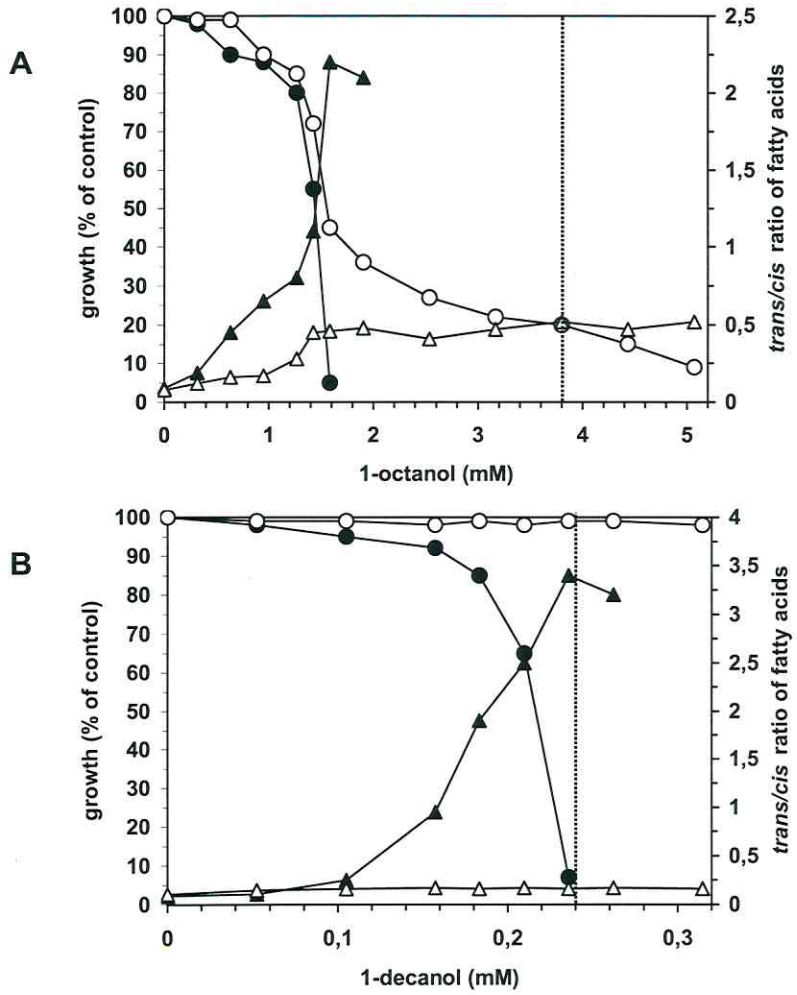

Fig. 4.1 Effect of 1-octanol (**A**) and 1-decanol (**B**) on growth (●, O) and *trans/cis* ratio of unsaturated membrane fatty acids (▲, △) of adapted (open symbols) and non-adapted (closed symbols) cells of *P. putida* DOT-T1E. The dashed lines mark the water solubility of the two solvents under the given conditions.

Effect of 1-octanol and 1-decanol on the cell membranes

Organic solvents are known to be toxic to cells, mainly due to their effect on the membranes as so-called "membrane-active compounds" (Heipieper *et al.* 1994, Sikkema *et al.* 1995). One of the first indications of membrane damage in bacteria caused by organic solvents is the efflux of potassium ions (Lambert & Hammond 1973, Heipieper *et al.* 1992). Different concentrations of 1-octanol and 1-decanol were added to resting cells. For a better comparison of the effects of the alkanols on membrane permeabilization, both compounds were added in concentrations that had caused similar growth inhibition effects on non-adapted cells of *P. putida* DOT-T1E (see **Fig. 4.1**).

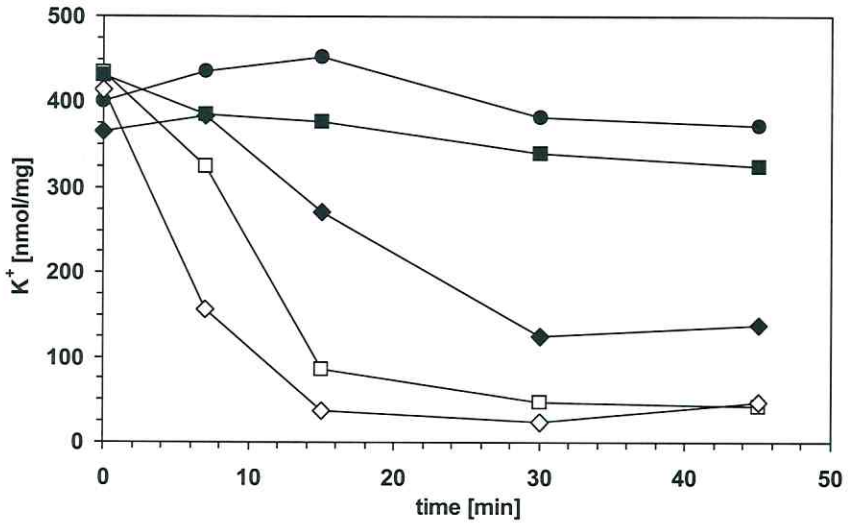

Fig. 4.2 Effect of 1-octanol and 1-decanol on the intracellular K^+-concentration of non-adapted resting cells of *P. putida* DOT-T1E. To allow a comparison of the effects of the alkanols on membrane permeabilization, both compounds were added in concentrations that caused a 50 % (1-octanol 0.0225 (□), and 1-decanol 0.004 (■) %, v/v) and 100 % (1-octanol 0.03 (◇) and 1-decanol 0.0045 (◆) %, v/v) growth inhibition of *P. putida* DOT-T1E. Control (●).

Fig. 4.2 shows the time scale of cellular potassium concentrations of resting cells after the addition of both alkanols. It is obvious that 1-octanol caused a much more drastic permeability increase, seen as a loss of cellular K^+ content, than those concentrations of 1-decanol that had similar effects on growth of the cells. Obviously, 1-decanol had a less dramatic effect on membrane fluidity than comparable

concentrations of 1-octanol. Although 1-decanol shows a much higher dose-dependent toxicity, the cell membranes seem to be less affected by this compound. This is another explanation for the easier adaptability of the cells to 1-decanol compared to 1-octanol.

Solvents in membranes

A relationship has been established between the toxicity of a solvent for an organism and the partitioning of that solvent to octanol from the water phase in a standard system (Weber & de Bont 1996). The logarithm of the octanol-water partition coefficient is designated as log P. This parameter was used to be an indicator of the solvent's partitioning from the aqueous medium into the membrane of microorganisms (Sikkema *et al.* 1994a). It is generally accepted that it is the solvent's effects on the cytoplasmic membrane, where it will preferentially accumulate, that result in the destruction of the organism (Sikkema *et al.* 1995, Weber & de Bont 1996). As a consequence of the high solvent concentrations in this compartment, the cell is no longer able to perform essential biochemical reactions and eventually loses its integrity. Various analytical techniques (Heipieper *et al.* 1994, Sikkema *et al.* 1994a) can be employed to determine the dose-response relationship of a solvent in a membrane (de Bont 1998). The different techniques reveal similar patterns, supporting the view that the actual membrane concentration of a solvent is an important parameter.

The solvent's membrane concentration depends on the concentration of the solvent in the water phase, the partitioning of the solvent from the water phase into the membrane, and the ratio of the volumes of the two liquid phases. Thereby, a correlation has been found between the log P value of a solvent and its partitioning between membrane and buffer (Sikkema *et al.* 1994a):

$$Log_{membrane/buffer} = 0.97 \times Log_{octanol/water} - 0.64$$

Using this equation, it is possible to calculate the actual concentration of a solvent in a membrane if its concentration in the water phase is known (de Bont 1998, Sikkema *et al.* 1994a). We performed these calculations that had originally been done for toluene now with all even-numbered aliphatic alkanols. Odd-numbered aliphatic

alcohols were not chosen because of their much higher price that would not allow an economic application. The results also presenting the data of de Bont (de Bont 1998) for toluene as reference compound and are summarized in **Table 4.1**.

Table 4.1 Relation between partitioning of several organic compounds in octanol/water (log P), membrane/buffer, water solubility and the maximum concentration in the membrane

Organic compound	log $P_{o/w}$ [a]	log $P_{m/b}$ [b]	$P_{m/b}$ [b]	Solubility [c] (mM)	MMC [d] (mM)	Adaptability
1-butanol	0.89	0.21	1.6	970.0	**1586**	-
1-hexanol	1.87	1.17	14.8	56.9	**841**	-
toluene	2.48	1.76	57.5	6.3	**368**	+
1-octanol	2.92	2.19	154.9	3.8	**588**	+/-
1-decanol	3.97	3.21	1621.8	0.23	**379**	+
1-dodecanol	5.02	4.23	16982.4	0.015	**254**	+
1-tetradecanol	6.07	5.25	177827.9	0.0008	**142**	+

[a] log $P_{octanol/water}$ calculated according to Rekker & de Kort (1979)
[b] log P membrane/buffer and P membrane/buffer calculated according to Sikkema *et al.* (1994a)
[c] Solubility in H_2O (%, v/v) at 30°C calculated according to Kinoshita (1958)
[d] MMC = maximum membrane concentration calculated according to de Bont (1998)

A direct relation between the concentrations of organic compounds in the membrane and their EC50 (Effective Concentration, inhibiting 50 % of cell growth) concentrations was already described by Heipieper *et al.* (1995). In this study, it was found that for every compound the actual concentration in the membrane necessary for an EC50 effect was nearly the same and ranged around 200 mM (Heipieper *et al.* 1995). So, for all compounds tested nearly the same concentration in the membrane is necessary to reach a comparable toxic impact. These results support the hypothesis of Sikkema *et al.* (1994a) who argued that the toxic effects of organic solvents are independent from the structural features of the molecules, but are strongly related to their ability to accumulate in the membrane. On the other hand, the specific chemical toxicity of a compound has to be taken into consideration. For

example, the nature and the degree of the substitution of N-aromatic substances were observed to have a profound effect on the toxicity of a compound (Donlon *et al.* 1995).

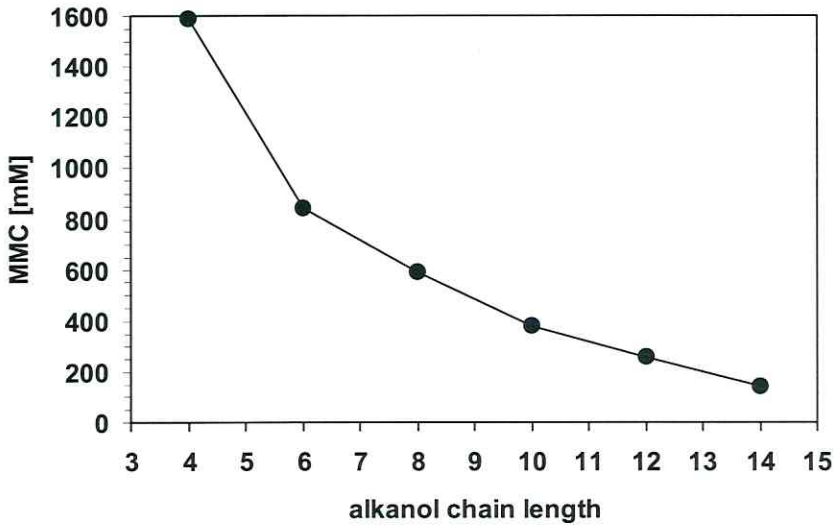

Fig. 4.3 Relation between the chain length of 1-alkanols and their maximum membrane concentration (MMC) calculated according to de Bont (1998) and as described in the text.

Calculating actual solvent concentrations in membranes helps us to understand the behaviour of solvents in biological membranes. Apparently, *P. putida* DOT-T1E can easily adapt to a second phase of those compounds that show a maximum membrane concentration (MMC) of around 400 mM or lower, such as toluene, 1-decanol, or 1-dodecanol. However, toluene was not tested as second liquid phase in this study. In case of 1-octanol, the MMC seems to be too high to allow a complete adaptation to the presence of a second phase. This compound is tolerated by the bacteria but does not allow non-affected stable growth in its presence. Similar results have already been reported by Rojas *et al.* (2004). Alkanols with short chain lengths (such as 1-hexanol and 1-butanol) show very high water solubility. Their MMC is too high so that no bacterium should be able to adapt to such a second phase. The lower MMC of 1-decanol compared to that of 1-octanol is not in contradiction with the higher toxicity of this compound towards non-adapted cells. The major parameter

that determines toxicity is the hydrophobicity, at least in a range up to a log P of around 4. Indeed, the MMC only allows a prediction of the toxicity of a compound when added in form of super-saturated concentrations.

The higher the chain length of an alkanol, the higher is its hydrophobicity and thus its tendency to accumulate preferentially in membranes (**Fig. 4.3**). However, as water solubility decreases with increasing chain length, a compound such as 1-dodecanol will not reach a high membrane concentration and is therefore not toxic to an organism. This is the reason, why solvents in a log P range of 1.5 - 4 are so extremely toxic: because they are relatively water-soluble and still partition well into the membrane. As a result, the actual membrane concentration of these solvents will be too high (Sikkema *et al.* 1994a, 1995, Weber & de Bont 1996). Thus, this calculation method is a useful tool to predict whether a solvent-tolerant bacterium is able to adapt to a second phase of a certain compound – in order to use it as solvent for two-liquid-phase biotransformations.

Effect of a second phase of 1-decanol on the toxicity of 3-nitrotoluene

In shake cultures, the toxicity of 3-nitrotoluene as a model substrate for biotransformations was estimated. The EC50 value of 3-nitrotoluene in cells cultivated in mineral medium without a 1-decanol phase was 1.75 mM and the minimal inhibitory concentration (MIC) was 3 mM. These values drastically increased with the application of a second phase of 1-decanol. Even in the presence of only a few percent (v/v) of 1-decanol, the bacteria showed a drastically increased tolerance towards 3-nitrotoluene (**Fig. 4.4**); the tolerance of the cells increased with the volume percentage of 1-decanol and thus with the amount of a second phase of the solvent. Contrary to that, the toxicity of 3-nitrotoluene was not affected by the presence of a second phase of 1-octanol (data not shown). This observed increase in tolerance is surely not only the effect of an adaptive process of the cells but mainly the result of a reduced bioavailability of the compound. For a detailed explanation of this effect of the solvent we measured the partition coefficient of 3-nitrotoluene in a 1-decanol-water-system (log $P_{d/w}$). The measured partition coefficient was 500 (log $P_{d/w}$: 2.72), which was very similar to the log $P_{o/w}$ value of 3-nitrotoluene (log $P_{o/w}$: 2.46). Because of this preferential partitioning of 3-nitrotoluene in 1-decanol, a second phase drastically reduces the concentration of the substrate within the medium phase, so

that much higher concentrations can be added without reaching the EC50 or MIC.

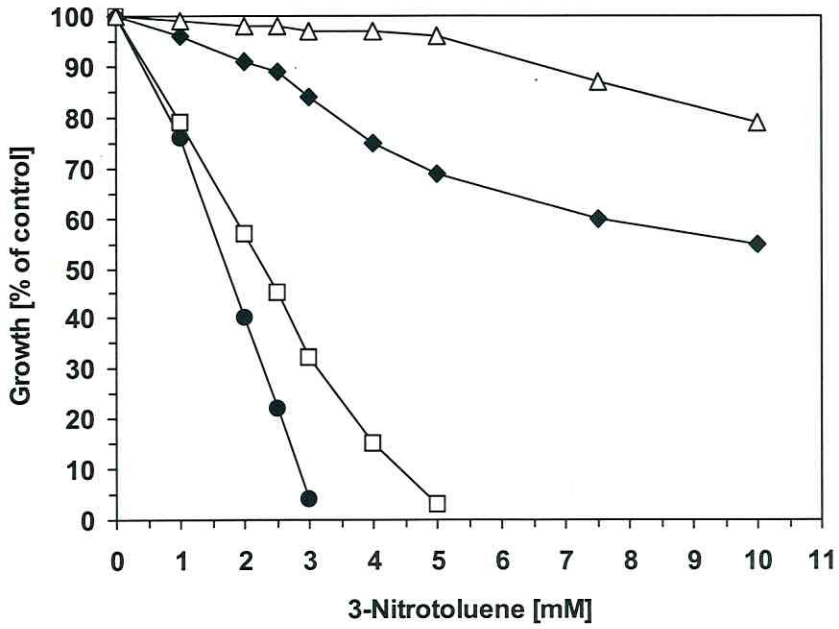

Fig. 4.4 Effect of the presence of a second phase of 0 (●), 1 (□), 5 (◆), and 10 (Δ) % (v/v) 1-decanol on growth inhibition caused by 3-nitrotoluene of shake cultures of *P. putida* DOT-T1E.

The volume dependent partitioning of a potential substrate (S) obeys a hyperbolic function, depending on the volumes of the two phases and the partition coefficient (K) of S in the solvent/water system:

$$\text{concentration of } S_{water} = \frac{\text{amount of S added to the system}}{\text{volume}_{water} + K_{solvent/water} \times \text{volume}_{solvent}}$$

Therefore, a phase of 10 % (v/v) of 1-decanol already contains more than 98 % of the added 3-nitrotoluene. Therefore, the application of a solvent such as 1-decanol allows the production of chemical compounds using whole cell biocatalysis in amounts that allow an economical competition with classical chemical synthesis.

To scale up this system, we continued our studies in 5 l fermentations with the presence of a 10 % (v/v) phase of 1-decanol. Also in this scale, the adapted cells of *P. putida* DOT-T1E showed the same growth behaviour in the presence as in the absence of the solvent (**Fig. 4.5**). Furthermore, the addition of up to 120 mM of 3-nitrotoluene did not cause any growth inhibition at all (**Fig. 4.5**).

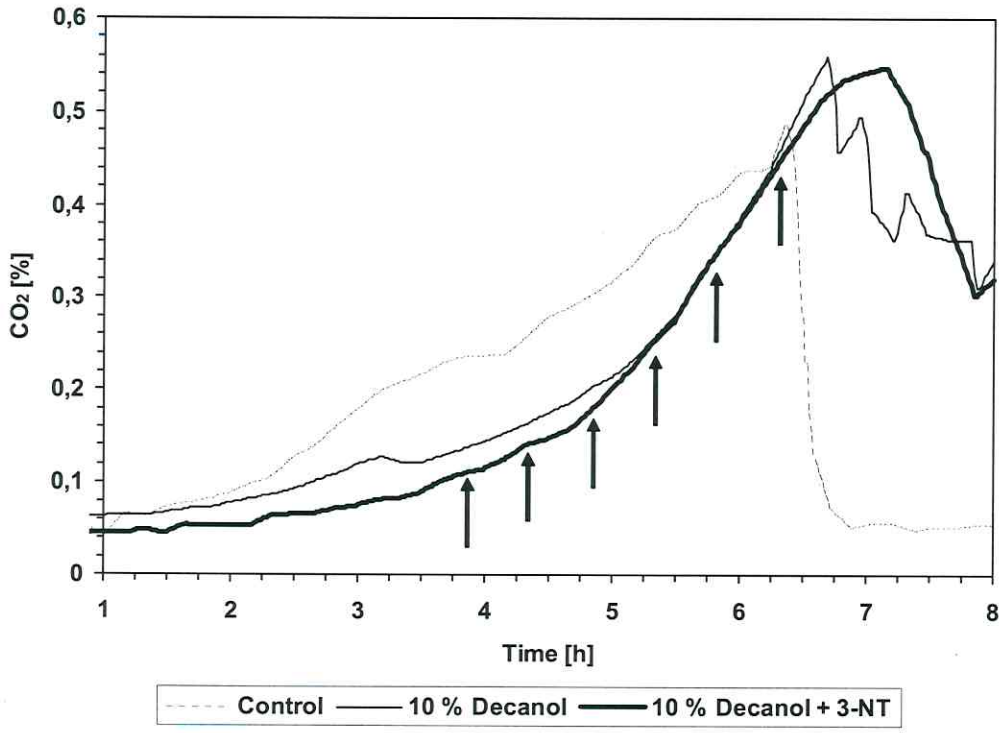

Fig. 4.5 Growth of *P. putida* DOT-T1E in a 5 l bioreactor in the absence (---) and in the presence (—) of a second phase of 10 % (v/v) 1-decanol, and in the presence of 10 % (v/v) 1-decanol with sub-sequential additions (as indicated by the arrows) of 20 mM 3-nitrotoluene (—).

A second solvent phase is beneficial as it allows a continuous extraction of the product from the aqueous medium phase as well as the presence of very high amounts of the substrate in the system. It keeps the concentration of both the substrates and the products in the aqueous phase below a level that would lead to growth inhibition or even to death of the bacteria. 1-Decanol, in which toxic compounds with a log P between 1 and 4 preferably partition, might be a suitable

alternative to hydrophobic solvents such as 1-dodecanol or hexadecane used as second liquid phase in biotransformations with *P. putida* DOT-T1E as biocatalyst. Additionally, the presented calculations of the maximum membrane concentrations (**Table 4.1**) allow a better prediction whether a potential solvent is tolerated by the biocatalysts. The partitioning of potential substrates and products between 1-decanol and water allows a prediction of maximum concentrations of fine chemicals within the whole fermentation system that will be tolerated by the bacteria. Thus, the application of a solvent-tolerant organism in such a 1-decanol water system offers new possibilities in coping with toxic products.

P. putida DOT-T1E and related strains such as *P. putida* S12 set an example for bacteria, which can tolerate solvents and are able to produce fine chemicals of interest in economically sound concentrations (Wery *et al.* 2000, Hüsken *et al.* 2001). These bacteria contain at least three different solvent efflux pumps that play a major role in adaptation (Segura *et al.* 2004) and are therefore the most effective potential biocatalysts among all *P. putida* strains. Additionally, these strains are very accessible to genetic manipulation and genes from other microorganisms can easily be introduced making it possible to obtain a wide range of products. Using this biotransformation system, many important fine chemicals with low molecular weight, including catechols, phenols, and ketones could be produced in concentrations and with volumetric productivities that will allow competing with classical chemical syntheses.

Acknowledgements

This work was partially supported by Contract No. QLRT-2001-00435 of the European Commission within its Fifth Framework Programme.

5 ENERGETICS AND SURFACE PROPERTIES OF *PSEUDOMONAS PUTIDA* DOT-T1E IN A TWO-PHASE FERMENTATION SYSTEM WITH 1-DECANOL AS SECOND PHASE

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ABSTRACT

The solvent-tolerant strain *Pseudomonas putida* DOT-T1E was grown in batch fermentations in a 5-liter-scale bioreactor in the presence and absence of 10 % (v/v) of the organic solvent 1-decanol. The growth behaviour, cellular energetics, such as the cellular ATP-content and the energy charge, as well as the cell surface hydrophobicity and charge were measured of cells growing in the presence and absence of 1-decanol. Although the cells growing in the presence of 1-decanol showed an about 10 % reduced growth rate and a 48 % reduced growth yield, no significant differences were measured, neither in the ATP and potassium content nor in the energy charge indicating that the cells completely adapted on the level of membrane permeability and energetics. Although the bacteria needed additional energy for their adaptation to the presence of the solvent, they were able to maintain or activate their electron transport phosphorylation allowing homeostasis of ATP level and energy charge in the presence of the solvent, at the price of a reduced growth yield. On the other hand, significantly enhanced cell hydrophobicities and more negative cell surface charges were observed in cells grown in the presence of 1-decanol. Both reactions occurred within about 10 minutes after addition of the

solvent and were significantly different after killing the cells with toxic concentrations of $HgCl_2$. This adaptation of the surface properties of the bacterium to the presence of solvents seems to be very similar to previously observed reactions on the level of lipopolysaccharides (LPS) with which the bacteria adapt to environmental stress such as heat shock, antibiotics or low oxygen content. The results gave clear physiological indications that the process with *P. putida* DOT-T1E as biocatalyst and 1-decanol as solvent is a stable system for two-phase biotransformations that will allow the production of fine chemicals in economically sound amounts.

INTRODUCTION

Solvent-tolerant bacteria such as *Pseudomonas putida* DOT-T1E are potential biocatalysts to be used in two-phase fermentation systems for the synthesis of high-yield, economically interesting, amounts of fine chemicals as they allow the application of solvents exhibiting chemical properties needed for a successful implementation of whole-cell two-phase biotransformations. In such kind of biotechnological process an organic solvent phase functions both as source and sink for toxic organic substrates and products, respectively (Vrionis *et al.* 2002a), allowing a continuous removal of the products (Laane *et al.* 1987b, Leon *et al.* 1998, Vrionis *et al.* 2002a, Wery *et al.* 2004). A second solvent phase is beneficial as it keeps the concentration of both the substrates and the products in the aqueous phase below a level that would lead to growth inhibition or even to the death of the bacteria.

One of the highly solvent-tolerant bacteria is *P. putida* DOT-T1E. This bacterium has been the subject of many investigations concerning the mechanisms responsible for these solvent tolerance properties (Segura *et al.* 1999, Ramos *et al.* 2002). Very recently, an extended proteomic survey was performed to identify all processes responsible for the strain's adaptation to toxic solvents such as toluene showing that a whole cascade of mechanisms is necessary to allow the bacterium to survive in the presence of such hazardous solvents (Segura *et al.* 2005). Additionally, a detailed description of the selection for the ideal solvent for two-phase fermentations with this bacterium was carried out (Neumann *et al.* 2005a). The major result of this investigation was that 1-decanol seems to be the ideal solvent for this bacterium in

such biotransformation processes (Rojas *et al.* 2004, Neumann *et al.* 2005a).

For a successful application of this system – also in competition with traditional chemical synthesis – the stability of the biocatalyst and high growth yields are necessary to guarantee high production rates. Therefore, a detailed characterisation of the growth behaviour (growth rates, yields) and the cellular energetics of the cells when grown in the presence of a solvent are necessary. Next to the well-described changes in the membrane composition (Härtig *et al.* 2005), also surface properties (de Bont 1998) are thought to be important to allow a complete adaptation to solvents.

The aim of this study was to investigate the exact growth parameters, energetics and cell surface properties of *P. putida* DOT-T1E in a two-liquid-phase system with 1-decanol to assess the stability and activity of this biocatalytic system for future biotechnological applications.

MATERIAL AND METHODS

Strain and chemicals

Pseudomonas putida DOT-T1E has previously been described (Ramos *et al.* 1995). All chemicals were reagent grade and obtained from commercial sources.

Culture conditions

P. putida DOT-T1E was cultivated in a mineral medium as described by Hartmans *et al.* (1989), with Na_2-succinate as carbon and energy source. Adaptation of the bacteria to the solvent was achieved by growing the cells in the presence of 10 % (v/v) 1-decanol in overnight cultures. Cells were grown in 50 ml shake cultures at 30°C in a horizontally shaking water bath with 180 rpm.

Preparation of resting cells

50 ml of exponentially growing cells were harvested by centrifugation and suspended in the same volume of potassium phosphate buffer (50 mM, pH 7.0) with 4 g/l Na_2-succinate as energy source. Experiments were started 45 min after suspension of cells, by which time growth had stopped completely.

Fermentations

The fermentations were carried out in a 5 l fermenter (ISF-205, INFORS GmbH,

Einsbach, Germany). Parameters such as temperature (°C), pH, CO_2-output (%), O_2-content (%), pO_2 (%), air flow (l/min), stirrer (rpm), and total weight (kg) of the fermenter were monitored online. The used IRIS software (INFORS CONTROL AG, Bottmingen, Switzerland) allowed the control of the measured parameters. Data were obtained and recorded with the Servomex Analyser Series 1400 (East Sussex, England). Standard parameters for fermentations were as follows: 30°C, pH 7.1, and 1500 rpm. The fermenter was inoculated with an overnight culture so that a starting OD_{560} of 0.08 was reached. Due to difficulties measuring the optical density in a dispersed two-phase system, growth data were obtained via measuring the protein content of the cells (Bradford 1976). In the computer controlled fermentation unit, growth was continuously determined as CO_2 production. For the fermentations carried out in this study, a direct correlation between CO_2 production and protein content was observed.

Cellular K^+ content

Five-millilitre samples were harvested at regular intervals before and after addition of solvent. Separation of cells from the supernatant was carried out by rapid centrifugation (Heraeus centrifuge, 10,000 rpm; 10 min). The cell pellet was disrupted by incubation in 5 % trichloroacetic acid at 90°C for 15 min, and debris was removed by centrifugation. The K^+ content of the supernatant was measured by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) using a Spectroflame P/M (SPECTRO Analytical Instruments, Kleve, Germany). All experiments were carried out three times. The standard deviation was less than 10 %.

ATP concentration

1 ml of cell suspension was added to 0.5 ml ice-cold 1.3 M perchloric acid (23 mM EDTA) in 2 ml sterile Eppendorf tubes. After mixing, the cell extract was incubated for 30 min at 4°C and subsequently centrifuged at 10,000 rpm for 15 min (4°C). 1 ml of the supernatant was transferred into sterile Eppendorf tubes and the pH was set to 7.5 using a 0.72 M KOH (0.16 M $KHCO_3$) solution. Again, the sample was centrifuged at 10,000 rpm for 15 min (4°C) and 0.5 ml of the supernatant was stored at -20°C for later analysis. The ATP concentration in the cells was determined using a luciferin-luciferase bioluminescence reaction (ATP Kit SL, BioThema AB, Sweden) (Lundin &

Thore 1975). In this reaction, light is formed from free ATP and luciferin by the enzyme luciferase from fireflies and measured in a Victor^2 Wallac 1420 multilabel counter spectrophotometer, Perkin Elmer Life Sciences GmbH (Germany). Data analysis was performed using the Wallac 1420 Workstation Software, Wallac 1420 manager, version 2.00 (release 8), Perkin Elmer Life Sciences GmbH. The photometer was equipped with two dispensers that were able to pump adjustable amounts of Tris-buffer and luciferin-luciferase solution into the wells of the microtiter plate, just before measurement of the samples.

Energy charge

The adenylate energy charge was measured using the energy charge kit of BioThema AB, Sweden. Besides the ATP measurement using the luciferin-luciferase reaction, the conversion of ADP to ATP by pyruvate kinase and the conversion of AMP to ADP by myokinase were necessary to determine the energy charge (Lundin *et al.* 1986). The adenylate energy charge is given by the following equation:

$$EC = \frac{(ATP) + 1/2(ADP)}{(ATP) + (ADP) + (AMP)}$$ (Atkinson & Walton 1967).

Characterisation of bacterial cell surface properties

Physico-chemical cell surface properties of bacteria were investigated using standard methods as described by others (van Loosdrecht *et al.* 1987b): The electrophoretic mobility (μ) of bacterial suspensions in 10 mM KNO_3 at pH 6.2 was determined in a Doppler electrophoretic light scattering analyser (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, Worcestershire, United Kingdom) at 100 V. The zeta potential (ζ) as an indirect measure of cell surface charge was approximated from the electrophoretic mobility according to the method of Helmholtz-von Smoluchowski (Hiementz 1986). The isoelectric point (IEP) of bacteria was determined from ζ-pH plots obtained by measuring μ in 10 mM HNO_3/KNO_3 solutions with pH varying between 2 and 6.5 using a MPT-2 autotitrator (Malvern Instruments Ltd., Malvern, Worcestershire, United Kingdom). Bacterial lawns needed for contact angle (θ_w) measurements were prepared by collecting cell suspensions in 10 mM KNO_3 on 0.45-μm pore-size Micropore filters (Schleicher & Schuell, Dassel, Germany),

mounting the filters on glass slides, and drying them for 2 h at room temperature. Cells exposed to 1-decanol were washed 6 times with 10 mM KNO_3. Cell surface hydrophobicities were derived from θ_w of water drops on the bacterial lawns using a Krüss drop shape analysis system DSA 100 (Krüss GmbH, Hamburg, Germany) (van Loosdrecht *et al.* 1987b). According to an earlier classification, cells exhibiting contact angles of $\theta_w < 20°$, $20° \leq \theta_w \leq 50°$ and $\theta_w > 50°$ are hydrophilic, intermediately hydrophilic and hydrophobic, respectively (Rijnaarts *et al.* 1995).

RESULTS AND DISCUSSION

Detailed growth behaviour of *P. putida* DOT-T1E in batch fermentations in the presence and absence of 1-decanol

5 l batch fermentations with a mineral medium and Na_2-succinate as energy and carbon source were performed in the presence or absence of a 10 % (v/v) phase of 1-decanol. The cells of *P. putida* DOT-T1E showed similar growth behaviour in the presence as in the absence of the solvent (**Fig. 5.1A**). The results for all growth parameters are summarised in **Table 5.1**. For comparison of the results, the data of Isken *et al.* (1999) for *P. putida* S12, as another highly tolerant strain and toluene as solvent were added to this table. The growth rates obtained in the presence of 1-decanol were about 90 % as high as for cells growing in the absence of the solvent, which contrasts to growth rates of *P. putida* S12 reduced to 76 % in the presence of toluene. In the presence of decanol, the amount of released CO_2 in the course of the fermentation was 85 % of the value obtained without the solvent (**Fig. 5.1A**). However, the cell yield was only about 52 % in the presence of 1-decanol, which corresponds to the value of 59 % measured for *P. putida* S12 in the presence of toluene. Those reductions of growth parameters can be explained by the far higher energy consumption that is used to run all adaptive mechanisms that are needed in order to maintain the cell physiology in the presence of a solvent.

Effect of 1-decanol on energetics

Organic solvents such as 1-decanol are known to be toxic to cells, mainly due to their permeabilising effect on the membranes (Heipieper *et al.* 1994, Sikkema *et al.* 1995).

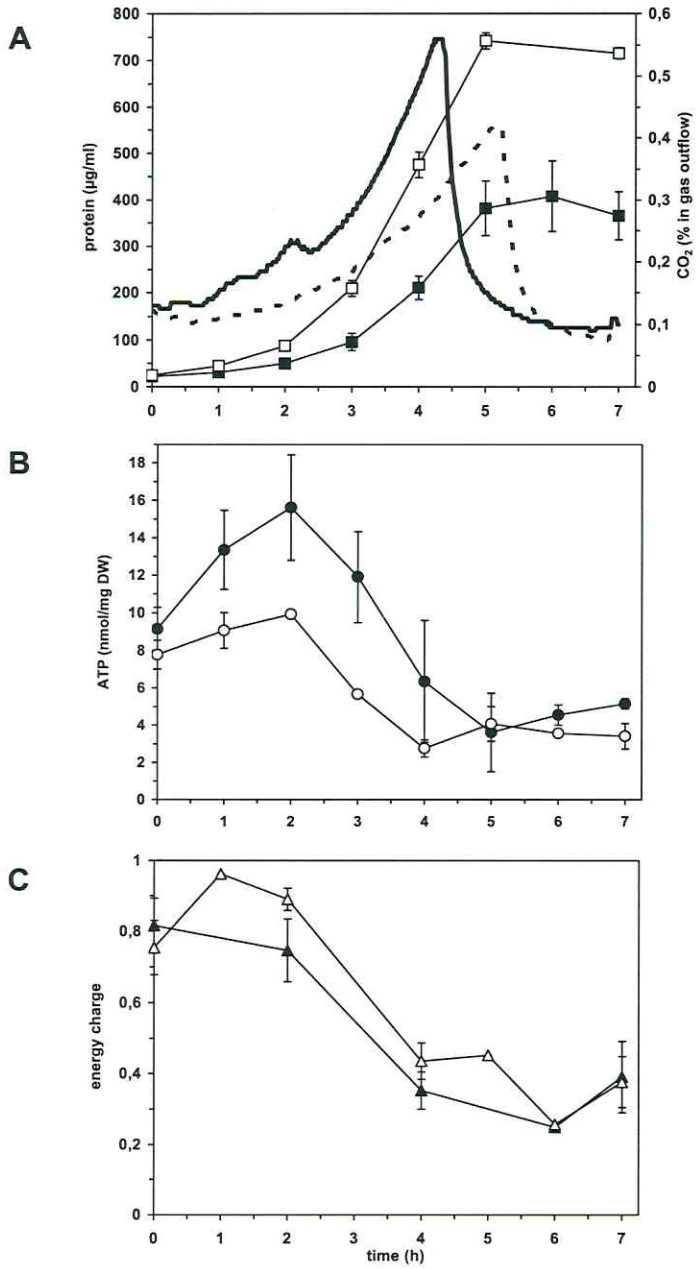

Fig. 5.1 Growth and energetics of *P. putida* DOT-T1E in a 5 l batch fermenter in the presence and absence of 10 % (v/v) 1-decanol. **(A)** CO_2 production in the presence (---) and absence (—) of 1-decanol. Protein concentrations in the presence (■) and absence (□) of 1-decanol. **(B)** Cellular ATP concentrations in the presence (●) and absence (○) of 1-decanol. **(C)** Cellular adenylate energy charge in the presence (▲) and absence (△) of 1-decanol.

This leads to a loss of important cellular components and ions (Lambert & Hammond 1973, Heipieper *et al.* 1992), including a decrease in the proton gradient (Sikkema *et al.* 1994a). Additionally, also the loss of cellular ATP after addition of phenols was described (Heipieper *et al.* 1991). Next to this loss of energetic potential caused by chemical effects of the solvents, the adaptive mechanisms, especially the activity of at least three energy-dependent efflux pumps (Segura *et al.* 2004), are consuming energy that cannot be used for growth. This is reflected in the lower growth yield that was observed in this and former investigations (Isken *et al.* 1999). Taking into consideration all these possible negative effects of the solvents on the bacteria one would assume a quite disturbed energetic level of the cells, which stands in strong contradiction to the growth rate of the bacteria in the presence of 1-decanol. In first experiments, the ATP content of the cells was measured in shake cultures. Here, non-adapted cells of *P. putida* DOT-T1E showed a dramatic loss of ATP (89-92 %) when exposed to the organic solvent 1-decanol in shake flask cultures as energised resting cells (data not shown).

Table 5.1 Calculated values for growth rates, doubling times and relative yield for the performed batch fermentations with *P. putida* DOT-T1E in the presence and absence of supersaturated concentrations of 1-decanol in comparison with literature values for chemostat cultures of highly solvent-tolerant *P. putida* S12 in the presence and absence of saturating concentrations (6.2 mM) of toluene (Isken *et al.* 1999).

Strain	*P. putida* DOT-T1E		*P. putida* S12	
Solvent	control	+ 1-decanol	control	+ toluene
C-source	Na_2-succinate		glucose	
Growth rate μ (h^{-1})	0.70	0.63	0.71[a]	0.54[a]
Doubling time t_d (min)	59	66	58[b]	76[b]
Yield (g protein/g C-source)	0.21	0.11	0.34	0.20
Relative cell yield (%)	100	52	100	59

[a] maximum dilution rate (= growth rate) possible in chemostat cultures not leading to washout of the cells
[b] maximum doubling time possible in chemostat cultures not leading to washout of the cells

Astonishingly, the ATP content in 5 l batch fermentations was, in contrast to the energised resting cells experiment, even higher in the presence of 10 % (v/v) 1-decanol. **Fig. 5.1B** shows the development of the ATP content of cells grown in 5 l batch fermentations in the presence and absence of 1-decanol. At time zero, similar ATP concentrations were measured showing an ATP content of 8-9 nmol/mg dry weight, which is similar to results found previously for aerobic bacteria (Tran & Unden 1998).

These lower values for cells grown in fermentations without 1-decanol can be explained by a decreasing specific ATP production at higher growth rates, indicating a higher energetic efficiency of carbon substrate utilization during fermentations in the absence of 1-decanol (Kayser *et al.* 2005).

The time-dependent pattern of the ATP content of the cells was very similar for both experiments. In the first 2 hours, the ATP concentration increased as the bacteria entered the exponential growth phase because of the abundance of Na_2-succinate and the relative low demand of ATP for anabolic processes at this stage. In the exponential growth phase upon limitation of the energy source for the ATP formation through catabolic activities, the ATP concentration declined rapidly. The time point at which the minimum ATP concentration was reached coincided with the time point at which the cells entered the stationary phase due to the exhaustion of the energy source (**Fig. 5.1B**). A similar curve behaviour was described by Müller *et al.* for the membrane potential ($\Delta\psi$) of cells of *Acinetobacter calcoaceticus* during growth on acetate as carbon and energy source. The membrane potential as a part of the proton motive force that drives the ATP synthesis can be used as an indicator for the energetic state of living systems as well (Müller *et al.* 1996).

Since the ATP content by itself is not always reflecting the actual energy status, also the concentrations of the other adenine nucleotides and the adenylate energy charge that allows an exact expression of the energetic levels of the cells under different growth conditions were measured and calculated, respectively (**Fig. 5.1C**). Also the energy charge of the cells showed no difference regardless of the presence or absence of 1-decanol. Apparently, the complete adaptation of the cells of *P. putida* DOT-T1E to 1-decanol is reflected in the absence of differences in the bioenergetics of these microorganisms during fermentations with and without 1-decanol.

A steep decline in the energy charge for both fermentations with and without

1-decanol was observed after 2 h. The reasons for this drop are the same as for the decrease of the ATP concentration. The decline to E.C. ≈ 0.3 - 0.45 is comparable to the values described in the literature at which viability of the microorganisms still is maintained before the cells die (Chapman *et al.* 1971, Loffhagen & Babel 1985, Lundin *et al.* 1986).

When the energy source got limiting, the ATP concentration decreased whereas the ADP and AMP concentrations kept increasing (data not shown). It could be concluded that ADP and AMP were formed from ATP because the sum of nucleotides was more or less constant. Chapman *et al.* described a stabilisation of the energy charge at this growth stage by reducing the sum of nucleotides in a short period of continued synthesis of ribonucleic acid (RNA) in *Escherichia coli* and a late drop in the energy charge when this process is not possible anymore (Chapman *et al.* 1971). However, this stabilisation of the energy charge was not observed in *P. putida* DOT-T1E, resulting in an earlier drop in the energy charge (from E.C. ≈ 0.8 down to E.C ≈ 0.3), when the cells were in the middle of their exponential growth phase, irrespective of the presence or absence of 1-decanol.

The results on the energetics of the cells were additionally supported by measurements of the cellular potassium concentration where no significant differences could be observed between fermentations with and without 1-decanol (data not shown). As leakage of potassium ions is an important parameter to determine membrane damage in the presence of toxins (Lambert & Hammond 1973, Neumann *et al.* 2005a) this indicated that also the cell membranes completely adapted to the presence of a second phase of 1-decanol. The cells are obviously able to maintain the appropriate ion gradients across the membrane that are necessary for an effective ATP synthesis. From these results, it can be concluded that although the bacteria need additional energy for their adaptation to the presence of the solvent, they are able to maintain or activate their electron transport phosphorylation allowing homeostasis of ATP level and energy charge in the presence of the solvent, at the price of a reduced growth yield.

Physico-chemical surface properties of cells adapted to the presence of 1-decanol

Already in 1998 (de Bont), modifications of the surface properties by changing the

composition of the very outer layer of Gram-negative bacteria were suggested as an adaptive response in solvent-tolerant bacteria to a second phase of a toxic organic compound. In order to evaluate the influence of a second phase of 1-decanol on physicochemical cell surface properties, the water contact angles (θ_w) (van Loosdrecht *et al.* 1987a) and zeta potentials (ζ) (van Loosdrecht *et al.* 1987b) were measured to describe cell surface charge and hydrophobicity of *P. putida* DOT-T1E. To guarantee the measurement of the cell properties and concomitantly to avoid measurement interferences by the physicochemical effect of 1-decanol adhering to or accumulating in the cells, six washing steps with the harvested cell pellets and 10 ml of 10 mM KNO_3 were carried out. After 6 and more washing steps, no changes of both θ_w and ζ were observed indicating that all reversibly bound 1-decanol was washed out from the cells (data not shown).

The results of the surface properties measured for 5 l fermentations are summarised in **Fig. 5.2**. Cells exposed to 1-decanol immediately showed significantly increased water contact angles (θ_w = 85°) being about 50° above θ_w of cells growing in the absence of the solvent (θ_w = 37°) (**Fig. 5.2A**). Contact angles of the cells in the lag and early exponential phase of both types of cultures, increased to 110° and 72° for cells grown in the presence and absence of 1-decanol, respectively. Cells grown in the presence and absence of 1-decanol were negatively charged and with the former (ζ = -30 mV) exhibiting (**Fig. 5.2B**) 15 mV more negative ζ-potentials than the latter (ζ = -15 mV). ζ-values decreased during the fermentations to ζ = -50 mV (in the presence of 1-decanol) and to -25 mV (in the absence of 1-decanol) correlating to corresponding changes of the water contact angle supporting earlier observations by others describing a negative correlation between cell hydrophobicity and surface charge (van Loosdrecht *et al.* 1987a, 1987b, Makin & Beveridge 1996a). Previous findings have demonstrated that the whole-cell isoelectric point (IEP, i.e. the pH at which ζ becomes zero) is a suitable indicator in predicting the biochemical surface composition of bacteria (Rijnaarts *et al.* 1995). Literature data demonstrate that an IEP $\leq$ 2.8 indicates the presence of significant amounts of cell surface polysaccharides inhibiting adhesion onto both hydrophobic and hydrophilic surfaces (Rijnaarts *et al.* 1995). Continuously more negative ζ values between pH 2 and pH 6.5 and no significant changes of the IEP were found in the absence and presence of the solvent (data not shown). The observed IEP of about 2.5 is lower than published

IEPs of other *Pseudomonas* strains (Rijnaarts *et al.* 1995), possibly indicating the presence of significant amounts of cell surface polymers such as lipopolysaccharides (LPS) at the cell surface of the strain *Pseudomonas putida* DOT-T1E.

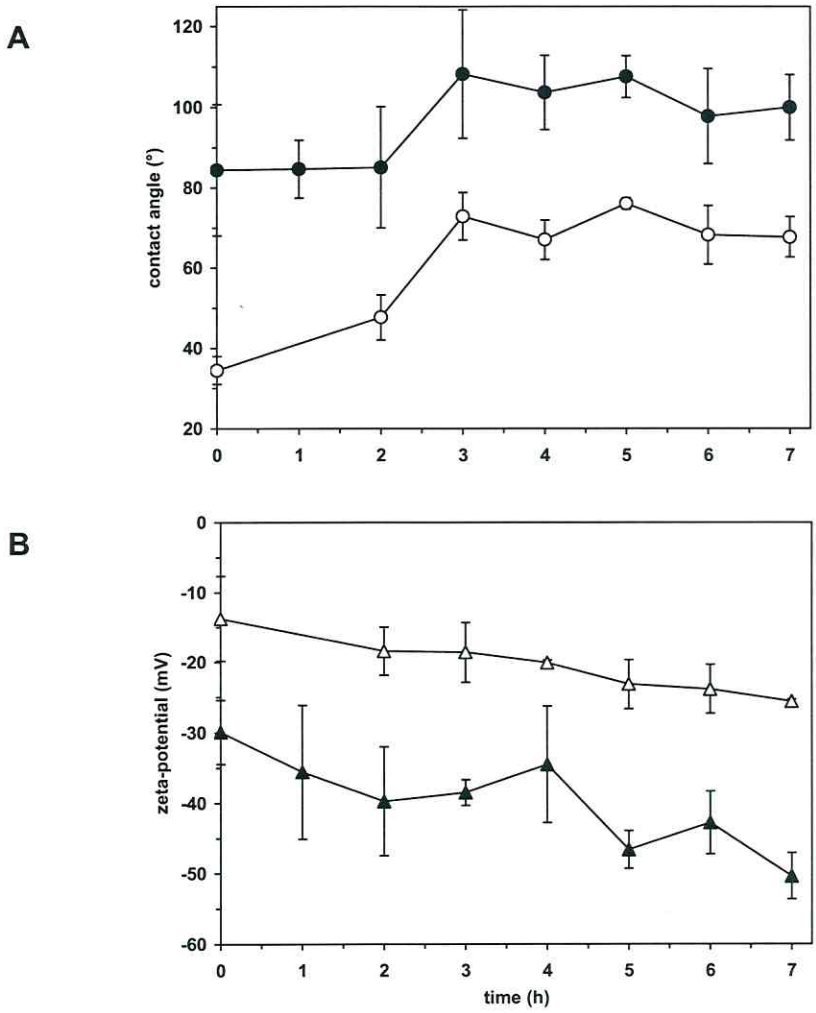

Fig. 5.2 Surface properties of *P. putida* DOT-T1E cells grown in a 5 l batch fermenter in the presence and absence of 10 % (v/v) 1-decanol. **(A)** Contact angle in the presence (●) and absence (○) of 1-decanol. **(B)** ζ-potential of cells in the presence (▲) and absence (△) of 1-decanol.

The steep increase of cell hydrophobicity (contact angle from about 30° to about 85°, **Fig. 5.2A**) and the increase in ζ-potential (from about -15 mV to about -30 mV, **Fig. 5.2B**) of cells grown in the presence of the solvent already at the beginning of the fermentations is surprising. Although it should be noted that in the fermenter experiments the first samples (t = 0) were taken after filling of the fermenter with medium that took about 30 min in which the cells were already in contact with 1-decanol. This led to the question whether the measured changes reflect physiological changes or if they were caused by (abiotic) physico-chemical interactions of 1-decanol with the cell wall. Therefore, a series of batch experiments with cells that had previously been treated with lethal concentrations of $HgCl_2$ (0.1 mM) were carried out. $HgCl_2$ is known to lead to cell death without having an effect on the physical and chemical properties of cell surfaces. $HgCl_2$ concentrations of 0.1 mM led to a complete loss of viability of the cells (data not shown). 1-decanol was added to the cells after 30 min incubation with $HgCl_2$ (**Fig. 5.3**). After addition of 1-decanol to living cells, the contact angle of the cells drastically increased from 27° to 85° after an initial delay of several minutes (**Fig. 5.3A**). Contrary to that, dead cells showed only a moderate but much more rapid increase to 48°. This is a clear indication that the observed increase in the hydrophobicity of cells grown in the presence of 1-decanol was caused by physiological changes that could only be carried out by living cells as opposed to the much faster physico-chemical effect, which is seen and expected in dead cells. Nearly the same effect was measured for the ζ-potential (**Fig. 5.3B**). Here, the living cells also show a slower response as compared to dead cells, but with a higher final decrease in the ζ-potential.

The contact angle and the zeta potential showed slight changes as a function of growth both in the presence and in the absence of 1-decanol (**Fig. 5.2**). Until the middle of the exponential phase, the contact angle increased and then stayed rather steady. The values for the zeta potential became constantly more negative in the course of growth until the beginning of the stationary phase.

Here, it was proven for the first time that changes in the surface properties as a cellular response to stress also occur as an adaptive mechanism to the presence of toxic organic solvents. The very fast physiological response can be explained by the formation of membrane vesicles mainly consisting of B-Band LPS that lead to fast and drastic increase in the hydrophobicity of the cells (Kadurugamuwa & Beveridge

1995, Sabra *et al.* 2003).

The major component in Gram-negative cells that affects surface properties such as charge and hydrophobicity is the composition of the LPS layer of the outer membrane. Here, especially the so-called O-specific region on the very outer cell surface has an effect on surface properties. In the LPS of *P. aeruginosa*, the O-specific region contains two major components. The A-band, a low molecular mass LPS consists of a homopolymer of D-rhamnose with only minor amounts of 2-keto-3-deoxyoctonic acid (KDO). The B-band, a high molecular mass LPS consists of a heteropolymer of mainly uronic acid derivatives and N-acetylfucosamine. The rapidly occurring complete loss of B-band LPS upon stress compared to the amounts of A-band LPS present on the surface have been shown to affect surface charge, surface hydrophobicity, adhesion to hydrophobic surfaces, biofilm formation as well as susceptibility to antimicrobial agents and host defence (Kelly *et al.* 1990). In addition, one investigated solvent-tolerant strain, *P. putida* Idaho, changed its LPS composition when grown in the presence of o-xylene. A higher-molecular-weight LPS band disappeared whereas it was replaced by a lower-molecular-weight band in the presence of the aromatic compound (Pinkart *et al.* 1996).

In *Pseudomonas aeruginosa*, the quantity of LPS was reported to be high in the initial phases of growth but then to decrease significantly to constant levels in the stationary phase. A strong increase in the yield of LPS in the mid and late exponential growth phase was observed (Weber-Frick & Schmidt-Lorenz 1988). *P. aeruginosa* is known for being able to alter the LPS composition of its surfaces very rapidly. This takes place as a response to high temperature (45°C) (Makin & Beveridge 1996b) but also to other environmental stress factors such as the presence of the membrane-active antibiotic gentamicin (Kadurugamuwa *et al.* 1993) and to low oxygen stress (Sabra *et al.* 2003).

Indeed, for mutants of *Escherichia coli* showing a higher tolerance towards solvents, hydrophobicity of cell surfaces has been reported to decrease (Aono & Kobayashi 1997). However, an explanation for the physiological advantage of a more hydrophobic cell surface as an adaptive response to the presence of a very hydrophobic solvent seems very difficult. This had already been discussed in 1998 by De Bont (1998) who had assumed a decrease in cell hydrophobicity in order to repel the solvent. The outer membrane is known to be a very good barrier for hydrophobic

compounds. This very low permeability for hydrophobic compounds is usually more affected by the outer membrane porins than by variations in the LPS content. However, taking into consideration that the major mechanism delivering the phenotype of highly solvent tolerant bacteria is the presence of at least three efflux pumps (Segura *et al.* 2004) that permanently remove the toxic solvents from the cytoplasmic membrane and transport them to the outer layer of the outer membrane, the observed modification of the surface properties make sense as this hydrophobic layer is able to take up more of the solvents.

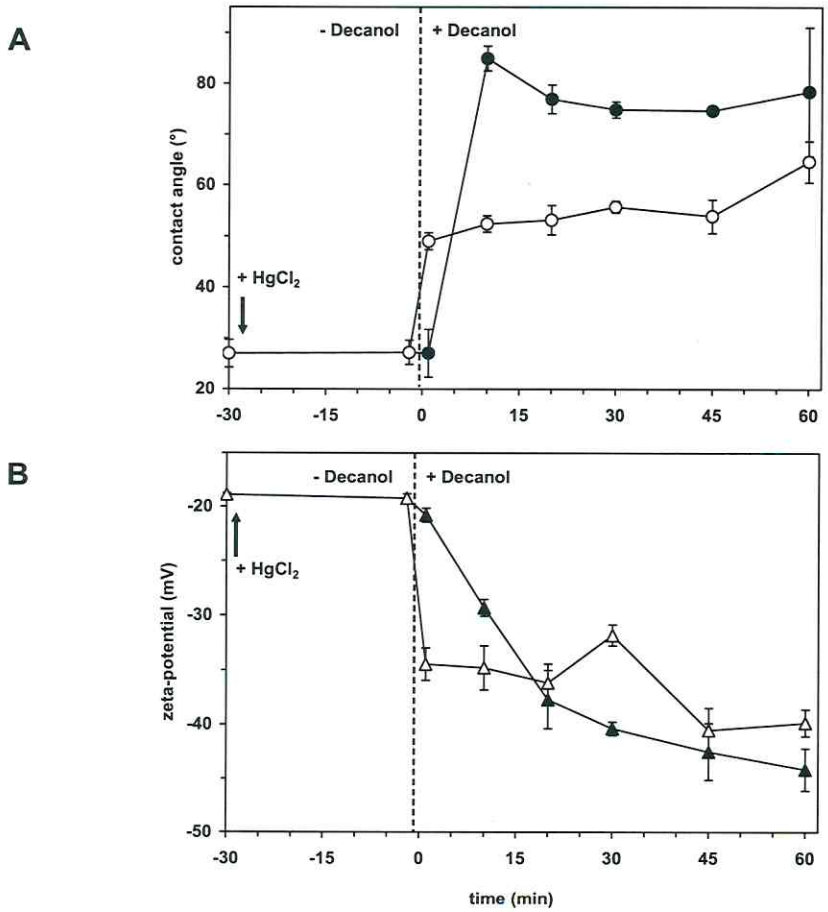

Fig. 5.3 Effect of a pre-incubation with toxic concentrations (0.1 mM) of $HgCl_2$ on surface properties of *P. putida* DOT-T1E cells in the presence of 10 % (v/v) of 1-decanol. **(A)** Contact angle of living (●) and dead (○) cells. **(B)** ζ-potential of living (▲) and dead (△) cells. The arrows indicate the addition of 0.1 mM $HgCl_2$ and the dashed lines mark the presence of 10 % (v/v) 1-decanol.

Additionally, the release of solvent containing membrane vesicles was discussed as a mechanism of adaptation to toxic solvents (Kobayashi *et al.* 2000). A detailed study of the LPS content of the strain growing in the presence and absence of 1-decanol, including antibodies for the different LPS bands, will be carried out in the near future.

Bacteria belonging to the genus *Pseudomonas* are not only famous because of their high solvent tolerance but mainly because of their capability to degrade a wide range of pollutants even at very low concentrations. Also in the presence of non-toxic crude oil components such as hexadecane, an increase in cell hydrophobicity has been observed (Norman *et al.* 2002). Major reason for this change in the surface properties seems to be an increased adhesion to the surface of the very poorly soluble compounds that leads to an increase in the bioavailability of the compounds (Wick *et al.* 2002, Wick *et al.* 2003). As in the adaptation to poorly water-soluble substrates, also uptake systems seem to be involved, a hydrophobic surface also works as a kind of source for the compounds that accumulates them at the cell surface and allows a better uptake (Arias-Barrau *et al.* 2005). Thus, a more hydrophobic surface can work as a kind of sink for toxic concentrations of solvents that are excluded by efflux pumps but also as a kind of source for less bioavailable substrates that are transported into the cells by several uptake systems.

P. putida DOT-T1E and other highly solvent tolerant bacteria were shown to be capable of adapting to the presence of very toxic solvents such as toluene or 1-decanol without being highly affected in their growth properties and cellular energetics. Thus, these bacteria can be handled in technical scales for the production of fine chemicals of interest in high, economically interesting concentrations (Wery *et al.* 2000, Hüsken *et al.* 2001). As these strains are also very accessible to genetic modifications and the introduction of foreign genes, a wide range of products can be synthesised using a two-phase biotransformation system.

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6 CONCLUDING REMARKS

Biological systems in general are interesting for industrial purposes since they are efficient and are able to catalyse reactions selectively. They synthesize stereo- and regio-specific compounds without the need for chemical protective groups. The goal of classic biotechnology is to substitute as many chemical processes as possible by biologically catalysed reactions. This sustainability approach is avoiding waste and saving energy as well as it serves preserving natural resources. Those so-called biotransformations are performed, if possible, in systems with isolated enzymes. For complex processes with the necessary regeneration of co-factors (e.g. NAD(P)H and ATP), systems with whole cells have to be implemented in order to ensure economic feasible processes. A big problem with biotechnological whole-cell processes is the product recovery from the aqueous medium since substrates and/or products can have a toxic or even deadly effect on the used cells, the biocatalysts.

A possible solution for this problem is the implementation of a second liquid phase consisting of a solvent. Thereby, toxic substrates are present in the water phase in only little concentrations – they are still bioavailable for the cells, but substrate toxicity is not a problem anymore. The product will preferentially dissolve into the solvent phase as well and so product toxicity is prevented.

Nevertheless, those organic solvents have to possess similar hydrophobicity properties (expressed as log P values) like the substrates and products of interest. That in turn means the solvents are toxic themselves to the biocatalysts.

This second problem can be solved by using solvent-tolerant microorganisms for two-phase biotransformation systems. Solvent-tolerant microorganisms able to adapt to toxic solvents offer new possibilities for the classical biotechnology. Up to now, mostly hydrophobic solvents with log P values of 4 and above were used as second phase, because they are not toxic to microorganisms. By using solvent-tolerant microorganisms, a broader variety of solvents with log P values appropriate for the providing of specific substrate and enabling product extraction could be applied. In this way, less hydrophobic products (log P 1 - 4) could get selectively extractable from the aqueous phase. Then, many important fine chemicals like catechols, phenols, benzoates, aromatic aldehydes, and other interesting compounds could be

synthesised biotechnologically. When chemical compounds can be produced in large amounts, only then is the biotechnological approach able to substitute the classical chemical syntheses.

Bacteria have to possess the ability to adapt to all kinds of environmental stresses. In order to enable and guarantee their survival they use different mechanisms. During this thesis, the main focus was on toxic solvents as stress factors for solvent-tolerant strains of *Pseudomonas putida* and their diverse adaptational potentials.
Changes in their morphology by increasing the cell size and therefore reducing their relative surfaces were detected. The cell surface and particularly the cytoplasmic membrane are the major targets for membrane-active compounds and therefore the reduction of the relative surface seems to be one example of an adaptive response to solvent stress. Additionally, the cells activated their potassium uptake systems and the *cis-trans* isomerisation of unsaturated fatty acids in the presence of solvents.
We detected decanol as the ideal solvent for *Pseudomonas putida* DOT-T1E within a two-phase biotransformation system. Although the cells show reduced growth rates and cell yields in the presence of decanol, they manage to adapt on the energetic level (ATP and potassium content, energy charge) as well as in terms of cell hydrophobicity (contact angle) and cell surface charge (zeta potential). This adaptation of the bacterial surface properties seems to be very similar to previously observed reactions on the level of lipopolysaccharides (LPS) with which the bacteria adapt to environmental stress such as heat shock, antibiotics or low oxygen content.
For our goal, the applicability of an economically sound biotransformation system with an organic solvent as second phase, all mentioned adaptational mechanisms are essential.

7 SUMMARY

The toluene-degrading and solvent-tolerant strain *Pseudomonas putida* DOT-T1E was investigated with respect to its suitability and economic efficiency as biocatalyst in aqueous-organic two-phase systems with aliphatic solvents as organic phase (Rojas *et al.* 2004, **chapter 4 and 5**) and to its adaptive responses to the solvent decanol.

The adaptive changes on the level of cell morphology (**chapter 2**), membrane fatty acids and permeability (**chapter 3**), as well as energetics and surface properties (**chapter 5**) of *P. putida* DOT-T1E have been investigated in order to ascertain information about the strain's suitability for two-phase biotransformation systems (**chapter 4**).

The morphological adaptation to the presence of solvents was observable in changes of the cell size of *P. putida* DOT-T1E. Those changes were dependent on the cellular activity and occurred only after addition of non-lethal solvent concentrations. The cells reacted to the presence of organic solvents by decreasing the ratio between surface and volume of the cells and therefore reducing their relative surfaces (**chapter 2**).

The cell surface and especially the cytoplasmic membrane are the major targets for toxic effects of membrane-active compounds like solvents. The mechanism of the *cis-trans* isomerisation of unsaturated fatty acids counteracts the fluidizing effect of solvents by increase the ordering of the membrane and therefore its rigidity. By comparing the responses of the cells to a series of stress factors (like solvents), a direct correlation between the activation of this mechanism and the well investigated K^+-uptake pumps was observed (**chapter 3**).

Huertas *et al.* (1998) reported that this strain tolerated concentrations of heptane, propylbenzene, octanol, and toluene of at least 10 % (vol/vol). 1-decanol is, in comparison to toluene, less hazardous and volatile, and it possesses good extraction properties for the desired fine chemical products. In further investigations of possible biotechnological processes, it was discovered that decanol is also a more suitable solvent as organic phase (**chapter 4**).

Although the cells of *P. putida* DOT-T1E needed additional energy for their

adaptation to the presence of the solvent decanol, they were able to maintain or activate their electron transport phosphorylation allowing homeostasis of ATP level and energy charge in the presence of the solvent, at the price of a reduced growth yield. On the other hand, significantly enhanced cell hydrophobicities converging with more negative cell surface charges were observed in cells grown in the presence of 1-decanol (**chapter 5**).

It is however important to note that all the cell's properties observed are closely linked to each other since they are all part of the adaptive response of the cells. It can be concluded that the easy adaptability and good growth properties of *Pseudomonas putida* DOT-T1E in the presence of the organic solvent 1-decanol make this system an excellent candidate for two-phase fermentation processes. Moreover, the absence of differences in the energetics of the bacteria during exposure to 1-decanol as compared to bacteria that grew in the absence of 1-decanol, support that this organism can be used for the industrial production of fine chemicals in an economically sound manner.

8 ZUSAMMENFASSUNG

Der Toluol-abbauende Stamm *Pseudomonas putida* DOT-T1E wurde hinsichtlich seiner Eignung und ökonomischen Effizienz als Biokatalysator in wäßrig-organischen Zwei-Phasen-Systemen untersucht, wobei aliphatische Lösungsmittel als organische Phase verwendet wurden (Rojas *et al.* 2004, **Kapitel 4 und 5**). Die adaptiven Antworten des Bakteriums auf das Lösungsmittel 1-Dekanol wurden dabei intensiv geprüft. Die adaptiven Veränderungen auf der Ebene der Zellmorphologie (**Kapitel 2**), der Membranfettsäuren und Membranpermeabilität (**Kapitel 3**) sowie der Zellenergetik und Oberflächen-Eigenschaften (**Kapitel 5**) von *P. putida* DOT-T1E wurden untersucht, um Informationen über die Eignung des Stammes für Zwei-Phasen-Biotransformationssysteme zu erhalten (**Kapitel 4**).

Die morphologische Adaptation an die Anwesenheit von Lösungsmitteln war anhand von Veränderungen der Zellgröße zu beobachten. Diese Veränderungen waren abhängig von der zellulären Aktivität und traten nur nach Zugabe von nicht-letalen Lösungsmittelkonzentrationen auf. Die Zellen reagierten auf die Anwesenheit von organischen Lösungsmitteln mit einer Erniedrigung des Verhältnisses zwischen Oberfläche und Volumen, d.h. mit einer Verringerung ihrer relativen Oberflächen (**Kapitel 2**).

Die Hauptwirkorte der toxischen Effekte von membranaktiven Verbindungen, wie beispielsweise Lösungsmitteln, sind die Zelloberfläche und insbesondere die Cytoplasmamembran. Der adaptive Mechanismus der *cis-trans*-Isomerisierung von ungesättigten Fettsäuren wirkt der fluidisierenden Wirkung von Lösungsmitteln entgegen, indem er die Ordnung der Membran und damit ihre Viskosität erhöht. Beim Vergleich der adaptiven Antworten von *P. putida* auf eine Reihe von Streßfaktoren (z.B. Lösungsmittel) konnte eine direkte Korrelation zwischen der Aktivierung des *cis-trans*-Mechanismus und der Aktivierung der Kalium-Aufnahme-Pumpen in der Membran festgestellt werden (**Kapitel 3**).

Huertas *et al.* (1998) zeigten für Heptan, Propylbenzol, Oktanol und Toluol, daß der Stamm *Pseudomonas putida* DOT-T1E Lösungsmittelkonzentrationen von mindestens 10 % (vol/vol) toleriert. 1-Dekanol ist im Vergleich zu Toluol weniger gefährlich und flüchtig und es besitzt gute Extraktionseigenschaften für die zu

produzierenden Feinchemikalien. In weiteren Untersuchungen von biotechnologischen Prozessen wurde bewiesen, daß Dekanol außerdem auch ein gut geeignetes Lösungsmittel für Zwei-Phasen-Biotransformationen ist (**Kapitel 4**).
Obwohl die Zellen von *P. putida* DOT-T1E zusätzliche Energie für ihre Anpassung an das Lösungsmittel Dekanol aufbringen mußten, waren sie in der Lage, ihre Elektronentransportphosphorylierung aufrecht zu erhalten. Das erlaubte eine Homöostasis von ATP-Level und Energieladung in Gegenwart des Lösungsmittels – auf Kosten eines reduzierten Biomasse-Ertrags. Allerdings waren in Gegenwart von Dekanol gleichzeitig eine signifikant höhere Hydrophobizität der Zellen und eine negativere Zelloberflächenladungen in wachsenden Zellen zu beobachten (**Kapitel 5**).
Es ist anzumerken, daß sämtliche beobachteten Zelleigenschaften eng miteinander verknüpft sind, da sie alle Teil der adaptiven Reaktion der Zelle sind. Aufgrund der guten Wachstumseigenschaften in Anwesenheit von organischen Lösungsmitteln und der problemlosen Anpassung an 1-Dekanol, ist *Pseudomonas putida* DOT-T1E in diesem System ein exzellenter Kandidat für Zwei-Phasen-Fermentationsprozesse. Das Fehlen von Unterschieden auf der energetischen Ebene der Zellen – in Ab- oder Anwesenheit von Dekanol – unterstützt die Tatsache, daß *Pseudomonas putida* DOT-T1E für eine industrielle und ökonomisch relevante Produktion von Feinchemikalien einsetzbar ist.

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DANKSAGUNG

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Hiermit erkläre ich, daß diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.
Ferner erkläre ich, daß ich diese Arbeit selbständig verfaßt und keine anderen als die darin angegebenen Hilfsmittel benutzt habe.

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