ACTIVE EFFLUX SYSTEMS IN THE SOLVENT–TOLERANT BACTERIUM *PSEUDOMONAS PUTIDA* S12

PROMOTOR

Dr. ir. J.A.M. de Bont
Hoogleraar in de Industriële Microbiologie
Wageningen Universiteit

PROMOTIECOMMISSIE

Prof. dr. W.M. de Vos
Wageningen Universiteit

Prof. dr. B. Witholt
Swiss Federal Institute of Technology, Zurich

Prof. dr. B. Poolman
Rijksuniversiteit Groningen

Dr. J. Frankena
Solvay Pharmaceuticals, Weesp

ACTIVE EFFLUX SYSTEMS IN THE SOLVENT-TOLERANT BACTERIUM *PSEUDOMONAS PUTIDA* S12

Jasper Kieboom

Proefschrift ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op 13 maart 2002
des namiddags te vier uur in de Aula.

ISBN 90-5808-596-1

The research described in this thesis has been carried out at the Division of Industrial Microbiology, Department of Food Technology and Nutritional Sciences, Wageningen University, The Netherlands.

Kieboom J. – Active efflux systems in the solvent-tolerant bacterium *Pseudomonas putida* S12 – 2002

Thesis Wageningen University, Wageningen, The Netherlands – With summary in Dutch – 88 p.

STELLINGEN

1 De conclusie van Ferrante *et al.*, dat een alkylhydroperoxide reductase betrokken is bij tolerantie voor oplosmiddelen in een *Escherichia coli* mutant, is onjuist gezien de waarneming van deze auteurs dat deze mutant tolerant is voor cyclohexaan.

Ferrante, A. A., J. Augliera, K. Lewis, and A. M. Klibanov. 1995. Cloning of an organic solvent-resistance gene in *Escherichia coli*: the unexpected role of alkylhydroperoxide reductase. Proc. Natl. Acad. Sci. USA **92:**7617-7621.

2 Het foutief gebruik van het woord 'resistentie', om de tolerantie van een organisme voor een chemische verbinding weer te geven, wordt door velen getolereerd.

Dit proefschrift

3 Voor een evenwichtige regionale ontwikkeling zijn gemeenten te klein en provincies te groot.

4 De conclusie van Kobayashi *et al.* dat met tolueen gevulde 'membrane vesicles' een significante bijdrage leveren aan tolueentolerantie in *Pseudomonas putida* IH-2000, wordt overschat.

Kobayashi, H., K. Uematsu, H. Hirayama, and K. Horikoshi. 2000. Novel toluene elimination system in a toluene-tolerant microorganism. J Bacteriol. **182:**6451-6455.

5 Bij het streven naar 'lichtgewicht' in de buitensport is alles wat men thuis laat meegenomen.

6 Efflux pompen van het 'RND' type hebben een verrassende substraat specificiteit.

Dit proefschrift

Stellingen behorende bij het proefschrift– Active efflux systems in the solvent–tolerant bacterium *Pseudomonas putida* S12 – Kieboom J. – Wageningen 2002

CONTENTS

1	GENERAL INTRODUCTION	7
2	MECHANISMS OF ORGANIC SOLVENT TOLERANCE IN BACTERIA	9
3	IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF AN EFFLUX PUMP INVOLVED IN *PSEUDOMONAS PUTIDA* S12 SOLVENT TOLERANCE	21
4	ACTIVE EFFLUX OF ORGANIC SOLVENTS IN *PSEUDOMONAS PUTIDA* S12 IS INDUCED BY SOLVENTS	33
5	IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF AN EFFLUX PUMP INVOLVED IN *PSEUDOMONAS PUTIDA* S12 MULTIDRUG RESISTANCE	41
6	TRANSPOSON MUTATIONS IN THE FLAGELLA BIOSYNTHETIC PATHWAY OF THE SOLVENT–TOLERANT *PSEUDOMONAS PUTIDA* S12 RESULT IN A DECREASED EXPRESSION OF SOLVENT EFFLUX GENES	53
7	CONCLUDING REMARKS	61
	REFERENCES	73
	SUMMARY	81
	SAMENVATTING	83
	DANKWOORD	85
	CURRICULUM VITAE	87
	LIST OF PUBLICATIONS	88

1

GENERAL INTRODUCTION

Pseudomonas putida is a Gram–negative, polarly flagellated, nonspore–forming rod, belonging to the family *Pseudomonadacea*. *P. putida* grows aerobically with an optimal growth temperature of 25–30°C and generally metabolize sugars via the Entner–Doudoroff pathway. Anaerobically *P. putida* is able to obtain its energy via nitrate respiration or denitrification but not by fermentation [105].

The metabolic and physiological properties of *P. putida* have been well characterized since its isolation in 1889 by Trevisan [139]. *P. putida* is able to use a wide spectrum of substrates and it can even metabolize a large number of heterocyclic and aromatic compounds that are not attacked by other bacteria. Due to their simple requirements, *P. putida* is ubiquitous. They are present in soil, water, wastewater and air [105].

The subject of this thesis is *P. putida* strain S12. This organism was isolated in 1990 on the basis of its ability to use styrene as sole carbon and energy source [33]. In 1993 it was observed that *P. putida* S12 was able to grow in the presence of high concentrations of styrene and other organic solvents [145]. Since 1993 this solvent–tolerant bacterium has been the object of several studies on the mechanisms of organic solvent tolerance. It has been observed that *cis* into *trans* isomerization of the unsaturated membrane phospholipids occur in solvent–tolerant cells [34, 146]. Moreover, at the level of the outer membrane changes have been found that could play a role in the solvent–tolerant properties of *P. putida* S12 [144]. In 1996 strong indications were obtained that toluene was actively transported from the membrane of *P. putida* S12 [55]. Interestingly, solvent–adapted *P. putida* S12 cells are more resistant to several antibiotics [58]. These putative export systems for solvents and for antibiotics were subsequently studied at the molecular level.

OUTLINE OF THIS THESIS

The objective of this Ph.D. study was to identify and to characterize the genes of *Pseudomonas putida* S12 involved in tolerance towards organic solvents and antibiotics.

In **Chapter 2** a short overview is given on organic solvent stress response and solvent tolerance mechanisms in Gram-negative bacteria. Chapter 2 has been published in the ASM book "Bacterial Stress Responses" [62].

In **Chapter 3** the isolation and sequencing of the first organic solvent transporter is described. Furthermore, a comparison is made between the organic solvent transporter from *P. putida* S12 and transporter proteins from bacteria. The role of proton-dependent efflux of organic solvents from the bacterial membrane in relation to solvent tolerance is discussed. Chapter 3 has been published in "The Journal of Biological Chemistry" [64].

In **Chapter 4** the characterization of the organic solvent transporter of *P. putida* S12 is extended by the analysis of the solvent transporter promoter. With the construction of a reporter plasmid we were able to identify the compounds responsible for the induction of solvent transport. Chapter 4 has been published in "The Journal of Bacteriology" [63].

In **Chapter 5** the isolation and sequencing of a proton-dependent multidrug transporter of *P. putida* S12 is described. A comparison with multidrug transporters and organic solvent transporters is made. The role of organic solvent transporters in the active efflux of antibiotics is discussed. Chapter 5 has been published in "Microbiology" [61].

In **Chapter 6** we describe the isolation of octanol-sensitive mutants of *P. putida* S12 and the partial characterization of the genes interrupted in these mutants. In this Chapter we discuss the role of flagellar genes in *P. putida* S12 solvent tolerance. Chapter 6 has been published in "FEMS Microbiology Letters" [65].

In **Chapter 7** the results presented in this thesis are discussed in general terms. Attention is given to future investigations in solvent tolerance mechanisms and to the application of these bacteria in biotechnological production processes.

2

MECHANISMS OF ORGANIC SOLVENT TOLERANCE IN BACTERIA

Jasper Kieboom and Jan A.M. de Bont

SUMMARY

The extreme toxicity of many organic solvents imposes a severe problem in the application of these chemical compounds in biotechnological production processes. The main reason for the toxicity of organic solvents is the accumulation of these molecules in the bacterial cell membrane, causing adverse effects on membrane structure and functioning. However, in the past decade several organic solvent–tolerant bacteria have been isolated from the environment that are able to survive in water–solvent two–phase systems. In these systems the concentrations of organic solvents reach levels that are lethal for normal bacteria. Solvent–tolerant bacteria are able to counterbalance the detrimental effects of organic solvents via several mechanisms that change the structure of the cell envelope. It has now been established that active efflux of solvent from the membrane plays a key role in organic solvent tolerance. We here review the current knowledge on these exceptional bacteria and the mechanisms for their survival in the presence of toxic solvents.

PUBLISHED IN THE ASM BOOK 'BACTERIAL STRESS RESPONSES' (2000) P. 393–402

INTRODUCTION

Organic solvents are generally regarded as extremely toxic to living microorganisms and therefore impose an important drawback in the application of these chemical compounds in biotechnology [125]. However, in the past decade efforts have been made to isolate microorganisms tolerant to organic solvents. These organisms have potential advantages in either the remediation of highly polluted waste–streams or biocatalytic applications for the production of specialty chemicals. The use of solvent–tolerant bacteria in biocatalysis would allow the introduction of an organic phase to dissolve water–insoluble substrates or to remove toxic product [17].

Toxicity of Organic Solvents

Because of their toxicity, organic solvents have been widely used as disinfectants, permeabilization agents, and food preservatives [16, 90]. The antimicrobial property of organic solvents correlates with its hydrophobic nature. The hydrophobicity of organic solvents is usually expressed in terms of P_{ow}, which gives the partitioning of a compound over an octanol/water two–phase system. It has been established that the common logarithm of P_{ow} is inversely correlated with the toxicity of the organic solvent [131]. This influence of the log P_{ow} on toxicity toward microorganisms has been described for different classes of organic solvents such as aromatic compounds, alkanes, alcohols, and phenols [72]. Organic solvents with a log P_{ow} value between 1 and 5 are generally regarded as highly toxic for microorganisms [103, 130]. It is generally accepted that the accumulation of organic solvents in the membrane is the main mechanism for toxicity, although other mechanisms cannot be ruled out because of specific chemical properties of each specific compound. The mechanism of membrane toxicity of organic solvents has been reviewed extensively by Sikkema *et al.* in 1995. To understand the organic solvent tolerance mechanisms of solvent–tolerant bacteria a short overview of organic solvent toxicity is given below.

In most cases, organic solvents, such as aromatic compounds and alcohols, target the bacterial cell membrane in which they preferentially partition [103, 130]. De Smet *et al.* first made this visible in 1978 by means of electron microscopic images of toluene–treated *Escherichia coli* cells [132]. While the outer membrane in these cells remained intact, considerable damage was done to the cytoplasmic membrane, resulting in the permeabilization of the cell membrane. This partitioning of organic solvents prevents the proper functioning of the membrane, which is a selective barrier for solutes between the cell and its external environment. The permeabilization of this barrier impaired the growth of *E. coli* in the presence of toluene and resulted in the leakage of macromolecules such as RNA, phospholipids, and proteins [59, 155]. The functioning of the membrane as a selective barrier is especially important for protons and some other ions. In a study

by Heipieper *et al.* in 1991, phenol–treated *E. coli* cells released ATP and potassium ions into medium [38]. The loss of ion gradients over the membrane prevents energy transduction in solvent–treated cells. Several other researchers demonstrated that the passive flux of ions destroys the gradients over the membrane, such as the ΔpH and the electrical potential $\Delta\psi$, which dissipates the electron motive force [13, 130].

The accumulation of organic solvents in the membrane of bacteria affects the physicochemical properties of the lipid bilayer by changing lipid ordering and bilayer stability [144]. An important aspect of the membrane is its fluidity, which is defined as the reciprocal of the viscosity. Sikkema *et al.* showed in 1994 that the fluidity increased due to the partitioning of organic solvents in biological membranes. This increased fluidity was the result of hydrophobic interactions of hydrocarbons within the membrane [130, 131]. In parallel to this, the accumulation of hydrocarbons resulted in the swelling of the membrane bilayer. As a result of these interactions of solvents with the membrane, its structure and stability were affected [129, 131], resulting in a decreased function of membrane–embedded proteins. This was demonstrated for a variety of organic solvents by measuring cytochrome c oxidase activity in artificial membranes [130]. In *E. coli* cells it was observed that partitioning of toluene resulted in the total inactivation of the galactose permease system [59].

In conclusion, the accumulation of organic solvents results in the disruption of the membrane structure and bilayer stability by increasing membrane fluidity. These adverse effects on the membrane will cause the loss of membrane functions, leading to cell death.

SOLVENT–TOLERANT BACTERIA

Despite the extreme toxicity of organic solvents, Inoue and Horikoshi in 1989 were able to isolate the first solvent–tolerant *Pseudomonas putida* strain that was able to grow in a two–phase water–toluene system [49]. This discovery was confirmed for several other *P. putida* strains [15, 116, 145] and also for other representatives of the genus *Pseudomonas* [47, 97, 99]. Furthermore, solvent tolerance has been found in other Gram–negative genera such as *Flavobacterium* [88] and *Alcaligenes* [99]. Solvent tolerance was also determined in Gram–positive genera such as *Rhodococcus* [104] and *Bacillus* [57, 87]. In addition to the naturally solvent–tolerant strains, several researchers were able to isolate solvent–tolerant mutants with enhanced resistance properties from non–tolerant strains. In this way, mutants of *E. coli* [4], *Pseudomonas aeruginosa* [69] and *P. putida* [128, 140] were obtained. An overview of organic solvent–tolerant bacteria, which are able to withstand organic solvents with a log P_{ow} between 2 and 5, is given in Table 2.1. It is

interesting to note that the naturally solvent–tolerant microorganisms were isolated from various sources such as polluted and nonpolluted soils, domestic wastewater, and the deep sea.

Solvent–tolerant strain	Solvent tolerated	log P_{ow}	Reference
P. putida IH–2000	Toluene	2.5	49
P. putida S12	Toluene	2.5	145
P. putida DOT–T1	Toluene	2.5	116
P. putida Idaho	Toluene	2.5	15
P. putida GM73	Toluene	2.5	67
P. putida KT2442	*p*–Xylene	3.1	27
P. putida F1	*p*–Xylene	3.1	99
P. putida CE2010	Toluene	2.5	99
P. putida PpG1–7T	Toluene	2.5	128
P. putida No.69–3	Heptanol	2.4	140
P. aeruginosa PST–01	Cyclohexane	3.2	98
P. aeruginosa PAO1	Hexane	3.5	78
P. aeruginosa LST–03	Toluene	2.5	97
P. aeruginosa PAK101	Hexane	3.5	69
P. aeruginosa PAK102	*p*–Xylene	3.1	69
P. mendocina K08–1	Dimethylphtalate	2.3	47
P. mendocina LF–1	Dimethylphtalate	2.3	47
Pseudomonas sp. strain LB400	Cyclohexane	3.2	99
A. xylosoxydans A41	*p*–Xylene	3.1	99
A. xylosoxydans ssp. *denitrificans* YO129	Cyclohexane	3.2	99
A. eutrophus H850	Hexane	3.5	99
Flavobacterium sp. strain DS–711	Benzene	2.0	88
Rhodococcus sp. strain 33	Benzene*	2.0	104
Bacillus sp. DS–994	Benzene	2.0	87
B. thuringiensis R1	Toluene	2.5	57
B. mycoides R3	Toluene	2.5	57
B. cereus R5	Toluene	2.5	57
E. coli JA300	*p*–Xylene	3.1	4
E. coli OST3121	*p*–Xylene	3.1	4

TABLE 2.1 Overview of organic solvent–tolerant bacteria. The tolerance to organic solvents is presented as the ability of the organism to survive in a water–organic solvent two–phase system. However, not in all cases (*) has this criterion been tested rigorously. Log P_{ow} values as published by Laane *et al.* in 1986.

Not surprisingly, many efforts were made to uncover the mechanisms behind solvent tolerance since the discovery of organic solvent–tolerant strains. Because the first mode of action of an organic solvent is to partition in the membrane, researchers initially focused on membrane changes. These membrane changes have also been discussed for non–tolerant strains such as *E. coli* [2, 48], but in this Chapter we will focus on solvent tolerance mechanisms of naturally solvent–tolerant Gram–negative bacteria. A schematic representation of the solvent tolerance mechanisms in solvent–tolerant bacteria is given Figure 2.1.

CYTOPLASMIC MEMBRANE CHANGES

Several response mechanisms at the level of the cytoplasmic membrane have been observed in reaction to the accumulation of organic solvents. These responses all

counteract the change in membrane fluidity and bilayer stability with the purpose of restoring membrane fluidity and membrane functions [144]. Mechanisms described include changes in the degree of saturation of membrane lipids [110, 146], changes in the composition of the membrane lipid headgroups [109, 115, 144], changes in the membrane lipid turnover [109], and *cis* into *trans* isomerization of unsaturated membrane lipids [35, 146]. Of these mechanisms, the isomerization of the membrane lipids has been studied in detail by several researchers.

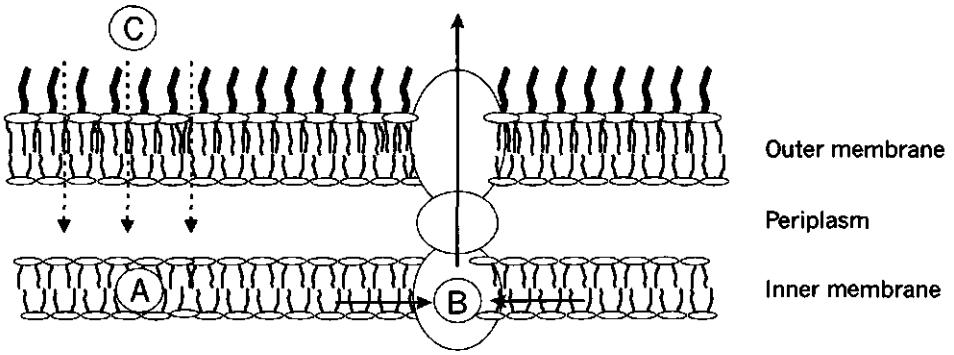

FIGURE 2.1 Schematic representation of organic solvent tolerance mechanisms of solvent-tolerant bacteria. To compensate for the accumulation of organic solvents in the membrane, solvent-tolerant bacteria modify the composition of their cytoplasmic membrane (**A**). This compensation will only be partial, and thus, in a dynamic process, solvents have to be removed continuously from the membrane by the active efflux of the organic solvent (**B**). In combination with a retarded influx of solvents due to modifications at the outer membrane (**C**), bacteria are able to withstand the organic solvents.

The isomerization of the *cis* double bond of an unsaturated fatty acid into the *trans* configuration increases the membrane ordering and subsequently decreases membrane fluidity. Isomerization of *cis*–unsaturated fatty acids as a reaction to hydrocarbons has been described by Heipieper *et al.* in 1992. In *P. putida* P8 cells, grown in the presence of phenols, *cis*–unsaturated fatty acids were isomerized into the *trans* configuration to survive high phenol concentrations. Isomerization of membrane lipids also takes place in solvent-tolerant strains as reaction to the addition of an organic solvent phase. In the solvent-tolerant bacterium *P. putida* S12 it was shown that the amount of *trans*–unsaturated fatty acids corresponds to the survival of the strain in the presence of organic solvents [146]. It seems that this energy-independent isomerization [21] of *cis*–unsaturated fatty acids plays an important role in organic solvent tolerance, allowing the cells to survive the initial cell damage by organic solvents.

The *cti* gene responsible for *cis* into *trans* isomerization of membrane lipids in the solvent-sensitive *P. putida* P8 has been isolated by Holtwick *et al.* by complementing an isomerase-negative mutant. The deduced amino acid sequence of the *cti* gene showed no homology with other proteins [41]. In studies by Heipieper *et al.* it has been shown that *trans*–unsaturated lipids were formed in the presence of

chloramphenicol, indicating a constitutive enzyme system for *cis* into *trans* isomerization in *P. putida* P8 [39]. This was later confirmed by sequence analysis of the *cti* promoter region, revealing a *Pseudomonas* constitutive *rpoD* promoter sequence in this strain [41].

The *cis–trans* isomerase from *Pseudomonas sp.* strain E–3 seems to be located in the cytosol [101], although the characteristics of the purified isomerase are not typical for cytosolic proteins [100]. In a recent study, the *cis–trans* isomerase of *Pseudomonas oleovorans* Gpo12 was purified and determined to be located in the periplasm [108], confirming earlier observations [14]. Both isomerases act *in vitro* only on free unsaturated fatty acids and not on membrane phospholipids [101, 108]. However, *in vivo cis*–unsaturated fatty acids are esterified to phospholipids and therefore an additional protein was anticipated to play a role in *cis* into *trans* isomerization [101]. In *Pseudomonas sp.* strain E–3 this protein was determined to be located in the cell envelope [101]. Moreover, in *P. oleovorans* Gpo12 it was shown that organic solvents induced this protein, and it was speculated that it is a membrane–bound phospholipase [108].

The isomerization of *cis*–unsaturated fatty acids appears to be heme–catalyzed [42]. Evidence from isomerase–negative mutants demonstrated the involvement of a protein of the cytochrome *c* type in the isomerization of *cis*–unsaturated fatty acids. It was speculated that this protein was involved in the transport of heme over the cytoplasmic membrane [42]. Transposon mutants in this study, which were disrupted in the cytochrome *c* operon, were unable to isomerize *cis*–unsaturated fatty acids. Interestingly, similar observations concerning the involvement of the cytochrome *c* operon in solvent tolerance had been obtained previously with solvent–sensitive transposon mutants of *P. putida* IH–2000. The absence of isomerase activity was not determined in this study [40], but a relation between these two aspects may exist.

Several mutants of *Pseudomonas* became solvent–sensitive because of the lack of isomerase activity [115]. However, other strains were shown to be sensitive toward organic solvents and still capable of isomerase activity [110, 115]. Therefore, the *cis–trans* isomerization of unsaturated fatty acids is unlikely to be the only mechanism for solvent tolerance. Isomerization of unsaturated fatty acids has also been reported as a reaction to starvation [30], elevated temperatures [102], heavy metals [35], antibiotics [58], and excess salinity [79]. Because an increased amount of *trans*–unsaturated fatty acids decreases membrane fluidity, it can be concluded that bacteria use isomerization of the unsaturated lipids to counter the increased fluidity due to general stress conditions, including organic solvents.

OUTER MEMBRANE CHANGES

The majority of the isolated solvent-tolerant bacteria are Gram-negative. This observation is in agreement with the fact that Gram-negative bacteria are more resistant to deleterious lipophilic compounds [31, 50, 141]. No differences between Gram-negative and Gram-positive bacteria were observed in regard to the critical concentration of organic solvent dissolved in the cytoplasmic membrane [141]. Therefore, the existence of an outer membrane in Gram-negative bacteria could explain the differences in tolerance toward organic solvents. The outer membrane is considered to be a highly porous shield that allows small hydrophilic molecules to pass via pores, and it has a surprisingly high transfer resistance toward hydrophobic molecules [96]. A few studies have described changes at the level of the outer membrane as a result of the presence of organic solvents. Several researchers described the stabilization of the outer membrane by magnesium and calcium ions resulting in an increased solvent tolerance [51, 116, 144]. Changes in the outer membrane, as a response to the presence of organic solvents, were described at the level of lipopolysaccharides (LPS) and proteins.

Modification of the outer membrane in Gram-negative bacteria has often been related to the LPS composition. Aono and Kobayashi suggested that an increased LPS content in solvent-tolerant mutants of *E. coli* K-12 resulted in a reduction of the cell envelope's hydrophobicity [3]. A similar reduction in the hydrophobicity of the cell envelope was observed in the organic solvent-tolerant *P. putida* S12 after adaptation to organic solvents [144]. These suggested changes in the LPS composition have indeed been found in the solvent-tolerant *P. putida* Idaho. Exposure of these cells to *o*-xylene resulted in the elongation of the saccharide chain of the LPS [110].

Other changes at the level of the outer membrane have been related to the membrane-embedded porins. OprL is a peptidoglycan-associated outer membrane lipoprotein (Pal) and was involved in the stabilization of the cell envelope and morphology [119, 120]. Mutants of *P. putida* DOT-T1E lacking the porin OprL became hypersensitive to organic solvents [119], most likely because their membrane structure was impaired. Similar results were observed in solvent-sensitive mutants of *P. aeruginosa* lacking the porin OprF [74]. It was suggested that in *P. aeruginosa* organic solvents normally should enter the cell by passing through the OprF porin. However, OprF and OprL both are bifunctional porins that not only serve as diffusion porin but are also required for maintaining the structural integrity of the bacterial membrane [156]. Thus, in analogy with the OprL porin in *P. putida*, solvent sensitivity in *P. aeruginosa* could be due to the decreased stability of the membrane. Similar results concerning the involvement of a porin were obtained by Aono *et al.* in 1997. A repressed OmpF synthesis resulted in solvent-tolerant mutants of *E. coli* [3]. Recently, however, Asako *et al.* showed that organic

solvent tolerance in *E. coli* was independent of OmpF levels and that the higher tolerance toward solvents was due to an elevated level of the *acrAB* efflux system [10]. In 1991, Hengge–Aronis and coworkers tested the resistance of *E. coli* to toxic compounds that affect the integrity of the membrane, proteins, and DNA. In their experiment stationary–phase cells were shown to exhibit better survival than log–phase cells after treatment with 0.1% toluene. Moreover, *rpoS* mutants were clearly more sensitive to toluene than wild–type cells were. From a present–day perspective, one could speculate that *acrAB* is involved since these genes are stationary phase induced [80] and are regulated by RobA [91], which in turn is under RpoS control [Hengge–Aronis, R., personal communication].

The specific roles of the various porins, the LPS content, and the composition of the outer membrane have not been clarified yet. It has been speculated that alterations at the level of the outer membrane prevent the rapid influx of organic solvent, allowing the cells to adapt to the extreme environment and to induce the necessary resistance mechanisms [17].

ACTIVE EFFLUX OF ORGANIC SOLVENTS

The exceptional resistance of solvent–tolerant bacteria toward a wide variety of deleterious organic solvents cannot be explained solely by the mechanisms as described above. These mechanisms would allow the cells to retard the rapid influx of solvents in the cytoplasmic membrane. Nevertheless, despite the decreased accumulation rate of organic solvents in the membrane, similar equilibrium concentrations will eventually be reached. Therefore, it was anticipated that a longer–lasting active efflux system had to play a critical role in organic solvent tolerance [144]. An active efflux system is able to extrude organic solvents from the membrane to decrease the toxin concentration below its critical concentration in the membrane. This allows the survival of the bacterium in the presence of high concentrations of organic solvents.

In 1996, Isken and de Bont were able to determine the active removal of nonmetabolizable ^{14}C–labeled toluene from the solvent–tolerant bacterium *P. putida* S12. It was found that the amount of toluene was 50% lower in toluene–induced cells compared to the amount in noninduced cells. Furthermore, it was determined that the addition of the respiratory chain inhibitor potassium cyanide and the proton conductor carbonyl cyanide *m*–chlorophenylhydrazone increased the accumulation of toluene, suggesting the energy–dependent export of toluene in *P. putida* S12 [55]. Similar results were obtained by Ramos *et al.* in 1997 by measuring the extrusion of ^{14}C–labeled 1,2,4–trichlorobenzene, as a nonmetabolizable analog for toluene, in the solvent–tolerant bacterium *P. putida*

DOT-T1E [115]. This confirmed the presence of energy-dependent efflux of organic solvents in solvent tolerance.

An attempt to clone the genes for the energy-dependent efflux of toluene in *P. putida* S12 was undertaken [see Chapter 3]. Transposon mutants that were sensitive to toluene were constructed, which made it possible to clone and sequence the genes responsible for toluene efflux. The deduced amino acid sequences of the isolated *srp* genes showed strong homology with an efflux system of the resistance/nodulation/cell division (RND) family [95, 107]. These results demonstrate that indeed an efflux system in a solvent-tolerant bacterium is involved in the resistance toward uncharged lipophilic compounds such as toluene and xylene.

The involvement of efflux in solvent tolerance was confirmed with the isolation of the *ttgB* gene and the *mepABC* genes in the toluene-tolerant *P. putida* DOT-T1E [117] and in the toluene-tolerant variant of *P. putida* KT2442 [27], respectively. Moreover, Kim *et al.* reported in 1998 that a transposon insertion in a protein of the RND family resulted in a *P. putida* mutant with a solvent-sensitive phenotype [67].

It is generally accepted that efflux pumps of the RND family are constructed in such a way that they traverse both the cytoplasmic and the outer membrane. Genetic and biochemical evidence supports the notion that the proton motive force is necessary for functioning of RND-type efflux pumps. In the reported efflux systems the inner membrane protein (RND protein) was thought to be a putative 12-transmembrane-segment transporter protein that is attached, via a membrane fusion protein (MFP) belonging to the MFP family, to an outer membrane protein (OMP) [22, 124]. The OMP is thought to be an outer membrane channel to circumvent the outer membrane barrier, allowing the pumped molecule to be released into the medium [22]. Various toxic substrates are excreted from the cell membrane by RND-type transporters [see Table 2.2 for an overview of some representatives of the RND family]. These efflux systems are involved in the export of antibiotics, metals, organic solvents, and oligosaccharides involved in nodulation signaling [95, 107].

SOLVENT EFFLUX SYSTEMS VERSUS MULTIPLE ANTIBIOTIC RESISTANCE

A close correlation between antibiotic and solvent tolerance has been demonstrated by Aono *et al.* in 1995. In this study cyclohexane-tolerant mutants of *E. coli* displayed an improved tolerance toward low levels of antibiotics [6]. A similar correlation between organic solvent tolerance and multiple drug resistance was observed by Isken *et al.* in 1997. In the naturally solvent-tolerant strain *P. putida* S12 it was demonstrated that cells, with a solvent-tolerant phenotype, were more

resistant toward multiple antibiotics. However, cells grown in the presence of antibiotic did not show an increased tolerance toward organic solvents [58]. These results can be explained in at least three different ways. First, the increased multiple antibiotic resistance can be the result of the active efflux as a result of the induction of the solvent transporter. Second, the solvents can be able to aspecifically induce an antibiotic efflux system. Third, the increased resistance can be the result of other mechanisms induced by organic solvents preventing the influx of the toxic compound.

Efflux protein			Organism	Substrate		Reference(s)
MFP	RND	OMP		Solvents	Antibiotics	
SrpA	SrpB	SrpC	*P. putida*	Toluene, *p*-xylene, octanol, ethylbenzene, propylbenzene, cyclohexane, hexane		63, 64
MepA	MepB	MepC	*P. putida*	Toluene, p-xylene, cyclohexane	Tc, PenG, Ery, Nov, Amp	27
TtgA	TtgB	TtgC	*P. putida*	Toluene, *m*-xylene, 1,2,4-trichlorobenzene	Tc, Cm, Amp	117
	Ttg3		*P. putida*	Toluene	ND	67
MexA	MexB	OprM	*P. aeruginosa*	Toluene, *p*-xylene, hexane	Tc, Cm, PenG, Ery, Nov, Carb, Rif	76, 77, 78, 112
MexC	MexD	OprJ	*P. aeruginosa*	*p*-Xylene, hexane	Tc, Cm	78, 114
MexE	MexF	OprN	*P. aeruginosa*	*p*-Xylene	Cm	78, 85
AcrA	AcrB	TolC	*E. coli*	Hexane, cyclohexane	Tc, Cm, PenG, Ery, Nov, Amp, Rif	81, 152

TABLE 2.2 RND-type efflux systems in Gram-negative bacteria involved in efflux of organic solvents and drugs. Some compounds are expected to be substrates based on solvent-sensitive phenotype or decreased MIC for transposon mutants. Abbreviations: MFP, membrane fusion protein; RND, inner membrane (transporter) protein; OMP, outer membrane protein; Tc, tetracycline; Cm, chloramphenicol; PenG, penicillin G; Ery, erythromycin; Nov, novobiocin; Amp, ampicillin; Carb, carbenicillin; Rif, rifampin; ND, not determined.

Indeed, the AcrAB efflux pump of *E. coli* was involved in resistance toward antibiotics and solvents [152]. It appears that the transcriptional activator *marA* [9], *soxS* [92], and *robA* [91] mediate this phenotype by upregulating the *acrAB* locus. Moreover, a toluene-sensitive transposon mutant of *P. putida* DOT-T1, with an insertional inactivation in the *ttgB* gene, was more susceptible to several antibiotics, including tetracycline, chloramphenicol, and ampicillin [117]. Similar results were obtained with an insertion mutant of a toluene-resistant variant of *P. putida* KT2442 [27]. Inactivation of the MepABC efflux system resulted in a solvent-sensitive phenotype with a concomitant increase of sensitivity toward antibiotics. It was demonstrated that resistance toward toluene and high concentrations of antibiotics in this strain occurs only in a toluene-tolerant variant and not in wild-type cells [27]. Toluene and multiple antibiotic resistance in this strain were suggested to be due to the upregulation of the MepABC efflux system. In *P. aeruginosa* the three efflux systems MexAB-OprM, MexCD-OprJ, and MexEF-OprN are involved in both multiple antibiotic resistance and solvent tolerance [78].

Moreover, organic solvent–tolerant mutants of *P. aeruginosa* were multidrug–resistant due to mutations in the regulator MexR [113]. This resulted in the overexpression of MexAB–OprM and an increased expression of MexEF–OprN.

Induction mechanisms of efflux systems for antibiotics in Gram–negative bacteria have not been studied in detail. Therefore, it is at present difficult to speculate on solvent–induced expression of multiple antibiotic efflux systems.

FUTURE PROSPECTS

Solvent–tolerant bacteria hold considerable promises in the field of applied biotechnology. Two possible applications, namely solvent–tolerant bacteria as cell factories [17, 116] and as the source for solvent–stable enzymes [97, 98], have been demonstrated. With the genetic uncovering of solvent tolerance mechanisms and their regulation, it becomes possible to use solvent–tolerant bacteria as production hosts in biotechnological processes. Furthermore, it has to be seen in the near future what the exact relation is between solvent tolerance and multiple drug resistance.

3

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF AN EFFLUX PUMP INVOLVED IN *PSEUDOMONAS PUTIDA* S12 SOLVENT TOLERANCE

Jasper Kieboom, Jonathan J. Dennis, Jan A.M. de Bont, and Gerben J. Zylstra

SUMMARY

Bacteria able to grow in aqueous–organic two–phase systems have evolved tolerance mechanisms to the toxic effects of solvents. One such mechanism is the active efflux of solvents from the cell, preserving the integrity of the cell interior. *Pseudomonas putida* S12 is tolerant to a wide variety of normally detrimental solvents due to the action of such an efflux pump. The genes for this solvent efflux pump were cloned from *P. putida* S12 and their nucleotide sequence determined. The deduced amino acid sequences encoded by the three genes involved show a striking resemblance to proteins known to be involved in proton–dependent multidrug efflux systems. Transfer of the genes for the solvent efflux pump to solvent–sensitive *P. putida* strains results in the acquisition of solvent tolerance. This opens up the possibilities of using the solvent efflux system to construct bacterial strains capable of performing biocatalytic transformations in normally toxic two–phase aqueous–organic systems.

PUBLISHED IN THE JOURNAL OF BIOLOGICAL CHEMISTRY (1998) 273: 85–91

INTRODUCTION

The microbial transformation of hydrocarbons is important not only in environmental applications such as soil remediation and waste stream purification, but also in biocatalytic applications for the production of specialty chemicals. The metabolic pathways by which many of these compounds are degraded in various bacteria have been elucidated and in many cases the genes coding for the enzymes involved have been cloned and sequenced [159]. A major problem in applying hydrocarbon–degrading bacteria to industrial processes is their susceptibility to the toxic effects of the very substrate that the organism is utilizing as a carbon source. This is often due to accumulation of the hydrophobic compound in bacterial membranes, which can cause devastating effects on membrane structure [130, 131]. A second problem in the application of catabolic pathways in the synthesis of fine chemicals is that many of the desired substrates of enzymatic reactions are sparingly soluble in water and thus may not be fully bioavailable to microorganisms. The use of solvent–tolerant bacteria allows the introduction of an organic phase to the medium, dissolving the desired substrate, and increasing the exposure of the cell to the substrate.

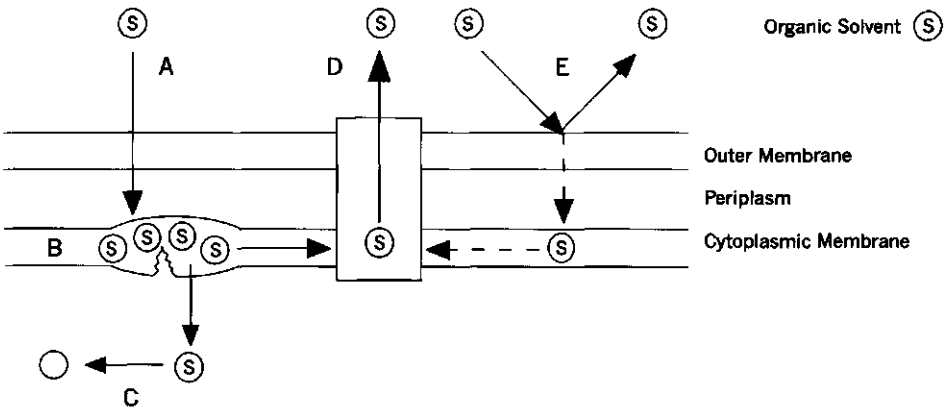

FIGURE 3.1. Schematic representation of essential features of solvent–tolerant bacteria. (**A**) Solvents diffuse to and preferentially partition to the cytoplasmic membrane where they cause disruptions in membrane functions by increasing membrane fluidity and affecting bilayer stability [131, 144]. (**B**) To compensate for these effects, solvent–tolerant bacteria modify the composition of the membrane either by isomerizing *cis*– into *trans*–unsaturated fatty acids from the membrane lipids [55, 146] or by changing the headgroup composition [115, 144]. This compensation will only be partial and thus in a dynamic process, solvents have to be removed continuously from the membrane. (**C**) Removal to a certain extent may be by degradation [116]. (**D**) Very recently, on the basis of whole cell experiments, evidence was obtained that an unprecedented energy–dependent export system for hydrophobic solvents is in operation [55]. (**E**) In combination with a retarded influx of solvents due to modifications at the outer membrane [74, 110], the new efflux pump functions as the key factor in solvent tolerance.

Many different mechanisms have been described that contribute to solvent tolerance [Figure 3.1] but despite these efforts no comprehensive overview is available to explain the physiological response of microorganisms to toxic organic solvents [see

Chapter 2]. We have been investigating the ability of *Pseudomonas putida* S12 to withstand toxic concentrations of toluene and other organic solvents [145]. This organism has evolved at least two mechanisms to resist the accumulation of hydrophobic solvents in the membrane or the interior of the cell. One key observation was the detection of *trans* rather than *cis*–unsaturated fatty acids in the membrane of the solvent–tolerant bacterium upon exposure to solvents [34, 36]. The conversion of *cis*– to *trans*–unsaturated fatty acids by a direct isomerization alters the packing of the phospholipids in the bacterial membrane. This results in a change in membrane fluidity, making the membrane less likely to allow solvents to partition into it, decreasing the detrimental effects on the membrane due to solvent partitioning, and thus increasing the solvent tolerance of the cell [60]. Recently it has been shown that a second mechanism of solvent tolerance is possessed by *P. putida* S12. This energy–dependent efflux system for solvents such as toluene [55] may function in a fashion similar to that for multidrug efflux pumps found in many antibiotic–resistant microorganisms. Thus, *P. putida* S12 employs at least two mechanisms for active defense against the detrimental effects of solvents: one functioning to keep solvents out of the interior of the cell and a second functioning to prevent solvents from partitioning into the cell membrane.

In this Chapter we describe the construction of transposon mutants of *P. putida* S12 that have lost the solvent–tolerant phenotype. This allowed the cloning of the genes responsible for a solvent efflux pump that is involved in the ability of *P. putida* S12 to withstand toxic concentrations of organic solvents. The nucleotide sequence of the genes involved was determined and their relationship to other bacterial efflux systems is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth of strains

P. putida S12 [33] is the wild–type strain capable of growth at saturated solvent concentrations [145] and is the object of the present investigation. *P. putida* JK1 is a solvent–sensitive mutant of *P. putida* S12 derived in the present work by transposon mutagenesis with Tn*Mod*–KmO. *P. putida* JJD1, also derived in the present work, contains a kanamycin gene/ColE1 origin cassette insertion in the genome of *P. putida* S12. The solvent–sensitive strain *P. putida* PPO200 is *P. putida* mt–2 cured of the TOL plasmid [160]. The artificial transposable element Tn*Mod*–KmO [18] contains a kanamycin resistance gene and the ColE1 origin of replication between Tn*5* inverted repeats. The Tn*5* transposase gene and an origin of transfer for conjugation are present outside the inverted repeats. *Escherichia coli* JM109 (*recA1 endA1 gyr A96 thi hsdR17 supE44 relA1* Δ(*lac–proAB*) [F' *traD36 proAB lacI*^{q}*ZΔM15*], [158]) was utilized as the host strain for all recombinant plasmids. The cloning vector pUC19 [158], the pGEM series of cloning vectors (Promega,

Madison, WI), and the broad host range vector pUCP22 [150] were used for the construction of subclones.

L broth [73] was used as complete medium. Minimal medium was prepared as described by Stanier *et al.* [135]. Solid media contained 2% agar. Ampicillin (100 μg/ml), kanamycin (50 μg/ml), or gentamicin (25 μg/ml) were added to the medium to maintain recombinant plasmids in *E. coli*. Gentamicin (25 μg/ml) was used to maintain the pUCP22 subclones in *P. putida* and kanamycin (250 μg/ml) was used in the selection of *P. putida* S12 transposon mutants. *E. coli* strains were routinely cultured at 37°C and *P. putida* strains were grown at 30°C. Growth of bacterial strains in the presence of various solvents (indicating solvent tolerance) was determined essentially as described by Weber *et al.* [145]. The experiments were conducted twice with duplicate samples each time.

*Generation and screening of Tn*Mod–*KmO insertion mutants*
The conjugatable suicide transposon donor Tn*Mod*–KmO was introduced into *P. putida* S12 by triparental mating using pRK2013 as the mobilizing plasmid by established procedures [23, 66]. Kanamycin–resistant colonies were tested for the ability to grow on L agar plates in the presence of saturating vapor amounts of toluene. This was accomplished by placing the agar plates in a sealed glass desicator along with a small beaker containing toluene. Growth was scored after 12 h at 30°C.

DNA techniques
Total genomic DNA from *P. putida* strains was prepared by the CTAB procedure [11]. Plasmid DNA was isolated by the alkaline–SDS lysis method of Birnboim and Doly [12]. DNA was digested with restriction enzymes and ligated with T4 ligase as recommended by the supplier (Life Technologies). DNA restriction fragment and PCR products were visualized by 0.7% or 1.0% agarose gel electrophoresis in 40 mM Tris, 20 mM acetate, 2 mM EDTA buffer. DNA from agarose gels was isolated using the method of Vogelstein and Gillespie [142]. Plasmid DNA was introduced into either *E. coli* JM109 or *P. putida* JK1 cells by electroporation [19] using a Gene Pulser (Bio–Rad).

All sequencing and PCR reactions were performed using a Gene Amp PCR System 9600 (Perkin–Elmer). Nucleotide sequencing reactions were performed with purified double–strand plasmid DNA or PCR products using AmpliTaq FS DNA polymerase fluorescent dye terminator reactions (Perkin–Elmer) as recommended by the supplier. Sequencing products were detected using an Applied Biosystems 373A stretch automated DNA sequencer (Applied Biosystems). Nucleotide sequence analysis was performed either with the Genetics Computer Group analysis package [20] or with the National Center for Biotechnology Information BLAST server [1]. PCR reactions for amplifying the region of genomic *P. putida* S12 DNA

containing the insertion point for the transposon were performed using *Taq* DNA polymerase (Perkin–Elmer). The reaction mixture (100 µl) was treated for 1 min at 94°C followed by 25 cycles of 1 min at 96°C, 1 min at 55°C and 1 min at 72°C before finishing for 10 min at 72°C. Primers for this reaction were 5'–CGTTTGCAACCGGTGAG–3' and 5'–TATCGGACGCAAACG–3' corresponding to positions 3735 to 3752 and 4238 to 4253 of the nucleotide sequence, respectively.

RESULTS

Isolation of solvent–sensitive mutants

P. putida S12 was chosen for a molecular study of the basis of solvent tolerance due to the extensive physiological studies that have been performed on the strain [34, 35, 37, 55, 144, 145, 146]. The organism can grow in the presence of a wide variety of normally toxic solvents with log P_{ow} values ranging from 2.3 to 3.5 [Table 3.1]. Initially, several solvent–sensitive transposon mutants were constructed using Tn*Mod*–KmO. *P. putida* S12 mutants which are no longer tolerant to solvents were detected by the inability of kanamycin–resistant exconjugants to grow on L medium in the presence of saturated vapor concentrations of toluene as described under "Materials and Methods". Several toluene–sensitive mutants were obtained and one of these, designated strain JK1, was chosen for further analysis. Besides toluene, JK1 is sensitive to a number of other solvents with log P_{ow} values less than or equal to 3.5 [Table 3.1], indicating that a single genetic trait is responsible for tolerance to all of the solvents tested.

Solvent	Log P_{ow}	Growth of *P. putida* strains on L/acetate and 1% solvent				
		S12	JK1	JK1 (pUCP22)	JK1 (pJD105)	JK1 (pJD106)
Hexadecane	8.8	+	+	+	+	+
Decane	5.6	+	+	+	+	+
p–Cymene	4.1	+	+	+	+	+
Propylbenzene	3.6	+	+	+	+	+
Hexane	3.5	+	–	–	+	+
Cyclohexane	3.2	+	–	–	+	+
Ethylbenzene	3.1	+	–	–	+	–
p–Xylene	3.0	+	–	–	+	–
Octanol	2.9	+	–	–	+	–
Toluene	2.5	+	–	–	+	–
Dimethylphtalate	2.3	+	–	–	+	–
Fluorobenzene	2.2	–	–	–	–	–
Benzene	2.0	–	–	–	–	–

TABLE 3.1. Growth of *P. putida* strains on L/acetate in the presence of various solvents. Strain JK1 is a transposon mutant of the genes for a solvent efflux pump. pJD105 and pJD106 are clones containing the three genes for the solvent efflux pump (*srpABC*) cloned into the vector pUCP22. pJD105 has the genes in the same orientation as the *lac* promoter on the plasmid while pJD106 has the genes in the reverse orientation. Different solvents (1% final concentration) were separately added to identical subcultures in L medium with 60 mM acetate during the early exponential growth phase. Growth of the culture was measured at various times after solvent addition with no continued growth indicating solvent sensitivity. A *plus* indicates growth with optical density (OD) > 0.7 after 24 h while a *minus* indicates no growth with OD < 0.1 after 120 h.

Cloning and analysis of the genes for solvent tolerance

To characterize the genes for solvent tolerance in *P. putida* S12 in more detail, the region of the genome containing the transposon insertion in mutant JK1 was cloned. This was aided by the fact that the Tn*Mod*-KmO transposable element utilized in the construction of the mutants contains an origin of replication derived from plasmid ColE1. Total genomic DNA from JK1 was cleaved with *Bam*HI, ligated to form circular molecules, and electroporated into *E. coli* JM109 with selection for kanamycin resistance. Since *Bam*HI does not cleave the transposon the resulting clone, pJD101 [Figure 3.2] must contain DNA from both sides of the transposon insertion. Approximately 11 kilobases of genomic DNA was cloned along with the transposon, which contains the origin of replication and the kanamycin resistance gene (2 kb). The point at which the transposon is inserted is only one kilobase away from one end of the *Bam*HI fragment cloned from strain JK1. This being the case, a second, overlapping 4 kb *Pst*I fragment was cloned from strain JK1 (designated pJD102, Figure 3.2).

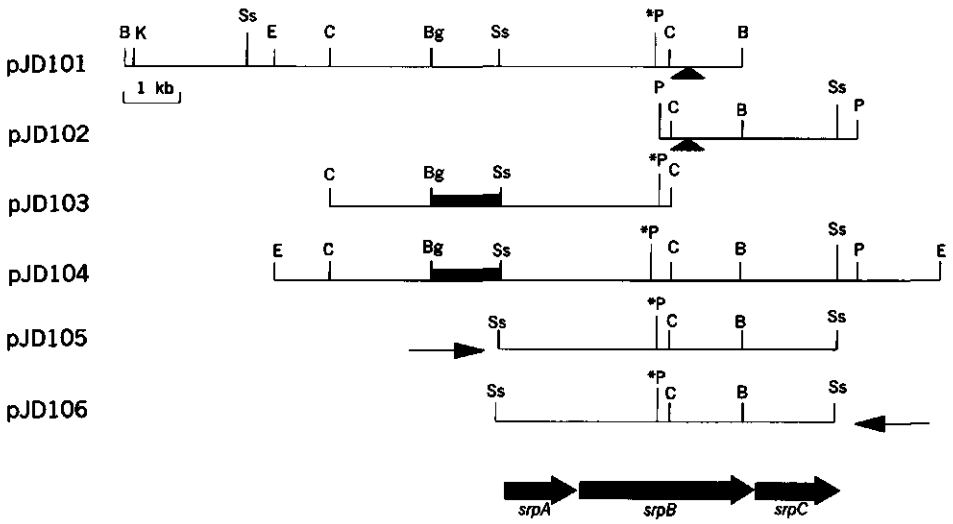

FIGURE 3.2. Restriction maps of the clones derived from the transposon and insertional mutant strains. A cartoon of the *srpABC* nucleotide sequence (GenBank accession number AF029405) is shown at the *bottom*, in proportion to the restriction maps and showing the positions of the genes relative to the restriction enzyme cutting sites. The *triangles* in the restriction maps of pJD101 through pJD102 and the *boxes* in the restriction maps of pJD103 and pJD104 indicate the location of the inserted kanamycin resistance gene and the ColE1 origin of replication in the cloned genomic DNA. The *arrows* next to pJD105 and pJD106 indicate the direction of transcription of the *lac* promoter from the vector pUCP22. The *asterisk* indicates that not all of the *Pst*I sites were mapped in these plasmids.

The cloned genomic DNA was initially analyzed by determining the nucleotide sequence to either side of the transposon insertion point using primers specific for either end of the transposon. Screening for similar nucleotide sequences in the GenBank database revealed a significant match with genes coding for multidrug resistance export pumps, consistent with the hypothesis that a solvent efflux pump

is a key solvent tolerance mechanism of *P. putida* S12. The complete nucleotide sequence of an operon into which the transposon had inserted was determined using a series of subclones and internal oligonucleotide primers. Both strands of a 6.5 kb *Sst*I fragment were sequenced in their entirety. To accurately determine the nucleotide sequence at the transposon insertion point, a 0.5 kb DNA fragment was amplified by PCR from the genome of the wild-type strain *P. putida* S12 using oligonucleotide primers flanking the transposon insertion point in strain JK1. A cartoon of the nucleotide sequence obtained is shown in Figure 3.2 to show the relationship of the open reading frames with the two clones pJD101 and pJD102. The three open reading frames show significant homology to the three proteins that assemble to form proton-dependent multidrug resistance efflux pumps [95, 107] and thus the genes were labeled *srp* for solvent resistance pump.

Complementation of P. putida JK1

To prove that the three open reading frames detected in the cloned fragment at the point of insertion of the Tn*Mod*-KmO transposon actually are responsible for solvent tolerance complementation experiments were performed. This required reconstruction of the operon since the clones obtained (pJD101 and pJD102) contain the transposon mutagenized DNA. A *Bgl*II-*Sst*I kanamycin resistance cassette also containing the ColE1 origin of replication from Tn*Mod*-KmO was inserted at the *Bgl*II and *Sst*I sites of a 6.7 kb *Cla*I fragment derived from pJD101. The resulting plasmid (pJD103, Figure 3.2) was electroporated into *P. putida* S12 to construct a new mutation by site-specific reciprocal recombination. The resulting strain, JJD1, contains a kanamycin resistance gene adjacent to the *srpABC* genes in the genome. A 12 kb *Eco*RI genomic fragment containing the kanamycin/ColE1 cassette was cloned from JJD1. This plasmid, designated pJD104 [Figure 3.2], contains the intact *srpABC* genes. A 6.5 kb *Sst*I fragment was cloned from pJD104 into the vector pUCP22 in both orientations with respect to the *lac* promoter. JK1 containing either of these two plasmids, designated pJD105 and pJD106 [Figure 3.2], regained solvent tolerance. JK1(pJD105), containing the *srpABC* genes in the same orientation as the *lac* promoter, regained tolerance to all of the solvents that the original strain, *P. putida* S12, was tolerant to [Table 3.1]. However, JK1(pJD106), containing the *srpABC* genes in the opposite orientation to the *lac* promoter, regained tolerance to only two solvents, hexane and cyclohexane, with log P_{ow} values near the border of the tolerance phenotype. These results are consistent with the solvent tolerance phenotype being dependent on the level of expression of the *srpABC* genes.

Transfer of the solvent tolerance phenotype

P. putida S12 displays multiple physiological responses to organic solvents [see "Introduction"]. Intuitively, a solvent efflux pump would be the most important mechanism of solvent tolerance since it would be involved in actively removing solvents from the cell. Experiments were therefore performed to determine whether the solvent efflux pump by itself is capable of imparting the solvent tolerance

phenotype on other *P. putida* strains. The two plasmids pJD105 and pJD106 were electroporated into the normally solvent–sensitive *P. putida* PPO200. The resulting recombinant strains are able to grow on rich medium in a toluene–saturated atmosphere whereas the parent strain PPO200 with the vector pUCP22 could not [Figure 3.3]. PPO200(pJD106) grew slightly slower than PPO200(pJD105), probably due to the fact that the *srpABC* genes are expressed from the *lac* promoter in pJD105. In liquid culture, PPO200(pUCP22) could withstand concentrations of toluene up to 2.8 mM while both PPO200(pJD105) and PPO200(pJD106) showed tolerance to the toxic effects of toluene up to a concentration of 4.9 mM [Table 3.2]. Neither of the recombinant strains was tolerant to a second phase of toluene. These experiments indicate that the solvent tolerance phenotype can be transferred to other bacterial strains and that higher levels of gene expression can enhance the tolerance.

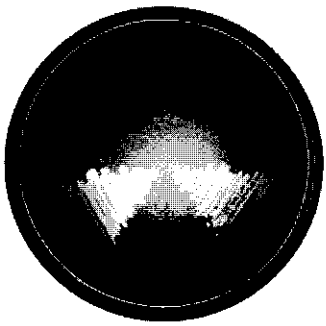

FIGURE 3.3. Photograph of the solvent–sensitive *P. putida* PPO200 plated on solid L medium in the presence of saturating vapor concentrations of toluene with and without the *srpABC* genes. *Upper: P. putida* PPO200(pUCP22), no growth. *Lower left: P. putida* PPO200(pJD105), good growth. *Lower right: P. putida* PPO200(pJD106), slow growth.

DISCUSSION

In the past several years solvent–tolerant microorganisms have been isolated directly from the environment [5, 15, 49, 110, 116, 145] or through the process of mutation of a solvent–sensitive strain [4, 6, 26]. It is evident that these organisms must therefore have specific adaptation mechanisms that impart the solvent tolerance. Physiological studies on microorganisms isolated from the environment that are naturally tolerant to high levels of organic solvents have revealed that many different factors may play a role [36, 144]. Naturally solvent–tolerant bacteria have been shown to alter the composition of the cell membrane by increasing the ratio of *trans*– to *cis*–unsaturated fatty acids [146] or by changing the headgroup composition [115]. This change in the cell membrane produces a physical barrier, preventing solvents from entering the cell by decreasing membrane fluidity. This would not entirely prevent solvents from entering the cell, only slow down their

diffusion into the cell and increase the time needed to reach equilibrium with the external environment. An intuitively better method of solvent tolerance would be to physically remove the solvent from the cell. One way of doing this would be to degrade the solvent but this would only be effective against low concentrations of solvents. Evidence was recently obtained that an energy–dependent export system for hydrophobic solvents functions to remove them from the interior of whole cells [55]. This solvent efflux pump should be a key element in solvent tolerance by naturally solvent–tolerant bacteria. The genes for such a solvent efflux pump in *P. putida* S12 were identified via transposon mutagenesis to construct a solvent–sensitive strain. The genes were cloned and sequenced and their role in solvent tolerance verified through complementation of the transposon mutation. The three genes involved were labeled *srpABC* for solvent resistance pump.

Toluene Concentration (mM)	Growth of *P. putida* strains on L Medium with Toluene		
	PPO200 (pUCP22)	PPO200 (pJD105)	PPO200 (pJD106)
0.0	+	+	+
0.7	+	+	+
1.4	+	+	+
2.1	+	+	+
2.8	+	+	+
3.5	–	+	+
4.2	–	+	+
4.9	–	+	+
5.6	–	–	–

TABLE 3.2. Growth of *P. putida* PPO200, containing the cloned *srp* genes, on L medium in the presence of toluene. Toluene was added at different concentrations to identical subcultures in L medium during the early exponential growth phase. A *plus* indicates growth with optical density (OD) > 0.7 and a *minus* indicates no growth with OD < 0.1 after 24 h.

The deduced amino acid sequences of the proteins encoded by the *srpABC* genes have extensive homology with those for proton–dependent multidrug efflux systems of the resistance/nodulation/cell division (RND) family [95, 107]. This family of efflux pumps is composed of three protein components that together span the inner and outer membranes of Gram–negative bacteria: an inner membrane transporter (SrpB analogues), an outer membrane channel (SrpC analogues), and a periplasmic linker protein (SrpA analogues). Members of this family are involved in export of antibiotics, metals, and oligosaccharides involved in nodulation signaling. Based on the work presented here, this family can be broadened to include a new class of efflux pump, involved in export of solvents. Dendrograms showing the phylogenetic relationship of SrpA, SrpB, and SrpC to other proteins involved in multidrug resistance are shown in Figure 3.4. The *srpABC*–encoded proteins show the most homology with those for the *mexAB/oprM*–encoded multidrug resistance pump found in *Pseudomonas aeruginosa* [28, 111]. SrpA, SrpB, and SrpC are 57.8, 64.4, and 58.5% identical to MexA, MexB, and OprM, respectively. The three dendrograms show that the Srp and the Mex proteins fall into a distinct class separate from but still closely related to the other members of the RND family of efflux pumps. The evolutionary relationship of the solvent tolerance pump to

multidrug resistance pumps is not surprising since they both function to export hydrophobic molecules from the cell. It is logical that a solvent efflux pump would have evolved since it would enable microorganisms to survive in close proximity to oil or coal deposits (a rich source of carbon and energy) in the environment.

There have been several attempts to clone genes that are involved in solvent tolerance leading in many cases to the identification of a protein that is somehow involved in making the cell tolerant to a single solvent or to a group of related solvents. Most of these studies took place using *E. coli* that was forced under selective pressure to become solvent-tolerant. Mutations in a number of different genes can result in an increased tolerance to a particular chosen solvent, any one of which can allow the cell to grow in the presence of the solvent. Genes implicated in increased organic solvent tolerance of *E. coli* include the uncharacterized *ostA* (for organic solvent tolerance) [7], *ahpC* encoding alkylhydroperoxide reductase [26], *robA* encoding a global regulatory protein [91], and *soxS* encoding regulatory proteins controlling the superoxide response regulon [92]. These genes enhance the survivability of the organism in the presence of a particular solvent but are not responsible for solvent tolerance *per se* since they do not aid in understanding the true mechanism(s) of solvent tolerance found naturally in environmental isolates. This paper, however, represents the first example of cloning and characterization of genes for a major solvent tolerance system: a proton–dependent solvent efflux pump.

We have shown that the cloned genes can be transferred to another *P. putida* strain with the concomitant gain of solvent tolerance by that organism. This has far reaching implications for industrial applications in the fine chemistry area. Existing and potential biocatalytic processes for compounds such as catechols, phenols, medium chain alcohols and enantiopure epoxides are suboptimal because the products formed are very toxic to normal microorganisms. Product accumulation to a concentration, which allows economic downstream processing is inherently prevented by the physical characteristics of these compounds. The ability, as demonstrated here, to take a normally solvent–sensitive strain and make it solvent–tolerant will greatly enhance the ability of a given strain to perform a desired biocatalytic reaction in aqueous–organic two–phase system.

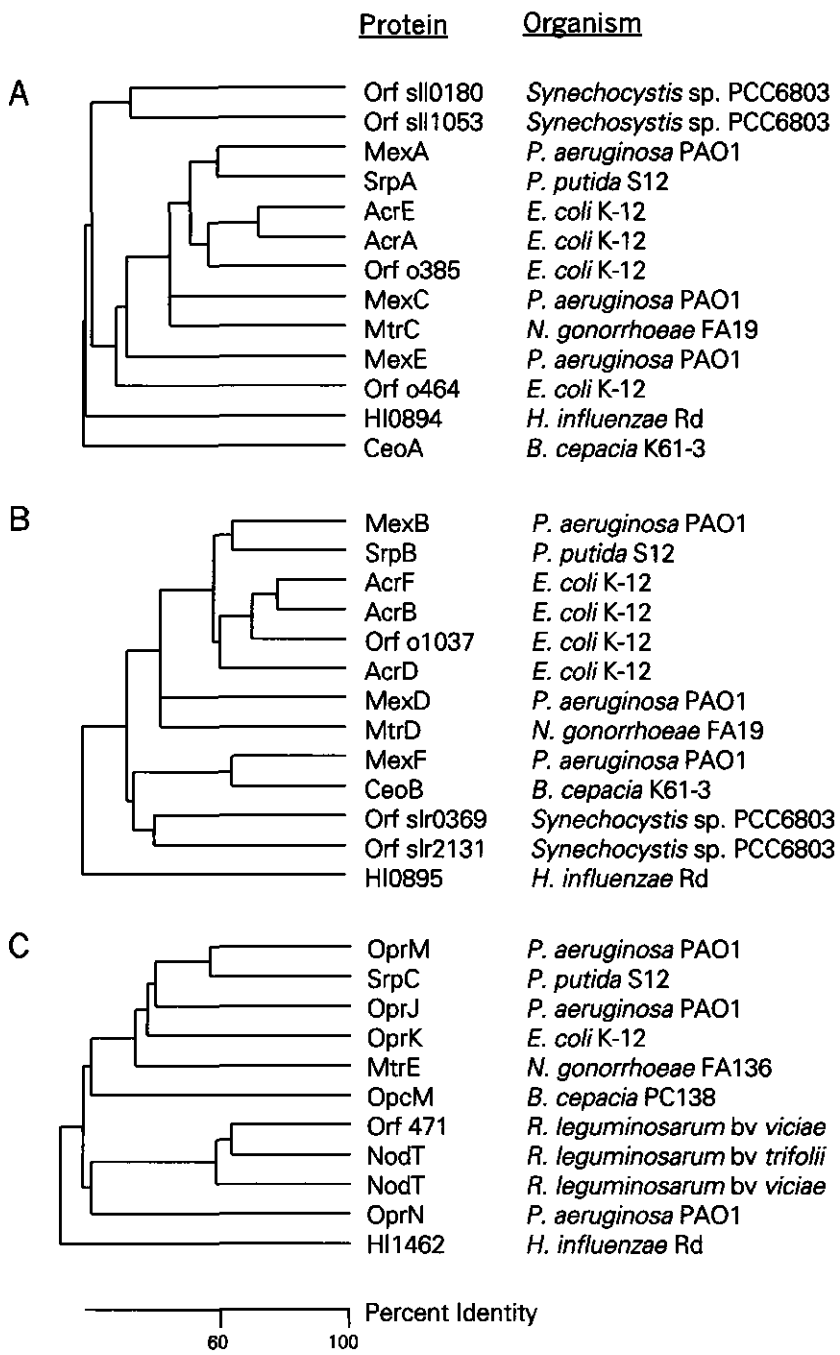

FIGURE 3.4. Dendrograms showing the levels of homology between the amino acid sequences of different proteins involved in proton–dependent efflux systems. **A.** Dendrogram of periplasmic linker proteins. **B.** Dendrogram of inner membrane transporter proteins. **C.** Dendrogram of outer membrane channel proteins.

4

ACTIVE EFFLUX OF ORGANIC SOLVENTS BY *PSEUDOMONAS PUTIDA* S12 IS INDUCED BY SOLVENTS

Jasper Kieboom, Jonathan J. Dennis, Gerben J. Zylstra, and Jan A.M. de Bont

SUMMARY

Induction of the membrane associated organic solvent efflux system SrpABC of *Pseudomonas putida* S12 was examined by cloning a 312 bp DNA fragment, containing the *srp* promoter, in the broad-host-range reporter vector pKRZ-1. Compounds that are capable of inducing expression of the *srpABC* genes include aromatic and aliphatic solvents and alcohols. General stress conditions such as pH, temperature, NaCl, or the presence of organic acids did not induce *srp* transcription. Although the solvent efflux pump in *P. putida* S12 is a member of the resistance/nodulation/cell division family of transporters, the *srpABC* genes were not induced by antibiotics or heavy metals.

PUBLISHED IN THE JOURNAL OF BACTERIOLOGY (1998) 180: 6769–6772

INTRODUCTION

Several *Pseudomonas putida* strains possess an intrinsic resistance to a wide variety of structurally unrelated hydrophobic solvents [15, 49, 110, 145] that are lethal for most other Gram–negative bacteria. The susceptibility of bacteria to hydrophobic solvents is due to the accumulation of these compounds in the membrane [130, 131], causing an adverse effect on its physicochemical properties. Solvent–resistant bacteria are able to counterbalance these effects through a variety of mechanisms mostly affecting the lipid content of the cell membrane: isomerizing *cis*–unsaturated fatty acids to the more rigid *trans*–unsaturated fatty acids [34, 36, 39], changing the head group composition of membrane fatty acids [144], increasing the phospholipid content [110], or increasing the basal rate of phospholipid synthesis [109]. These adaptations of the membrane are static, acting as a physical but still permeable barrier, and cannot explain the exceptional resistance of some *P. putida* strains to organic solvents. Therefore, it was anticipated that a dynamic system for exporting a broad range of structurally unrelated organic solvents from the bacterial membrane had to play an essential role in solvent tolerance [144]. Such an efflux system was indeed identified in *P. putida* S12 by means of an assay based on radiolabeled toluene [55]. The genes (*srpABC*) for this solvent efflux system were subsequently cloned, sequenced, and shown to impart the solvent–resistant phenotype to solvent–sensitive *P. putida* strains [Chapter 3]. This efflux system shows strong homology to those of the resistance/nodulation/cell division family of transporters known to be involved in the extrusion of hydrophobic antibiotics, dyes, detergents, bile salts, heavy metals, and fatty acids from the membrane [95, 107].

Recently, several researchers also reported the involvement of active organic solvent efflux in solvent–resistant strains of *Pseudomonas* [27, 67, 78, 117]. Induction in *Pseudomonas* species of efflux systems involved in either multidrug resistance or solvent tolerance has not been studied in detail.

RESULTS

Construction of the lacZ *reporter plasmid pKRZ–srp*

Plasmid isolation, restriction analysis, ligations, electroporation, sequencing and PCRs were performed according to standard methods as described in Chapter 3. The region of DNA encompassing the putative promoter region of the *srp* operon was amplified by PCR from pJD101 [Chapter 3]. Primers for the PCR, were 5'–GGGTCGACGCTGCTCTGGCGATGACC–3' and 5'–GGTCTAGATCTGTCTCACGGTTTG·GC–3', which amplify a 312 bp fragment corresponding to the region immediately upstream of the *srpABC* genes (positions –285 to +8, where 0 is the G of the GTG codon of *srpA*). The primers contain added recognition sites for *Sal*I and *Xba*I, respectively. The PCR fragment was cloned into the *lacZ* reporter plasmid pKRZ–1

[122] cut with *Xba*I and *Sal*I. The resulting plasmid, containing the sequence shown in Figure 4.1, was designated pKRZ–srp.

```
      SalI
  1   GTCGACTCGACGCTGCTCTGGCGATGACCTGGATGCCCCCGGCCTTGCCAACATTTTCAT   60

 61   CTGATTGGTTCATATCTTTCCTCTGCGCGTACGGCTGCGACCTATCACATTGGCCGCCAC  120
          SstI
121   TGTAGAGCTCTCTGCCTCCGCTAGCCAGCCACACATCGCGAACGCCCCAATCGGTAAACC  180

181   TCGACCAACGGCGGCTCAGGCTTGCTTGCATCGAGAGTATCGCATAATGGTAGACTCTAC  240

241   CGCATTACGATTCAGCAATAGCCCCCGTCATGCGGGCAGAGAGATGGAGCCAAACCGTGA  300
            XbaI                                          srpA  M
301   GACAGATCTAGA                                                  312
      R  Q  I  *
```

FIGURE 4.1. Nucleotide sequence of the PCR–amplified *Sal*I–*Xba*I fragment containing the *srpABC* promoter region. The terminal *Sal*I and *Xba*I sites were added by incorporating their cutting sites into the PCR primers. The amino acid sequences of *srpA* are shown extending outward on the right side of the PCR product. This fragment was inserted into pKRZ–1 so that the promoterless *lacZ* gene is downstream of the start codon of *srpA*. An *asterisk* indicates the stop codon inserted into *srpA* by the added *Xba*I site.

Basal levels of srp promoter activity

P. putida S12 strains were grown to late log phase at 30°C in 200 ml of Luria–Bertani broth [126] supplemented with 50 μg of kanamycin per ml. Cells were harvested by centrifugation at 4°C (16,000 × *g*, 10 min) and washed twice with 100 mM potassium phosphate buffer (pH 7.0). The washed pellet was resuspended in 2 ml of the same buffer and lysed by sonication for 5 min. Cell debris was removed by centrifuging the crude cell extract at 4°C and 20,000 × *g* for 20 min. β–Galactosidase activity in the extracts was determined in triplicate by the method of Miller [86]. Total protein content in the extracts was determined in triplicate by the bicinchoninic acid method [133]. *P. putida* S12 containing either the promoter probe vector or pKRZ–srp was grown with no added inducers. ß–Galactosidase activity for the vector only was 0.9 ± 0.1 nmol min^{-1} mg^{-1} while the basal activity for the clone containing the promoter region was 3.8 ± 0.1 nmol min^{-1} mg^{-1}. Similar levels of basal activity were observed when the strains were grown on minimal medium D [32] supplemented with 20 mM glucose as sole carbon and energy source and 50 μg of kanamycin per ml (0.8 and 3.9 nmol min^{-1} mg^{-1}, respectively).

Activation of the srp promoter over time

To determine the time course for induction of the *srp* operon, ß–galactosidase activity was measured as a function of time following exposure of *P. putida* S12 containing either the promoter probe vector or pKRZ–srp. *P. putida* S12(pKRZ–srp) cells were grown in LB broth, and 3 mM toluene was added when the cells reached an optical density of 0.3 [Figure 4.2]. Induction of *srp–lacZ* expression was observed 30 min after the addition of toluene and gradually increased over time. The maximum level of induction of the *srp* operon was observed when the cells reached

stationary phase. This clearly shows that exposure to one organic solvent result in a significant increase in transcription of the *srp* operon.

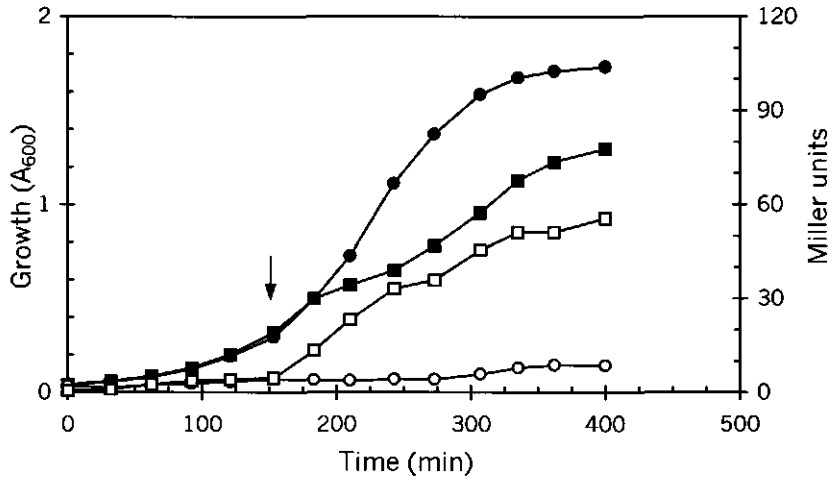

FIGURE 4.2. Activation of *P. putida* S12 *srp* promoter over time. The *arrow* indicates the addition of 3 mM toluene. Cell density of *P. putida* S12(pKRZ–srp) in LB broth (●, control; ■, induced cells) and β–galactosidase activity (O, control; □, induced cells) were monitored over time. β–Galactosidase activity in the time scale experiments was determined in triplicate cultures by the method of Miller [86] by using chloroform and SDS to permeabilize the cells.

Activation of the srp promoter by organic solvents

The influence of toluene concentration on gene induction was determined by varying the amounts of toluene added to the cells in the early exponential phase. ß–Galactosidase activity was measured in the late exponential phase of growth (optical density of 1.0 at 600 nm). As shown in Table 4.1, an increased amount of toluene added to the growth medium increases the level of *srp–lacZ* gene induction reaching a maximum at 6.0 mM. (The saturating limit of toluene in aqueous solutions at 30°C is 6.2 mM.) Induction by toluene thus results in a 15– to 17–fold increase in induction over basal levels.

Several hydrophobic organic solvents including aromatic compounds, aliphatic compounds, and aliphatic alcohols were tested for their ability to induce the *srp–lacZ* construct. As can be seen by the data presented in Table 4.1, all of the compounds tested are able to induce the *srp* genes. Certain aromatic compounds and aliphatic alcohols showed the highest levels of induction. The level of induction seems to correlate with increasing side chain length in the case of the alkyl–substituted aromatics and with chain length in the case of the aliphatic alcohols (up to a 15– to 17–fold induction).

Chemical	Concentration (mM)	β–Galactosidase activity (nmol min^{-1} mg of $protein^{-1}$)	Induction (fold)
None	–	3.8 ± 0.1	1.0
Aromatic solvents			
Toluene	1.0	13.3 ± 0.4	3.5
	2.0	36.5 ± 0.6	9.6
	3.0	48.6 ± 5.4	12.8
	4.0	57.0 ± 7.5	15.0
	5.0	59.1 ± 3.3	15.6
	6.0	64.3 ± 6.6	16.9
Benzene	3.0	36.6 ± 0.9	9.6
Styrene	3.0	32.2 ± 3.4	8.5
p–Xylene	3.0	36.0 ± 4.9	9.5
Ethylbenzene	3.0	47.3 ± 4.1	12.4
Propylbenzene	3.0	59.0 ± 2.0	15.5
Aliphatic solvents			
Pentane	1.0	30.1 ± 0.8	7.9
Hexane	1.0	25.2 ± 0.7	6.6
Heptane	1.0	34.2 ± 1.2	9.0
Octane	1.0	26.8 ± 0.1	7.1
Nonane	1.0	23.7 ± 1.0	6.2
Alcohols			
1–Propanol	3.0	22.2 ± 1.2	5.8
1–Butanol	3.0	25.1 ± 0.5	6.6
1–Pentanol	3.0	38.1 ± 1.2	10.0
1–Hexanol	3.0	60.3 ± 2.1	15.9
1–Heptanol	3.0	66.1 ± 0.2	17.4
1–Octanol	3.0	46.2 ± 1.2	12.2

TABLE 4.1. Induction of β–galactosidase expression in *P. putida* S12(pKRZ–srp) by solvents. *P. putida* S12(pKRZ–srp) cells were grown to late exponential phase (optical density of 1.0 at 600 nm) in LB broth. β–Galactosidase was determined in cell extracts by the method of Miller [86].

Antibiotics and heavy metals

In Chapter 3 we showed that the *srp* operon shows a high level of similarity to proton–dependent multidrug efflux systems, which are know to be involved in the efflux of a variety of antibiotics and heavy metals [95, 107].

Firstly, the ability of certain antibiotics to induce the *srp* genes was tested. In initial experiments, we determined the MIC of each antibiotic by twofold serial dilution in LB broth. The inoculum was 2% of an overnight culture and growth at 30°C was determined after 12 h by measuring the optical density at 600 nm. The level of ß–galactosidase activity was measured in the late exponential phase of cultures exposed to a level of each antibiotic, which resulted in approximately 50% decrease in growth rate. Growth of *P. putida* S12(pKRZ–srp) in the presence of 128 μg chloramphenicol per ml, 128 μg ampicillin per ml and 4 μg tetracycline per ml resulted in only a twofold increase in induction over basal levels. No increase in induction over basal levels was observed in the presence of the other antibiotics tested: 256 μg penicillin G per ml, 256 μg novobiocin per ml and 4 μg rifampin per ml.

Secondly, the ability of heavy metals to induce the *srp–lacZ* fusion was determined. Cells of *P. putida* S12(pKRZ–srp) were grown in LB broth in the presence of six different heavy metals (added as chloride salts in a final concentration of 1 mM). Zinc, chromium, cobalt, nickel, and copper had no detectable effect on the *srp* promoter, while cadmium resulted in only a 1.6–fold increase in induction.

Environmental factors

General stress conditions such as NaCl, ethanol, and stationary phase are known to induce the AcrAB efflux system in *Escherichia coli* [80]. On this basis, it was suggested that a general regulatory mechanism exist in *E. coli* to prevent hydrophobic compounds from entering the bacterial cell. Such a general response was also observed in *P. putida* S12 in the case of the induction of *cis–* to *trans–* isomerization of the membrane unsaturated fatty acids by environmental stress such as pH and heavy metals [37]. This change in fatty acid profile coincided with an increased resistance to organic solvents. To investigate whether the *srp* promoter activity was induced by these environmental factors, cells of *P. putida* S12(pKRZ–srp) were grown in LB broth under different conditions. Varying the growth temperature between 15 and 37°C and varying the pH between 6.0 and 8.0 did not result in a change in *srp* promoter induction. High levels of inorganic ions (50 g of NaCl per liter) did not affect *srp–lacZ* expression, although the growth of *P. putida* S12(pKRZ–srp) under the conditions tested was severely inhibited by high levels of NaCl.

Weber *et al.* [145] previously showed that incubating *P. putida* S12 with high acetic acid concentrations increased the survival of the strain after these cells were exposed to organic solvents. To determine if this was due to induction of the *srp* operon, the ability of acetate to induce this active efflux system for organic solvents was tested. *P. putida* S12(pKRZ–srp) was grown in LB broth in the presence of 20, 40, and 60 mM acetic acid (pH 6.5). Under each concentration of acetate, only a twofold induction of the *srp–lacZ* construct was observed. These results suggest that the enhanced survival of acetic–acid–adapted cells is not due to activation of the SrpABC efflux system.

DISCUSSION

The data in this Chapter clearly shows that lipophilic, aromatic, and aliphatic solvents and alcohols induce the *srpABC* operon. The data suggests that neither aromaticity nor charge is required for organic solvents to act as an inducer. Unlike other efflux systems, the *srpABC* operon is not induced by environmental stress or heavy metals, demonstrating that the genes are specifically induced and are not the result of a general regulatory mechanism as described elsewhere for *E. coli* [80].

The specific induction by solvents is underlined by the observation that hydrophobic antibiotics do not induce the SrpABC efflux system. *P. putida* S12 only becomes more resistant to hydrophobic antibiotics by culturing cells in the presence of toluene, while *P. putida* S12 grown in the presence of antibiotics does *not* elicit the solvent-tolerant phenotype [58]. These observations suggest that hydrophobic antibiotics are removed from the membrane in solvent induced cells, while these hydrophobic antibiotics do not induce SrpABC-mediated efflux of hydrophobic organic solvents.

Interestingly, Ramos *et al.* very recently reported the existence of at least two efflux pumps in the solvent-resistant *P. putida* DOT-T1E. One system apparently was expressed constitutively, while a second system was inducible [117].

Although the natural function of these resistance/nodulation/cell division-type of efflux systems has not been clarified yet, our data suggests that the SrpABC system plays a protective role in resistance to a wide variety of structurally unrelated hydrophobic compounds. Multidrug efflux systems in *P. aeruginosa* are not induced by known substrates of these efflux pumps [113], suggesting that the extrusion by these pumps of hydrophobic compounds is a nonspecific action. At present, the natural substrate(s) of these systems remains unknown. The SrpABC efflux system apparently does not have a natural function in excreting hydrophobic antibiotics because (i) solvent-sensitive mutants of *P. putida* S12 have normal levels of antibiotic resistance [Chapter 7] and (ii) the *srpABC* operon is induced solely by solvent stress [this Chapter].

5

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF AN EFFLUX SYSTEM INVOLVED IN *PSEUDOMONAS PUTIDA* S12 MULTIDRUG RESISTANCE

Jasper Kieboom and Jan A.M. de Bont

SUMMARY

In Chapter 3 we have described *srpABC*, an operon involved in proton–dependent solvent efflux in the solvent–tolerant *Pseudomonas putida* S12. In Chapter 4 we have shown that organic solvents and not antibiotics induce this operon. In this Chapter we have characterized a new efflux pump, designated ArpABC, on the basis of two isolated chloramphenicol–sensitive transposon mutants. The *arpABC* operon is involved in the active efflux of multiple antibiotics such as tetracycline, chloramphenicol, carbenicillin, streptomycin, erythromycin, and novobiocin. The deduced amino acid sequences encoded by the three genes involved show a striking resemblance with proteins of the resistance/nodulation/cell division family, which are involved in both organic solvent and multiple drug efflux. These findings demonstrate that ArpABC is highly homologous to the MepABC en TtgABC efflux systems for organic solvents and multiple antibiotics. However, ArpABC is not contributing to organic solvent tolerance in *P. putida* S12 but is solely involved in multidrug resistance.

PUBLISHED IN MICROBIOLOGY (2001) 147:43–51

INTRODUCTION

It is well known that several strains within the family *Pseudomonadacea* show significant intrinsic resistance to a wide variety of structurally unrelated compounds such as antimicrobial agents and organic solvents [for reviews see 56, 95, 107, and Chapter 2]. In the case of *Pseudomonas aeruginosa* it was thought that a very low nonspecific permeability is the main cause of resistance to antibiotics [94]. In the case of *Pseudomonas putida* several changes at the level of the membrane contribute to organic solvent tolerance [56, 144]. Nevertheless, the exceptional resistance of *Pseudomonadacea* cannot be explained solely by low permeability of the outer membrane and changes at the level of the membrane. Not surprisingly, efflux systems have been identified that contribute to the exceptional resistance of these strains. These efflux systems, which belong to the resistance/nodulation/cell division (RND) family of transporters, consist of three components: the inner membrane transporter protein (RND protein) that is attached via a membrane fusion protein (MFP) to an outer membrane protein (OMP) [124]. The OMP is thought to be an outer membrane channel by which to circumvent the outer membrane barrier, allowing the pumped molecule to be released into the medium [22].

In Chapter 3 it was shown that the energy–dependent efflux of organic solvents was the key factor in organic solvent tolerance via the organic solvent transporter SrpABC [64]. Similar efflux systems for the active removal of organic solvents have been found in other *P. putida* strains. These RND–type of efflux systems, encoded by *ttgABC* [117], *ttgDEF* [89] and *mepABC* [27] are involved in the active efflux of toxic compounds such as toluene, *p*–xylene and styrene. In the case of the multidrug–resistant *P. aeruginosa* four efflux systems have been described: MexAB–OprM [112], MexCD–OprJ [113], MexEF–OprM [68] and AmrAB [151]. These systems contribute to the energy–dependent efflux of a wide variety of antimicrobial agents such as β–lactams, tetracycline, fluoroquinolones and chloramphenicol. The interesting question now arises whether the RND–type transporters are able to export both antibiotics and solvents. Indications so far confirm that these pumps have dual pumping capacity. The *mex*–encoded efflux systems have recently been shown to be involved in the efflux of organic solvent in *P. aeruginosa* [78]. Moreover, solvent–sensitive mutants of *P. putida* DOT–T1E and *P. putida* KT2442 became more sensitive to antibiotics such as tetracycline, chloramphenicol and ampicillin, suggesting the active removal of multiple antibiotics by these efflux systems [27, 117].

In this Chapter we have studied this aspect at the molecular level and have found a new efflux system in *P. putida* S12 that is involved in the intrinsic resistance of this strain to a wide variety of structurally unrelated antibiotics. Sequence analysis showed that this efflux system is highly homologous to the solvents/antibiotics

transporters MepABC and TtgABC. Moreover, we demonstrate that ArpABC in *P. putida* S12 is only involved in multidrug resistance and not in tolerance towards organic solvents.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth of the strains

P. putida S12 (ATCC 700801) [32], a wild-type strain capable of growth at saturated solvent concentrations [145], was the object of the present investigation. Mutant strains of *P. putida* S12 generated in this study and those generated previously are shown in Table 5.1. *E. coli* DH5α [158] was utilized as the host strain for all recombinant plasmids. The cloning vectors used and the plasmids generated in this study are shown in Table 5.1. Luria–Bertani (LB) broth [126] was used as complete medium. For electroporation cells were grown in 10 g/l trypthone and 5 g/l yeast-extract. Solid media contained 2% agar. Ampicillin (100 μg/ml), kanamycin (50 μg/ml), gentamicin (10 μg/ml), and tetracycline (50 μg/ml) were added to the medium to maintain recombinant plasmids in *E. coli* and *P. putida*. The *E. coli* strains were routinely cultured at 37°C and *P. putida* strains were grown at 30°C.

Strain/plasmid	Relevant characteristics	Source or reference
Strains		
P. putida S12	Ap^r Cm^r *srpABC*$^+$ *arpABC*$^+$	32
P. putida JK1	Ap^r Cm^r *srpB*∇Tn*Mod*–KmO	64
P. putida CM1	Ap^r Cm^s *arpB*∇Tn*Mod*–KmO	This study
P. putida CM2	Ap^r Cm^s *arpC*∇Tn*Mod*–KmO	This study
P. putida DM1	Ap^r Cm^s *srpB*∇Tn*Mod*–KmO *arpA*::*tetA–oriR*::*arpB*	This study
E. coli DH5α	recA1	158
Plasmids		
pTn*Mod*–KmO	Km^r, pMB1 replicon	18
pUC18	Ap^r, cloning vector	Life Technologies
pUC19	Ap^r, cloning vector	Life Technologies
pBBR1MCS	Cm^r, cloning vector, REP replicon	70
pUCP22	Ap^r Gm^r, cloning vector, pRO1600 replicon	150
pKRZ–1	Km^r, promoter probe cloning vector	122
pACYC184	Tc^r, cloning vector	Calgene
pJD105	pUCP22 containing *srpABC*	64
pCM1P	Km^r *arpBC* from chromosomal DNA of *P. putida* CM1	This study
pCM2P	Km^r *arpBC* from chromosomal DNA of *P. putida* CM2	This study
pCM1B	Km^r *arpABC* from chromosomal DNA of *P. putida* CM1	This study
pCMPE	pUC18 containing *arpRA*	This study
pCMC1	pUCP22 containing *arpAB*	This study
pCMC2	pUCP22 containing *arpABC*	This study
pKRZ-arp	pKRZ–1 containing P_{arp} *lacZ* fusion	This study
pTO1	Tc^r Cm^s pBBR1MCS containing *tetA–oriR*	This study
pSC1	Tc^r *arpA*::tetA–oriR::*arpB*, pMB1 replicon	This study

TABLE 5.1 Strains and plasmids used in this study. Ap, Cm, Km, Gm and Tc represent resistance (r) or sensitivity (s) to ampicillin, chloramphenicol, kanamycin, gentamicin and tetracycline, respectively.

*Generation and screening of Tn*Mod–*KmO insertion mutants*

The plasposon Tn*Mod*–KmO was introduced into *P. putida* S12 by electroporation [19]. Kanamycin–resistant transposon mutants were tested for the ability to grow on LB agar plates supplemented with 100 μg/ml chloramphenicol and 50 μg/ml kanamycin. Mutants were incubated at 30°C for 24 h. Mutants unable to grow in the presence of 100 μg/ml chloramphenicol were selected.

DNA techniques

Total genomic DNA from *P. putida* strains was prepared by the CTAB procedure [11]. Plasmid DNA was isolated by the alkaline–SDS lysis method of Birnboim and Doly [12]. DNA was digested with restriction enzymes and ligated with T4 ligase as recommended by the supplier (Life Technologies). DNA restriction fragment and PCR products were visualized by 0.8% agarose gel electrophoresis in 45 mM Tris/borate, 1 mM EDTA buffer. DNA from agarose gels was isolated using the method of Vogelstein and Gillespie [142]. Southern analyses of chromosomal DNA and colony hybridizations were carried out according to Sambrook *et al.* [126]. Probes were labeled with DIG–dUTP using the PCR DIG Probe Synthesis Kit (Boeringher Mannheim) with the appropriate primers. Southern blot hybridizations were carried out by chemiluminescent detection under high–stringency conditions as described by the supplier (Boeringher Mannheim). Plasmid DNA was introduced into either *E. coli* DH5α or *P. putida* cells by electroporation [19] using a Gene Pulser (Bio–Rad).

DNA sequences of both strands of the *arpABC* operon were determined by a combination of subcloning and primer walking. All sequencing and PCR reactions were performed using a Gene Amp PCR System 9700 (Perkin–Elmer). Nucleotide sequencing reactions were performed with purified double–strand plasmid DNA using AmpliTaq FS DNA polymerase fluorescent dye terminator reactions (Perkin–Elmer) as recommended by the supplier. Sequencing products were detected using an Applied Biosystems 373A stretch automated DNA sequencer. Nucleotide sequence analysis was performed either with the Lasergene analysis package (DNASTAR) or with the National Center for Biotechnology Information BLAST server [1].

Construction of pCMC2 for complementation

For the complementation experiments we reconstructed the *arpABC* operon since the clones obtained from *P. putida* CM1 and CM2 contain the transposon mutagenized DNA. Therefore, a 2.2 kb *Bam*HI–*Pst*I fragment from pCM1B was cloned into the broad host range vector pUCP22, the resulting plasmid was designated pCMC1. A 2.3 kb *Pst*I–*Eco*RI fragment from pCM2P and a 2.0 *Eco*RI–*Pst*I fragment from pCM1P were ligated into *Pst*I–digested and alkaline dephosphatase treated pCMC1. The resulting plasmid pCMC2, containing the *arpABC* genes in the

same orientation as the *lac* promoter and was transferred to the mutant strains CM1 and CM2.

Construction of suicide plasmid pSC1

We amplified a 1289 base pair fragment containing the tetracycline resistance gene *tetA* from pACYC184. The primer 5'–CGGAATTCTCATGTTTGACAGCT–3' and 5'–GCGGTACCTCAGGTCGAGGTGG–3' contain added recognition sites for *Eco*RI and *Kpn*I, respectively. The reaction mixture (50 µl) was treated for 10 min at 94°C followed by 35 cycles of 1 min at 94°C, 2 min at 55°C and 1 min at 72°C before finishing for 10 min at 72°C. This 1.3 kb *Eco*RI and *Kpn*I fragment and a 0.7 kb *Kpn*I–*Sst*I fragment from TnMod–KmO, containing the ColE1 origin of replication, were ligated in *Eco*RI and *Sst*I–digested pBBR1MCS, resulting in pTO1. The suicide plasmid pSC1 was constructed by ligating a 2.0 kb *Eco*RI–*Sst*I fragment from pTO1, a 3.0 kb *Pst*I–*Sst*I fragment from pCM1P and a 3.0 kb *Pst*I–*Eco*RI fragment from pCMPE.

Construction of pKRZ–arp and determination of β–galactosidase activity

PCR reactions for amplifying the region of genomic *P. putida* S12 DNA containing the *arp* promoter were performed using Super *Taq* DNA polymerase (SphearoQ). The reaction mixture (50 µl) was treated for 10 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C before finishing for 10 min at 72°C. Primers for this reaction were 5'–CCGCTCGAGTACAACCTCATCTGGCCC–3' and 5'–CGCTCTAGAATTGCATGAGGATCCTCG–3', which amplify a 276 base pair fragment corresponding to the region immediately upstream of the *arpABC* genes. The primers contain added recognition sites for *Xho*I and *Xba*I, respectively. β–Galactosidase activity in *P. putida* S12(pKRZ–arp) was determined by growing the cells to late–log phase at 30 °C in 10 ml LB broth supplemented with inducer. The β–galactosidase activity in these experiments was determined in triplicate by the method of Miller [86] using chloroform and SDS to permeabilize the cells.

Determination of solvent tolerance and the minimal inhibitory concentration of antibiotics

Solvent tolerance of the *P. putida* strains was determined in duplicate by growing the cells in 10 ml liquid LB medium in 100 ml flasks supplemented with 1 mM magnesium chloride. Toluene and *p*–xylene (1% final concentration) were separately added to identical subcultures in LB/$MgCl_2$ medium during the early exponential growth phase. The maximal aqueous benzene concentration was determined by adding increasing amounts of benzene. Growth of the cultures was measured 24 h after solvent addition with no continued growth indicating solvent sensitivity. The MIC for various antibiotics was determined by threefold serial dilution in LB broth in microtitre plates. The inoculum was 1% of an overnight culture and growth was determined by measuring the optical density at 600 nm after 36 h at 30°C.

RESULTS

Isolation of chloramphenicol–sensitive mutants

P. putida S12 is able to grow in the presence of a wide variety of normally toxic solvents and is relatively resistant to various antibiotics. Chloramphenicol–sensitive mutants were obtained by electroporating the transposon Tn*Mod*–KmO in *P. putida* S12. Over 4000 kanamycin–resistant transposon mutants were constructed with an overall transformation efficiency for Tn*Mod*–KmO of $1{,}5\times10^3$ per μg DNA. These mutants were tested for growth on solid LB medium supplemented with 100 μg/ml chloramphenicol. Two chloramphenicol–sensitive mutants were isolated and were designated strain CM1 and CM2, respectively.

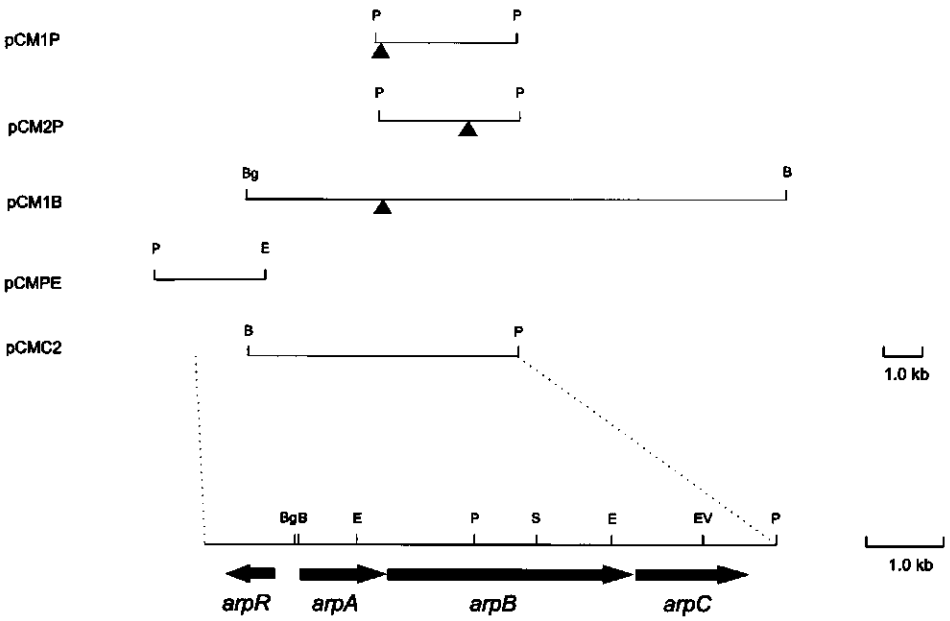

FIGURE 5.1. Restriction maps of the clones derived from the transposon mutants CM1 and CM2. A diagram of the *arp* nucleotide sequence is shown at the bottom, in proportion to the restriction maps and showing the positions of the genes relative to the restriction enzyme cutting sites. The triangles in the restriction maps indicate the location of the inserted plasposon Tn*Mod*–KmO in the cloned genomic DNA.

Cloning and analysis of the genes for chloramphenicol resistance

To characterize the genes for chloramphenicol resistance in *P. putida* S12, the regions of the genome containing the transposon insertion in mutants CM1 and CM2 (both 5.8 kb) were cloned. From both mutants a clone was obtained from *Pst*I–digested chromosomal DNA; the clones were designated pCM1P and pCM2P, respectively. Genomic DNA cut with *Bam*HI from strain CM1 was used to construct the 14.0 kb plasmid pCM1B. Plasmid pCM1B was shown to contain a complete operon, including the inserted Tn*Mod*–KmO [Figure 5.1]. The nucleotide sequence of the operon was determined and screening for similar nucleotide sequences in the

GenBank database revealed a significant match with genes encoding for multidrug and solvent export pumps. We labeled the genes *arp* for antibiotic resistance pump. A diagram of the nucleotide sequence obtained is presented in Figure 5.1 to show the relationship of the open reading frames with the three clones pCM1P, pCM2P and pCM1B. The deduced nucleotide sequence of *arpA*, *arpB* and *arpC* encode for proteins of 371, 1050 and 484 amino acid residues with a calculated molecular mass of 40.3, 112.8 and 52.8 kDa, respectively. Putative ribosome–binding sites precede the *arpABC* genes and a stable stem–loop structure was found downstream *arpC* and that may function as transcriptional terminator [Figure 5.2].

Construction of an arpABC mutant of P. putida JK1

The presence of the solvent efflux pump SrpABC in *P. putida* CM1 and CM2 could mask the possible efflux of organic solvent by ArpABC. Therefore, to determine the role of ArpABC in solvent tolerance we decided to inactivate the *arpABC* gene cluster by site–specific reciprocal recombination in the solvent–sensitive transposon mutant *P. putida* JK1, creating a *srp–arp* double mutant. We electroporated *P. putida* JK1 with the suicide plasmid pSC1 [Table 5.1] replacing part of *arpA* and *arpB* with the ColE1 origin of replication and tetracycline resistance gene, resulting in the *srp–arp* double mutant *P. putida* DM1. Recombination was confirmed by hybridization of *P. putida* DM1 chromosomal DNA with a probe for the *tetA* gene (data not shown).

Antibiotic	MIC in μg/ml for strain:				
	S12	CM1	CM2	CM1 (pCMC2)	CM2 (pCMC2)
Tetracycline	16	1	2	16	16
Chloramphenicol	64	16	16	128	128
Ampicillin	256	64	128	ND	ND
Carbenicillin	1024	4	4	ND	ND
Streptomycin	32	8	8	64	64
Erythromycin	128	8	4	128	256
Rifampicin	64	32	32	32	32
Polymyxim B	16	8	8	8	8
Nalidixic Acid	256	128	128	256	256
Novobiocin	1024	64	128	1024	1024
Penicillin G	>4094	2048	4094	ND	ND

TABLE 5.2 Determination of the MIC for various antibiotics for the *P. putida* strains. The MIC for antibiotics was determined in duplicate by threefold serial dilution in LB broth in microtitre plates. The inoculum was 1% of an overnight culture and growth was determined by measuring the optical density at 600 nm after 36 h at 30°C. ND; not determined.

Complementation of P. putida mutants

Complementation experiments were performed to prove that the Tn*Mod*–KmO inserted open reading frames detected in *P. putida* CM1 and CM2 are actually responsible for chloramphenicol resistance. These complemented strains regained chloramphenicol resistance similar to the wild–type levels [Table 5.2]. These results are consistent with the chloramphenicol–resistant phenotype being dependent on expression of the *arpABC* genes. We tested a variety of structurally unrelated antibiotics, which are known substrates for homologous efflux systems of the RND

family of transporters [95, 107]. Mutants CM1 and CM2 were not only sensitive to chloramphenicol but also to a number of other antibiotics [Table 5.2], demonstrating that a single genetic trait in *P. putida* S12 is responsible for resistance to the antibiotics tested.

Solvent resistance of the P. putida strains.

P. putida S12 is resistant to organic solvents mainly through the presence of a solvent efflux system [55, 64]. Therefore, it was determined whether the ArpABC efflux pump was involved in solvent resistance in *P. putida* S12. The antibiotic–sensitive mutant strains CM1 and CM2 were as resistant as the wild–type strain to benzene, toluene and *p*–xylene, whereas the *srp–arp* double mutant DM1 was as sensitive to organic solvents as the solvent–sensitive JK1 strain [Table 5.3]. These results indicate that the solvent resistance phenotype of *P. putida* S12 is not dependent on expression of the *arpABC* genes.

Solvent	Strain				
	S12	CM1	CM2	JK1	DM1
Benzene	10 mM	10 mM	10 mM	6 mM	6 mM
Toluene	+	+	+	–	–
p–Xylene	+	+	+	–	–

TABLE 5.3 Solvent tolerance of the *P. putida* strains. Solvent tolerance of the *P. putida* strains was determined in LB broth supplemented with 1 mM magnesium chloride. A *plus* indicates growth with 1% organic solvent after 24 h at 30°C.

Cloning and induction of the arpABC promoter

To clone and sequence the promoter region and possible regulatory genes of the *arp* operon, we examined the region upstream the *Bam*HI site of pCM1B. Therefore, we isolated approximately 3.0 kb *P. putida* S12 chromosomal DNA fragments digested with *Pst*I and *Eco*RI. These fragments were cloned into pUC18 cut with *Pst*I and *Eco*RI. We were able to isolate the plasmid from one colony out of 1200 by colony hybridization with a 0.7 kb *Bam*HI–*Eco*RI fragment from pCM1B. This plasmid, pCMPE, contained the complete promoter region and an ORF (*arpR*) that is transcribed in the opposite direction to that of *arpABC* [Figure 5.1]. The *arpR* gene product (ArpR) is most similar to MepR (98.6% amino acid identity), which is a putative regulator of the MepABC organic solvent transporter [27]. The deduced nucleotide sequence of *arpR* encodes for a protein of 210 amino acid residues with a calculated molecular mass of 23.8 kDa. Sequence analysis of ArpR revealed a TetR family signature motif. A putative ribosome–binding site precede the *arpR* gene and a stable stem–loop structure was found downstream *arpR* and may function as transcriptional terminator [Figure 5.2]. Moreover, a putative σ^{70}–dependent promoter consensus motif was found upstream the start op *arpA*.

The *P. putida* S12 solvent transporter is solely induced by organic solvent [63]. In this light it is interesting to examine the induction of the multidrug transporter ArpABC. Therefore, the putative promoter region of the *arpABC* operon was

amplified by PCR and cloned in the *lacZ* reporter plasmid pKRZ–1 [122] cut with XbaI and *Sal*I. The resulting plasmid, designated pKRZ–arp, was electroporated to *P. putida* S12. *P. putida* S12(pKRZ–arp) was grown in LB broth in the absence and presence of the antibiotics chloramphenicol, carbenicillin and erythromycin and the solvents toluene, p–xylene and hexane. ß–Galactosidase activity was subsequently assayed in late exponential phase (OD of 1.0 at 600 nm). No significant induction was observed by any of these compounds.

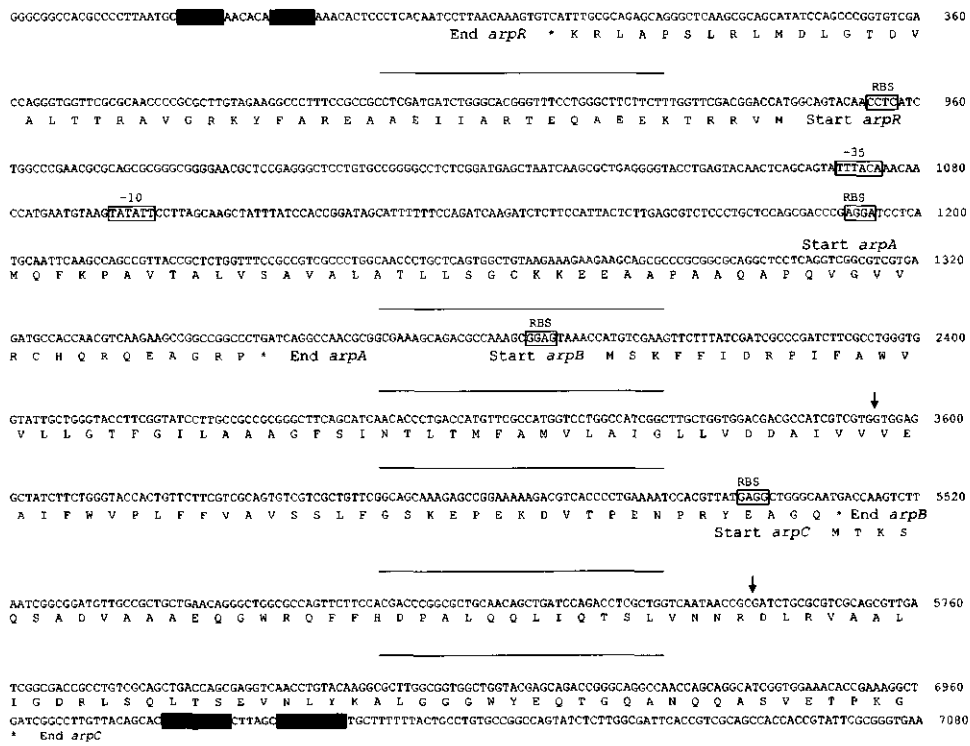

FIGURE 5.2. Nucleotide sequence of the relevant portions of a 7313 bp fragment containing the *arp* genes. The deduced amino acid sequences of the encoded proteins are shown beneath the nucleotide sequence. Termination codons are indicated with an *asterisk* and putative ribosome binding sites and a putative σ^{70}–dependent promoter motif that resembles the *E. coli* consensus motif 5`–TTGACA–$N_{(15-19)}$–TATAAT–3` have been marked with *boxes*. Inverted repeat sequences are indicated in *black boxes*. An *arrow* indicates the position of Tn*Mod*–KmO in *P. putida* CM1 and *P. putida* CM2 (position 3594 and 5738 of the *arpABC* sequence, respectively). The GenBank accession number for the *arp* sequence is AF183959.

DISCUSSION

In this Chapter we describe the isolation and characterization of chloramphenicol–sensitive mutants from the solvent–tolerant and multidrug–resistant bacterium *P. putida* S12. Two chloramphenicol–sensitive mutants were isolated and sequence analysis showed that both mutants were disrupted in the same operon. Multidrug

efflux systems play an important role in the intrinsic resistance of members of the family of *Pseudomonadacea* to a variety of antibiotics [see Table 5.4 for homology between ArpABC and other representatives of the RND–family of transporters]. At present four antibiotic efflux systems have been described in *P. aeruginosa*: MexAB–OprM [112], MexCD–OprJ [113], MexEF–OprM [68] and AmrAB [151]. These systems are involved in the proton–dependent efflux of antimicrobial agents such as β–lactams, tetracyclines, fluoroquinolones and chloramphenicol. In *P. putida* TtgABC [117], MepABC [27] and the ArpABC efflux system described in this Chapter are involved in tolerance towards antibiotics such as tetracycline, chloramphenicol, carbenicillin, streptomycin, erythromycin and novobiocin. Our findings demonstrate that the ArpABC efflux system is a key mechanism in multidrug resistance in *P. putida* S12.

Organisms	Efflux system proteins							
	MFP	Identity	RND	Identity	OMP	Identity	RP	Identity
P. putida	ArpA	100	ArpB	100	ArpC	100	ArpR	100
	TtgA	99.7	TtgB	99.9	TtgC	99.6	–	–
	MepA	99.2	MepB	99.9	MepC	99.8	MepR	98.6
	TtgD	58.0	TtgE	62.2	TtgF	56.6	–	–
	SrpA	57.1	SrpB	63.4	SrpC	57.3	SrpR	30.9
							SrpS	27.3
P. aeruginosa	MexA	67.6	MexB	78.1	OprM	69.4	MexR	37.5
	AmrA	37.4	AmrB	48.7	–	–	AmrR	32.2
	MexC	43.1	MexD	48.4	OprJ	41.6	–	–
	MexE	29.6	MexF	41.4	OprN	28.4	MexT	22.0
E. coli	AcrA	53.8	AcrB	65.4	TolC	19.3	AcrR	37.6
	AcrE	50.6	AcrF	64.4	–	–	EnvR	30.3
N. gonorrhoeae	MtrC	43.3	MtrD	46.6	MtrE	39.7	MtrR	33.0

TABLE 5.4 Amino acid sequence identity (in %) between ArpABC and multidrug and solvent efflux proteins. MFP, membrane fusion protein; RND, efflux protein; OMP, outer membrane protein; RP, regulatory protein. Identity (matches over matches, mismatches and gaps) was calculated with the Lipman–Pearson algorithm using the deduced amino acid sequences of ArpA, ArpB, ArpC, and ArpR (GenBank accession no. AF183959); TtgA, TtgB and TtgC (AF031417); MepA, MepB, MepC, and MepR (AB008909); TtgD, TtgE and TtgF (PPY19106); SrpA, SrpB and SrpC (AF029405): SrpR and SrpS (AF061937); MexA and MexB (L11616); OprM (L23839); MexR (U23763); AmrA, AmrB and AmrR (AF147719); MexC, MexD, and OprJ (U57969); MexE, MexF and OprN (X99514); MexT (AJ007825); AcrA and AcrB (U00734); TolC (X00016); AcrR (U00734); AcrE and AcrF (AE000405); EnvR (X57648); MtrC (U14993); MtrD (U60099); MtrC (X95635) and MtrR (Z25797).

Proton–dependent efflux systems also play an important role in organic solvent tolerance in *Pseudomonas putida* strains [27, 64, 117]. The first RND–type efflux system for toluene was isolated by Kieboom *et al.* in 1998 and was shown to be responsible for organic solvent tolerance in *P. putida* S12 [64]. The involvement of efflux systems in solvent tolerance was confirmed with the isolation of the *ttgABC*, *ttgDEF* genes from *P. putida* DOT–T1E [89, 117] and the *mepABC* genes from *P. putida* KT2442 [27]. Moreover, Kim *et al.* reported that a transposon insertion in a protein of the RND–family resulted in a *P. putida* mutant with a solvent–sensitive phenotype [67]. Surprisingly, other studies suggested that the isolated solvent transporter in *P. putida* was also involved in the active efflux of multiple antibiotics. A toluene–sensitive *mepB*∇Tn5 mutant of *P. putida* KT2442 was also sensitive to

ampicillin, penicillin G, erythromycin, novobiocin, and tetracycline [27]; and a toluene-sensitive *ttgB*∇mini-Tn*5*'*phoA*-Km^r mutant of *P. putida* DOT-T1E was sensitive to chloramphenicol, ampicillin and tetracycline [117]. With the construction of a *srp-arp* double mutant we were able to demonstrate that the ArpABC efflux system was not involved in organic solvent tolerance in *P. putida* S12.

An interesting but yet not completely clear picture is now emerging from the studies on efflux pumps from pseudomonads with regard to both their substrate recognition and their induction patterns. The inherent problem in studying these aspects is that responses to doses of antibiotics or organic solvents must be monitored at the whole cell level. Both wild-type cells and mutants may or may not contain additional, and often unknown, pumps. Moreover, the rate of influx of compounds will depend on their chemical structure, while the cell may alter the composition of its cell envelope, thus further obscuring the explanation of results. Nevertheless, on the basis of this type of experimentation it is now possible to distinguish three types of proton-dependent efflux systems in pseudomonads on the basis of compounds expelled from the cell. To date it seems two efflux pumps have been described that are involved solely in the efflux of solvents and that appear not to be able to export antibiotics. This type of efflux pump includes SrpABC in *P. putida* S12 and TtgDEF in *P. putida* DOT-T1E and was shown to be induced by organic solvents [64, 89]. Most of the efflux systems characterized in pseudomonads, however, export both antibiotics and solvents. These systems include the constitutive TtgABC pump in *P. putida* DOT-T1E [117], the MepABC pump in *P. putida* KT2442 [27], and the Mex efflux systems in *P. aeruginosa* [78]. The third type is the ArpABC system reported here, which is involved in antibiotic resistance but not in solvent tolerance as shown by the phenotype of the *srp-arp* double mutant. However, a note of caution in identifying ArpABC as an antibiotic and not as a solvent-removing system is appropriate. A solvent pump has to operate at a higher speed than an antibiotic pump due to considerable higher influx of hydrophobic solvents compared to the influx of antibiotics. Toluene may be present at 5 mM in the aqueous phase, while antibiotics usually remain below 1 mM and it is to be expected that antibiotics will diffuse more slowly into the cell than uncharged, small solvent molecules do. Consequently, solvent efflux pumps may have to generate an efflux that is probably 10–100 times higher than the efflux created by the antibiotic pumps. If the expression level is not affected by the presence of solvent as substrate, as is the case for the ArpABC system, then the solvent-pumping ability may not be registered at the whole cell level. We have tried to overcome this pitfall by using benzene as test solvent and by employing it at different concentration. This method should be relatively suitable to detect minor contributions to solvent pumping, but nevertheless, no effect of the presence of ArpABC on benzene sensitivity was observed.

The grouping of the known *Pseudomonas* efflux pumps according to their pumping activity for either solvent and/or antibiotics is not supported by the molecular structure of these pumps. The ArpB, TtgB and MepB proteins are almost identical at the amino acid level, which is quite surprising because they have been identified in strains isolated from 3 very different locations. Also their A and C components are almost identical. ArpB differs only in one amino acid from TtgB (Ala–544 to Gly–544), while compared to MepB it differs 3 amino acids (Ala–533 to Thr–533, Ala–544 to Gly 544, and Glu–692 to Ala–692). On the basis of the MexB membrane topology [29] it is expected that Ala–544 is located in a putative transmembrane segment, whereas Ala–544 and Glu–692 are periplasmatic. These observations would lead to the conclusion that Gly–544 might be a key amino acid in interactions with antibiotics. However, till date the substrate–binding domain of RND–type of proteins has not been identified.

Alternatively it might be argued that MepABC and TtgABC are present at higher levels in their respective hosts. For *P. putida* KT2442 it was anticipated that the *mep* operon was overexpressed in the toluene–tolerant variant TOL. Possibly a similar adaptation phenomenon has taken place already during the isolation of strain *P. putida* DOT–T1E. Therefore, in both strains, a high transcription level of the efflux operons may result in a high solvent efflux, required for the cell to survive in the presence of toluene. Similar results were reported for the *mexAB–oprM* efflux system in *P. aeruginosa*. In this strain a single substitution in the *mexR* regulator resulted in the overexpression of MexAB–OprM resulting in an increased multidrug resistance and organic solvent tolerance [75, 78, 113].

In summary, the intrinsic resistance of *P. putida* S12 to multiple antibiotics is due to the efflux of these components by ArpABC. Whether, ArpABC in *P. putida* S12 is unable to interact with solvent due to an amino acid substitution or due to the lack of overexpression, the extrusion of organic solvents by this efflux system is too low to prevent their influx but sufficiently high to overcome the antibiotic influx. *P. putida* S12 does not have to rely on ArpABC–mediated solvent efflux for its solvent–tolerant phenotype, since it possesses the SrpABC efflux system that is induced under solvent stress.

6

TRANSPOSON MUTATIONS IN THE FLAGELLA BIOSYNTHETIC PATHWAY OF THE SOLVENT–TOLERANT *PSEUDOMONAS PUTIDA* S12 RESULT IN A DECREASED EXPRESSION OF SOLVENT EFFLUX GENES

Jasper Kieboom, Riekje Bruinenberg, Ineke Keizer–Gunnink and Jan A.M. de Bont

SUMMARY

Fourteen solvent–sensitive transposon mutants were generated from the solvent–tolerant *Pseudomonas putida* S12 by applying the Tn*Mod*–KmO mutagenesis system. These mutants were unable to grow in the presence of octanol and toluene. By cloning the region flanking the transposon insertion point a partial sequence of the interrupted genes was determined. Comparison of the deduced amino acid sequences with a protein database revealed the following interrupted putative gene products: organic solvent efflux proteins SrpA and SrpB, the flagellar structural proteins FlgK, FlaG, FliI, FliC, and FliH, the transcriptional activator FleQ, the alternative RNA polymerase sigma factor RpoN, and the flagellum specific RNA polymerase sigma factor FliA (RpoF). The transposon mutants, except for the organic solvent efflux mutants, were nonmotile as determined by a swarm assay and the formation of the flagellum was totally impaired. Expression studies with a *srp* promoter probe showed a decreased expression of the SrpABC efflux pump in the nonmotile mutants.

PUBLISHED IN FEMS MICROBIOLOGY LETTERS (2001) 198:117–122

INTRODUCTION

In the last decade several solvent–tolerant *Pseudomonas putida* strains [15, 49, 110, 116, 145] have been isolated that are able to grow in two–phase water organic solvent systems that are lethal for normal bacteria. Solvent–tolerant strains are able to counterbalance the toxic effects of organic solvents, at the level of the bacterial cell membrane, through a variety of mechanisms. The head group formation of the membrane phospholipids may be changed [144], the *cis*–unsaturated fatty acid may be isomerized to the more rigid *trans* configuration [39], the phospholipids content or the basal rate of phospholipid biosynthesis may be increased [109, 110]. At the level of the outer membrane, changes in the lipopolysaccharide (LPS) content [3], LPS composition [109], and cell hydrophobicity have been observed [144].

These modifications do not fully explain the high organic solvent tolerance levels of several *P. putida* strains. Therefore, it was anticipated that a dynamic efflux system for organic solvents had to play a role in solvent tolerance. Such a mechanism was indeed identified in *P. putida* S12 [55] and the genes (*srpABC*) for the active efflux of organic solvents were subsequently cloned and sequenced [64]. This energy–dependent export system is involved in the resistance to a wide variety of organic solvents in *P. putida* S12. The SrpABC efflux system was induced by organic solvents [63] and not by other environmental stress factors. Recently, several efflux pumps have been described in other strains and underline the importance of organic solvent efflux in solvent tolerant pseudomonads [27, 67, 78, 117].

This study was carried out to identify alternative mechanisms involved in *P. putida* S12 solvent tolerance by isolating solvent–sensitive mutants. The solvent–sensitive mutants were physiologically characterized and the interrupted genes were partially sequenced. Moreover, we determined the effect of these transposon insertions on the expression of the organic solvent transporter SrpABC and discuss the relationship between the interrupted gene products and organic solvent efflux.

MATERIALS AND METHODS

Bacterial strain, plasmids and growth of strains

P. putida S12 [33] is the wild–type organic solvent–tolerant strain capable of growth at saturated solvent concentrations [145]. The *P. putida* mutants were derived in the present work by transposon mutagenesis. *E. coli* DH5α [158] was utilized as the host strain for all recombinant plasmids. The transposon Tn*Mod*–KmO [18] was used for the construction of *P. putida* S12 mutants. The promoter probe pKRZ–srp [63] and the cloning vector pJWB1 [148] were used to construct the promoter probe pLF1. Luria–Bertani (LB) broth [126] was used as complete medium. Solid media contained 2% agar. Kanamycin (50 μg/ml) and gentamicin (10 μg/ml) was

added to the medium to select for Tn*Mod*–KmO integrands and to maintain recombinant plasmids in *E. coli*. *P. putida* strains were routinely cultured at 30°C and *E. coli* strains were grown at 37°C.

Transposon mutagenesis

The transposon Tn*Mod*–KmO was introduced into *P. putida* S12 by electroporation [19] using a Gene Pulser (Bio–Rad). Kanamycin–resistant transposon mutants were tested for the ability to grow in the presence of 10% (v/v) octanol. This was accomplished by inoculating 200 µl LB broth in 96–wells microtitre plates supplemented with 10% (v/v) octanol and subsequently placing them in a sealed glass desicator. Growth of the cultures was determined after 12 h at 30°C. Mutants unable to grow in the presence of 10% (v/v) octanol were selected and designated O1 through O14.

Cloning and partial sequencing of chromosomal DNA

Isolation of total genomic DNA from *P. putida*, plasmid DNA isolation from *E. coli*, restriction endonuclease analysis, ligations, and sequencing were preformed as described previously [64]. To elucidate the possible functions of the genes interrupted in the isolated mutants, we determined the nucleotide sequence flanking the transposon insertion using the primers 5'–TTCCCGTTGAATATGGC–3' and 5'–ACGCTCAGTGGAACG–3'. Therefore, chromosomal DNA was isolated, digested with the restriction enzyme *Pst*I or *Sst*I, ligated at room temperature and electroporated to *E. coli* DH5α. Plasmids from kanamycin–resistant colonies were isolated and sequenced. The obtained nucleotide sequence was translated and the amino acid sequences, which had been interrupted by Tn*Mod*–KmO, were compared to known protein sequences in the PRF database using the GenomeNet BLAST server [1].

Physiological characterization of transposon mutants

Solvent sensitivity of the *P. putida* strains was determined in LB/acetate medium. *P. putida* cells were grown in 10 ml LB medium containing 60 mM sodium acetate, until the cells reached the early exponential growth phase. The growing cells were 10 times diluted with fresh LB/acetate medium. Organic solvents were added to 10 ml inoculated media in a final concentration of 2% (v/v). Growth was determined overnight with mild agitation at 30°C. The experiments were conducted twice with duplicate samples each time.

Motility and electron microscopy

The swarming ability of the *P. putida* strains was tested using semisolid agar plates containing LB medium and 0.3% agar. Motility was scored overnight at 30°C. For light microscopy *P. putida* cells were grown in LB broth for 4 h at 30°C to mid–exponential phase. For electron microscopy, motile and nonmotile *P. putida* strains were negatively stained using 0.5% uranyl acetate. A droplet of the culture was

applied on a formvar–coated grid. In ca. 20 s the fluid was removed and the grids were washed with a droplet of distilled water, air dried and examined in a Philips CM10 transmission electron microscope operating at 80 kV.

Induction studies

To determine the *srp* promoter activity in the kanamycin–resistant transposon mutants we constructed a gentamicin–resistant promoter probe. PCR reactions for amplifying a fragment (containing the promoter region of *srp* already fused to the promoterless *lacZ* gene) were preformed using the promoter probe pKRZ–srp as template [63]. PCR reactions were performed as described previously [63] using the primers 5'–CGGATCCTCGACGCTGCTCTGGCGATGA–3' and 5'–CGCGGCCGCTT-ATTTTTGACACCAGACCAAC–3', which contained added restriction sites for *Bam*HI and *NotI*, respectively. This 3.3 kb PCR fragment was digested with *Bam*HI and *Not*I and ligated into pJWB1 creating the promoter probe pLF1.

To determine the induction of the *srp* promoter *P. putida* cells harboring the promoter probe pLF1 were grown in LB broth at 30°C. 3 mM toluene was supplemented when the cells reached an optical density of 0.3 at 600 nm. At an optical density of 1.4 at 600 nm β–galactosidase activity was determined in triplicate using the method of Miller with chloroform and sodium dodecyl sulphate to permeabilize the cells [86].

RESULTS

Isolation of octanol–sensitive transposon mutants

Octanol–sensitive mutants of *P. putida* S12 were generated using Tn*Mod*–KmO, allowing the rapid cloning of the genomic region adjacent to the Tn*Mod*–KmO insertion point. From a screening of 4000 kanamycin–resistant colonies 14 octanol–sensitive mutants, designated O1 through O14, were isolated. Southern hybridization of *Pst*I–digested genomic DNA of these mutants with a probe for the kanamycin resistance cassette in Tn*Mod*–KmO revealed single hybridization bands suggesting single transposon insertion (data not shown).

Sequence homologies

In all octanol–sensitive mutants the region adjacent to the transposon insertion was cloned and sequenced (Table 6.1). The deduced amino acid sequences from the inactivated genes of mutant O6, O8 and O14 revealed that the transposon was integrated in the energy–dependent efflux mechanism SrpABC. This operon is responsible for the active efflux of organic solvents and was shown to play a role in organic solvent tolerance in *P. putida* S12 [64].

The interrupted genes in mutant O1, O2, O7, O9 and O12 showed homology with structural proteins of flagella. The deduced amino acid sequences showed high

sequence homology with structural proteins such as FlgK, the flagellar hook–associated protein (HAP1) [43], FlaG and FliC flagellin [134, 153], FliI, a putative flagellum–specific ATPase subunit of a protein translocase for flagellum–specific export, and FliH, a flagellar assembly protein [143].

Strain	Inactivated gene	Number of deduced amino acids	Identity	Homology (%)	Organism	Reference
O1	*flgK*	104	40	64	*S. typhimurium*	43
O2	*flaG*	166	94	96	*P. putida*	153
O3	*fleQ*	150	70	84	*P. aeruginosa*	8
O4	*rpoN*	158	98	99	*P. putida*	52
O5	*fliA*	173	97	98	*P. putida*	24
O6	*srpA*	163	100	100	*P. putida*	64
O7	*fliI*	156	78	87	*S. typhimurium*	143
O8	*srpB*	148	100	100	*P. putida*	64
O9	*fliC*	122	24	51	*P. aeruginosa*	134
O10	*fleQ*	158	84	89	*P. aeruginosa*	8
O11	*fleQ*	140	97	97	*P. aeruginosa*	143
O12	*fliH*	74	20	54	*S. typhimurium*	8
O13	–‡	–	–	–	–	–
O14	*srpB*	162	100	100	*P. putida*	64

TABLE 6.1. Sequence homology the Tn*Mod*–KmO inactivated genes in the solvent–sensitive *P. putida* strains. The PRF database accession numbers are as follows; FlgK, 1613338B; FlaG, 2106279B; FleQ, 2320269A; RpoN, 1604231A; FliA, 2410250D; SrpA, 2408344A; FliC, 2301229A; FliI, 1713317B; FliH, 1713317A; and SrpB, 2408344B. ‡ No homology was found.

The inactivated genes in mutant O3, O4, O5, O10 and O11 revealed three inactivated regulators. The deduced amino acid sequence revealed strong homology with the transcriptional activator FleQ [8]. The other regulators found were the RNA polymerase σ^{54} factor RpoN [52] and the flagellum specific RNA polymerase factor σ^{F} FliA (RpoF) [24]. The putative function of the inactivated gene in mutant O13 could not be determined since its deduced amino acid and nucleotide sequences did not show any significant homology with protein or gene sequences in databases.

	P. putida strain														
Solvent	S12	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	O12	O13	O14
Decane	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hexane	+	+	+	+	+	+	–	+	–	+	+	+	+	+	–
Cyclohexane	+	+	+	+	+	+	–	+	–	+	+	+	+	+	–
p–xylene	+	+	+	+	+	+	–	+	–	+	+	+	+	+	–
Octanol	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Toluene	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fluorobenzene	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Benzene	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

TABLE 6.2. Growth of *P. putida* strains on LB/acetate in the presence of various solvents. Different solvents (2% final concentration) were separately added to identical subcultures in LB medium with 60 mM acetate during the early exponential growth phase. Growth of the culture was measured at various times after solvent addition with no continued growth indicating solvent sensitivity. A *plus* indicates growth with OD > 0.7, while a *minus* indicates no growth with OD < 0.1 after 24 h.

Physiological properties of the solvent–sensitive mutants

General growth characteristics, susceptibility to organic solvents, and motility of all solvent–sensitive strains were investigated. The growth curves of the mutants in the absence of organic solvents were similar to the wild–type *P. putida* S12 strain (data not shown). This indicated that organic solvent sensitivity was not due to altered growth characteristics. The sensitivity to various organic solvents was determined by adding 2% (v/v) organic solvent to growing cells in the early exponential growth phase. Growth was determined by measuring the optical density after overnight incubations at 30°C. As indicated in Table 6.2 the transposon mutants were not able to grow in the presence of 2% octanol and neither in the presence of 2% toluene. In addition, mutant O6, O8, and O14 were sensitive to *p*–xylene, cyclohexane, and hexane, which is similar to the previously described phenotype of the *srpB* mutant *P. putida* JK1 [64].

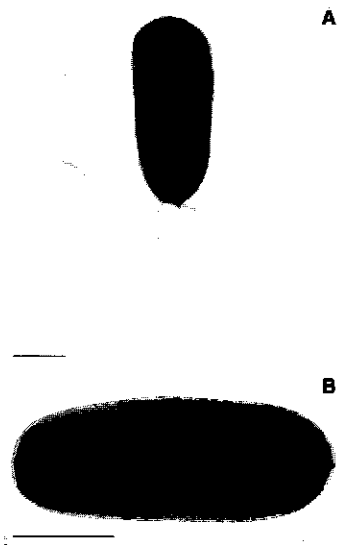

FIGURE 6.1. Electron micrographs of representative cells of the (A) wild–type *P. putida* S12 and the (B) nonmotile mutant *P. putida* O3. Bars, 1 μm.

As a result of homologies that were found with the proteins involved with bacterial flagella we determined whether the mutant strains were motile. The motility was tested by a swarm test using semisolid agar plates. Mutant O6, O8, and O14, which were lacking the energy–dependent export system for organic solvent, were motile. All other organic solvent–sensitive mutant strains were nonmotile, including the unidentified mutant O13 (data not shown). These results were confirmed with electron microscopy. While the *P. putida* S12 and mutant O6, O8, and O14 were extremely motile, as determined by phase contrast microscopy, the other mutants were nonmotile. The electron microscopy experiments showed that *P. putida* S12 contains 4 to 6 polar flagella, whereas a similar examination of several mutants

failed to detect any flagella [Figure 6.1]. Thus, the lack of motility in the mutants was due to their inability to form flagella.

Induction of the solvent efflux transporter SrpABC

Because the solvent transporter SrpABC is the main determinant for solvent tolerance in *P. putida* S12 [64], efforts were made to determine the expression of the solvent transporter in the nonmotile solvent–sensitive mutants. We constructed the promoter probe pLF1 to measure expression of the efflux pump. As shown in Figure 6.2 the *srp* promoter activity decreased in all nonmotile mutants when 3 mM toluene was used as inducer. From these results we can conclude that mutations in the flagella biosynthetic pathway in *P. putida* S12 result in decreased expression of the *srpABC* genes.

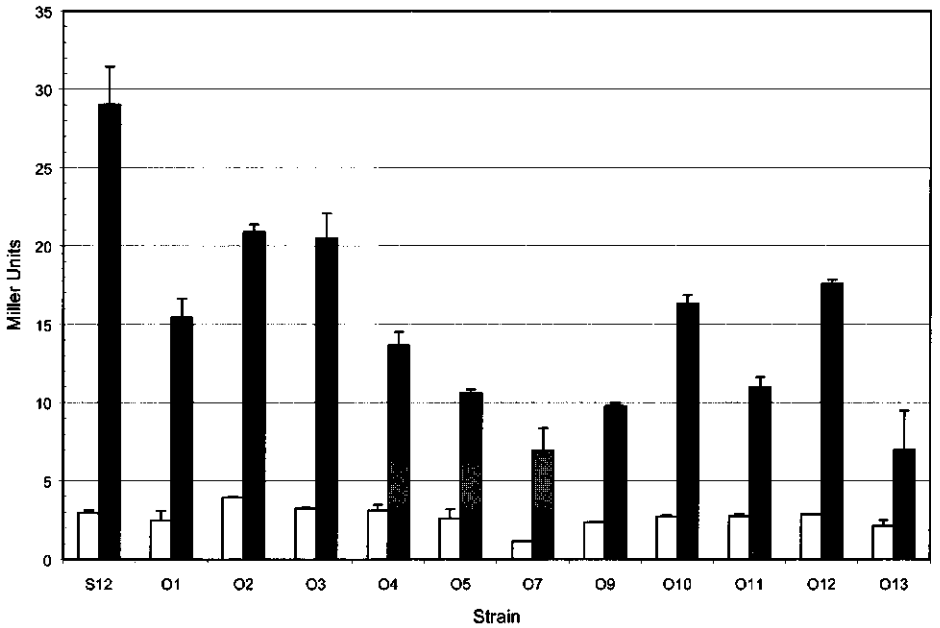

FIGURE 6.2. Induction of *P. putida* S12 *srp* promoter. *P. putida* strains containing the promoter probe pLF1 were grown in LB broth. At an optical cell density of 1.4 at 600 nm, 3 mM toluene was added. β–galactosidase activity in uninduced (white bars) and toluene–induced (gray bars) cells was determined in triplicate cultures at an optical density of 1.4 at 600 nm.

DISCUSSION

Here we report the isolation of solvent–sensitive mutants from *P. putida* S12 by transposon mutagenesis. Sequence analysis showed that three out of fourteen mutants were disrupted in the previously described efflux mechanism SrpABC for organic solvents [64]. Five mutants were interrupted in the structural proteins of the bacterial flagella, while the others were interrupted in the regulatory proteins RpoN, FliA (RpoF) and FleQ. As determined by a swarming assay these 11 mutants

were nonmotile and were totally impaired to form flagella (Fig. 6.1). These results are intriguing since the role of the flagella biosynthetic pathway in solvent tolerance is unexpected. Other researchers have recently isolated organic solvent–sensitive mutants. Eight solvent–sensitive mutants were isolated from the toluene–tolerant *P. putida* GM7 [67]. It appeared that, in addition to proton–dependent efflux of organic solvents, an ABC–transporter, and enzymes possibly involved in repair mechanisms of damaged membranes, were disrupted in these toluene–sensitive transposon mutants. Moreover, two toluene–sensitive mutants from *P. putida* IH–2000 were interrupted in cytochrome o, although the exact role of the *cyo* gene cluster in solvent tolerance has not been determined [40]. Surprisingly, toluene–sensitive mutants of *P. putida* DOT–T1E were isolated that were also interrupted in an operon for the flagella biosynthetic pathway [123].

In mutant O4 the RNA polymerase σ^{54} factor RpoN was interrupted. In *P. putida* RpoN is required for the expression of a broad range of genes involved in the formation of pili, the utilization of nitrate, amino acids as nitrogen source, C_4–carboxylates as carbon source and the degradation of toluene [53, 71, 138]. However, the *srp* promoter seems to be σ^{54}–independent for no RpoN consensus sequence could be found in the *srp* promoter [63]. Therefore, other regulatory pathways have to play a role in the downregulation of the *srp* locus in the nonmotile mutants. We speculate that the strictly regulated cascade of the flagella biosynthetic pathway play a role herein.

In *Salmonella typhimurium* the flagella biosynthetic pathway is organized into a transcriptional hierarchy of classes [83]. Each later class of operons is only transcribed when previous classes are completely functional. The expression of class 3 operons is mediated by the *fliA* gene product. In the solvent–sensitive mutants the formation of flagella was totally impaired, as demonstrated by electron microscopy. Therefore, it seems that the expression of class 3 operons is not only blocked in the FliA transposon mutant O5, but also in the other mutants. This regulatory mechanism has been shown to be controlled by anti–σ factors, which bind to the to the flagella–specific σ factor FliA (RpoF) [44]. From our results one could speculate that this regulatory mechanism also affects the expression of the *srp* operon, resulting in solvent–sensitivity. In this light it is interesting to note that FlhF, a protein of the flagella biosynthetic pathway in *P. putida*, was found to be involved in general stress response [106].

The regulatory mechanisms of the *srp* operon have not been clarified yet. In contrast to a repressor upstream the organic solvent transporter locus found in other solvent tolerant strains, two putative regulators were sequenced upstream *srpA* (SrpR and SrpS, GenBank Accession Number AF061937), although their role remains unclear [147]. It seems that with the isolation of the flagella biosynthetic insertion mutants and the unexpected downregulation of the *srp* locus in these

solvent–sensitive mutants, another level of transcriptional regulation has to be accounted for in unraveling the molecular basis of organic solvent efflux and its regulation.

7

CONCLUDING REMARKS

Organic solvents are generally extremely toxic to living microorganisms. Nevertheless, in the past decade several microorganisms have been isolated that are tolerant to organic solvents. One such exceptional organic solvent–tolerant bacterium is *P. putida* S12. Solvent tolerance and antibiotic resistance in this bacterium was the object of investigation reported in this thesis. The results presented in this thesis are discussed here along with recent progress in the field of solvent tolerance. Moreover, some concluding remarks are made in view of future investigations in solvent tolerance and the possible applications of these bacteria in biotechnological production processes.

SOLVENT TOLERANCE MECHANISMS

Organic solvents target the cell envelope of Gram–negative bacteria, in which they preferentially partition. The accumulation of solvents in the cell envelope and the concomitant destruction of membrane fluidity, resulting in a deterioration of membrane functions, is the main reason for toxicity of organic solvents [see Chapter 2]. However, organic solvent–tolerant bacteria, like *P. putida* S12, are able to induce a variety of defense mechanisms to withstand the toxic effects of organic solvents [see Chapter 2].

The initial response to organic solvents in a bacterium is a stress response related mechanism. Fast changes in the membrane allow the bacterium to survive the initial shock of organic solvents. For instance the *cis* into *trans*–isomerization of membrane fatty acids is such a fast response mechanism [35, 146]. These rapid changes alone are not sufficient to resist the long–term toxic effects of organic solvents. Therefore, longer–lasting mechanism have to be induced to overcome the toxic levels of organic solvents. These long–lasting mechanisms, mainly at the level of the cytoplasmic and outer–membrane, can be divided into two groups. Firstly, mechanisms that strengthen the bacterial membrane, such as changes in the phospholipid headgroups [109, 115, 144], changes in the degree of saturation of membrane lipids [110, 146], and the presence of membrane embedded proteins [119, 120]. Secondly, mechanisms that retard the rapid influx of organic solvents, such as changes in the content of lipopolysaccharides or its composition [3, 110].

However, these mechanisms alone cannot explain the exceptional resistance of some solvent–tolerant strains. An equilibrium concentration will eventually be reached in the membrane that is lethal for the bacterium, despite the strengthened membrane and the decreased influx of organic solvents. Therefore, a long–lasting mechanism has to be present in solvent–tolerant strains that actively removes the deleterious organic solvents from the membrane.

ACTIVE EFFLUX OF ORGANIC SOLVENTS

In 1996 strong indications were obtained that organic solvent was actively removed from the membrane of the solvent–tolerant *P. putida* S12 [55]. These results were confirmed in 1997 in another solvent–tolerant *P. putida* strain [115]. These findings supported the notion that efflux of organic solvents may be a key mechanism in solvent tolerance. However, it should be noted that only the interplay between efflux and a number of mechanisms that strengthen the membrane and retard the influx of organic solvents would result in solvent tolerance.

With the assumption that efflux is the key mechanism in solvent tolerance we anticipated that we were able to isolate solvent–sensitive transposon mutants of *P. putida* S12. Indeed, in early 1998 we were able to isolate a solvent–sensitive mutant of *P. putida* S12. As described in Chapter 3 we isolated the *srpABC* genes from this mutant that encoded for an efflux pump able to actively remove organic solvents from the membrane. With this report we demonstrated that organic solvent efflux was a key mechanism in solvent tolerance. Since this initial observation several other research groups isolated organic solvent efflux systems in solvent–tolerant strains. The Ttg efflux systems from *P. putida* DOT–T1E [89, 117, 121], and the MepABC efflux system from *P. putida* KT2442 [27] all confirmed that active removal of organic solvents was the key mechanism in solvent tolerance. Interestingly, the efflux mechanisms for organic solvent were consequently linked to efflux of multiple antibiotics. The SrpABC efflux mechanism could not be linked to multidrug resistance since the SrpB lacking mutant was still resistance to various antibiotics. Therefore, we anticipated a second efflux mechanism in *P. putida* S12. Indeed, we were able to isolate the multidrug transporter ArpABC [see Chapter 5]. Although almost identical to the solvent transporter MepABC from *P. putida* KT2442 [27], ArpABC was not contributing to solvent tolerant phenotype of *P. putida* S12.

MULTIDRUG RESISTANCE VERSUS SOLVENT–TOLERANCE

The efflux systems for organic solvents were all very homologous to efflux systems of the resistance/nodulation/cell division family (RND). RND–family transporters are involved in the transport of antibiotics, heavy metals, and oligosaccharides [95,

107]. Several interesting questions arise in view of the homology between solvent transporters and multidrug transporters. Is the SrpABC efflux system involved in antibiotic resistance? Is the antibiotic efflux system ArpABC involved in solvent tolerance?

Is SrpABC involved in multidrug resistance?
It had already been observed that solvent tolerance and antibiotic resistance were closely correlated in the solvent-tolerant *P. putida* S12. In 1997 it was demonstrated that solvent-adapted cells were more resistant to multiple antibiotics, but cells grown in the presence of antibiotics were not tolerant to organic solvents [58]. These results can be explained in at least three different ways. First, the increased antibiotic resistance can be the result of active efflux by the solvent transporter. Second, the solvents can be able to induce an antibiotic efflux system. Third, the increased antibiotic resistance can be the result of other mechanisms induced by organic solvents preventing the influx of the toxin.

The solvent transporter SrpABC seems not to be involved in multiple antibiotic resistance despite its homology to multidrug transporters. It was observed that a solvent-sensitive mutant of *P. putida* S12, which was inactivated in the SrpABC efflux system, was as resistant as the wild-type to antibiotics such as tetracycline, chloramphenicol, and β-lactams. These results indicated that other mechanisms have to be involved in multidrug resistance in *P. putida* S12. Indeed, as described in Chapter 5 an efflux system (ArpABC) for antibiotics is present in *P. putida* S12. This efflux system is involved in the efflux of tetracycline, chloramphenicol, novobiocin, erythromycin, streptomycin, and β-lactams. However, organic solvents were not able to induce the *arp* locus. Moreover, we demonstrated that the ArpABC efflux system was not involved in solvent tolerance in *P. putida* S12. Therefore, the enhanced antibiotic resistance of solvent-adapted *P. putida* S12 had to be due to a decreased influx of these chemicals. Isken has recently demonstrated that the permeability of the cell envelope for several growth substrates decreased due to the presence of organic solvent [54]. This decreased permeability of the cell envelope due to organic solvents might be the main reason for the increased multidrug resistance of solvent-adapted *P. putida* S12.

Are antibiotic efflux systems involved in solvent tolerance?
The ArpABC efflux system for antibiotics in *P. putida* S12 is involved in multidrug resistance. However, despite its high homology to the solvent/antibiotic transporters TtgABC and MepABC, ArpABC was not involved in efflux of organic solvents [Chapter 5]. In the multidrug-resistant *P. aeruginosa*, 4 efflux pumps have been described that are involved antibiotic efflux: MexAB-OprM [112], MexCD-OprJ [113], MexEF-OprM [68] and AmrAB [151]. Surprisingly, the *mex* encoded multidrug efflux systems of this pathogen were able to transport organic solvent

[78]. It has not yet been determined if the AmrAB efflux system in *P. aeruginosa* is able to extrude organic solvent from the membrane.

REGULATION AND INDUCTION OF EFFLUX

In *P. putida* S12 the expression of the solvent transporter SrpABC is induced by aromatic and aliphatic solvents and alcohols, whereas general stress conditions such as pH, temperature, NaCl and the presence of organic acids do not induce *srp* transcription [Chapter 4]. Upstream the *srpABC* operon two putative regulators were found. The putative regulator SrpR was most homologous to AcrR, the operon repressor of the AcrAB in *E. coli* [82]. The putative regulator SrpS was homologous to IclR, the glyoxylate bypass operon repressor in *E. coli* [137]. As demonstrated by Wery *et al.* interruption of *srpS* by IS*S12* upregulates expression of SrpABC [147]. In *P. putida* DOT–T1E a homologous transporter, TtgGHI, was found. The organic solvent substrate range of this transporter was similar to SrpABC but in contrast, TtgGHI was able to transport antibiotics [121]. TtgGHI was expressed from a single constitutive promoter in the absence of toluene, while cells exposed to toluene expressed TtgGHI from two promoters, a constitutive one and an inducible one [121].

The ArpABC efflux pump is identical to TtgABC and MepABC [see Chapter 5]. The TtgABC system is constitutively expressed and is involved in tolerance to toluene, styrene, *m*–xylene, ethylbenzene and propylbenzene [121, 117]. Like MepABC and ArpABC, TtgABC is involved in resistance towards tetracycline, chloramphenicol, and ampicillin. In contrast to its homologue in *P. putida* S12, TtgABC and MepABC are able to transport organic solvents. The regulator of the *ttgABC* operon, TtgR, is identical to MepR and ArpR and was recently shown to repress *ttgABC* transcription in the presence of toluene [25]. As described in Chapter 5, induction of the *arp* locus in *P. putida* S12 could not be assessed.

TtgDEF efflux system in *P. putida* DOT–T1E was only involved in toluene and styrene tolerance [89, 121]. This efflux system was induced by organic solvents and the transcription start of the TtgDEF operon in *P. putida* DOT–T1E was determined [89]. Moreover, a regulatory protein belonging to the IclR family of transcriptional regulators, TtgT, was shown to be involved in regulation of TtgDEF expression in *P. putida* DOT–T1E [25].

It goes without saying that more detailed experiments are needed to fully understand the complex regulation of RND–efflux systems in *P. putida*. As demonstrated in Figure 7.1 the complete picture of regulation of efflux in *P. putida* is not yet complete. Especially the pathways of solvent or antibiotics stress signals need to be determined. The downregulation of the SrpABC system due to insertions

in the genes for flagella biosynthesis [see Chapter 6] and the finding that a global regulatory protein TtgX in *P. putida* DOT–T1E was involved in *ttgDEF* and *ttgABC* transcription [25], already indicated that a complex picture of regulation pathways will emerge in the future.

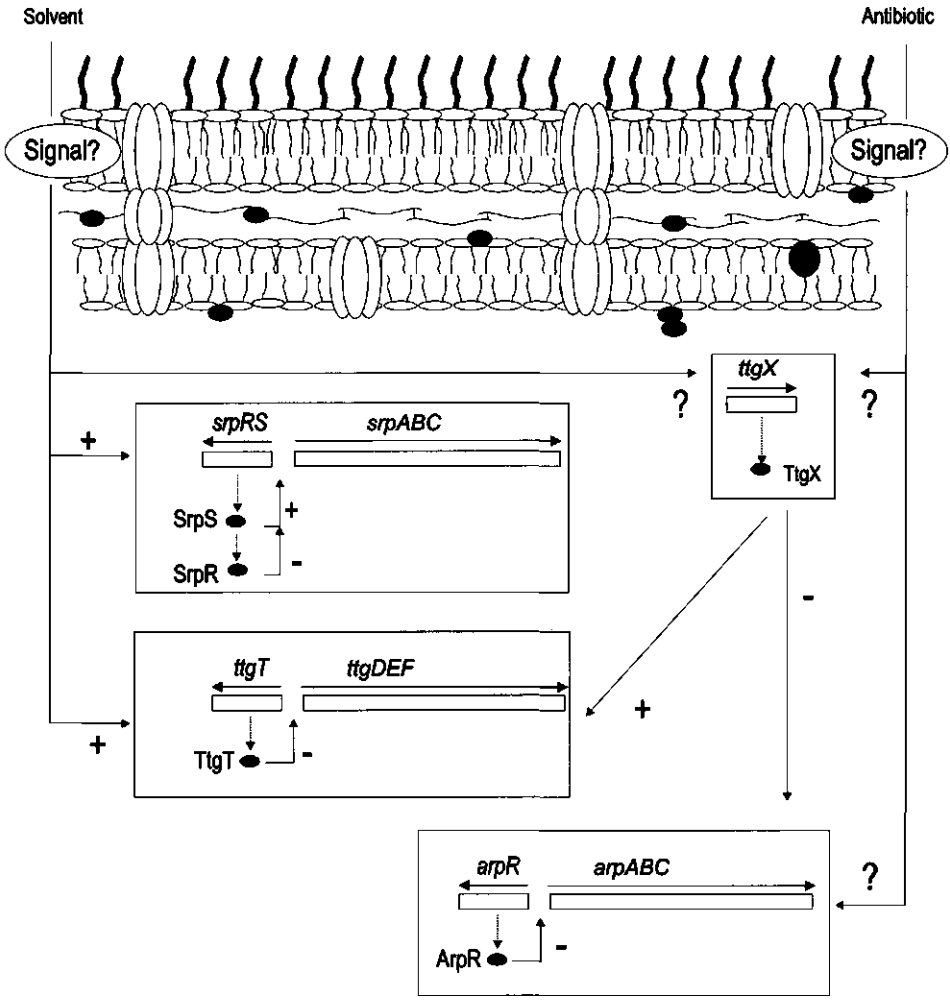

FIGURE 7.1. Overview of regulation of organic solvent and antibiotic transporters in solvent tolerant *P. putida*. A *plus* indicates positive control and a *minus* indicates negative control.

SCREENING FOR ORGANIC SOLVENT TOLERANCE MECHANISMS

The physiological approach

Active efflux might not be the only longer-lasting response to organic solvents. Researchers until now only focussed on the screening for toluene-sensitive mutants using transposon mutagenesis. This approach implies that the knocked out gene(s)

have to be crucial for the survival of the wild-type strain in the presence of organic solvents. In my opinion additional efflux systems might be present in *P. putida* S12 but the isolation of these systems, based on transposon mutagenesis and subsequent physiological characterization, could be hindered by the activity of efflux mechanisms. Therefore, already solvent-sensitive mutants should be used to screen for efflux systems. For instance the absence of SrpABC, which is a key mechanism in solvent tolerance, would allow the isolation of other mechanisms (non-efflux mechanisms) that are involved in strengthening the membrane or in retardation of organic solvent influx.

An important factor in the isolation of solvent-sensitive mutants is the medium composition. The solvent-sensitive phenotype of the transposon mutant *P. putida* JK1 was shown to be strongly dependent on the medium used [see Table 7.2]. It was demonstrated that this strain became more sensitive to organic solvents due to the presence of high concentrations of sodium acetate. Conversely, such a high concentration of sodium acetate was thought to induce the organic solvent-tolerant phenotype of *P. putida* S12, allowing this strain to survive the sudden shock of organic solvent [145]. Moreover, the use of rich medium increased the solvent-tolerant phenotype of *P. putida* JK1. This could be due to the presence of magnesium or calcium ions, which were thought to have a stabilizing effect on the cell membrane [51, 116, 144].

Solvent	Medium composition			
	MM 60 mM acetate	MM, 0.01% YE 60 mM acetate	LB 60 mM acetate	LB 5 mM acetate
Hexadecane	+	+	+	+
Decane	+	+	+	+
p-Cymene	–	+	+	+
Propylbenzene	–	–	+	+
Hexane	–	–	–	+
Cyclohexane	–	–	–	+
Ethylbenzene	–	–	–	+
p-Xylene	–	–	–	–
Octanol	–	–	–	+
Toluene	–	–	–	–
Dimethylphtalate	–	–	–	+
Fluorobenzene	–	–	–	–
Benzene	–	–	–	–

TABLE 7.1. Growth of the solvent-sensitive *P. putida* JK1 on different media in the presence of various solvents. MM; mineral medium, LB; Luria-Bertani broth, YE; yeast extract.

From the results presented in Table 7.1 one could too easily conclude that only *p*-xylene and toluene are substrates for SrpABC. However, at the whole cell level medium composition determines the tolerant or sensitive phenotype. Substrate specificity can only be examined using radiolabeled organic solvents as described by Isken and de Bont in 1996 [55].

In conclusion, selection of optimal medium composition and the appropriate organic solvent could prove the most important factor in finding other genes involved in solvent tolerance. As demonstrated in Chapter 6, regulatory genes have been found by isolating solvent-sensitive mutants using a rich medium and a less toxic organic solvent.

THE GENOMICS APPROACH

In the past decade microbiologist have moved from describing a single physiological phenomena to describing a whole genome. The completion of microbial genomes is continuing with 43 bacterial genomes completed and an additional 130 bacterial genome sequencing projects in progress worldwide (www.tigr.org). With the sequencing of bacterial genomes a new science called bioinformatics emerged. Bioinformatics is composed of many different, yet interrelated scientific fields such as functional genomics, proteomics, and transcriptomics. Genomic studies uncovered many genome variations in closely related bacteria and revealed basic principles involved in bacterial diversification, improving our knowledge of the evolution of bacteria. Proteomic studies deals with proteins separated by two-dimensional gel electrophoresis. Selected protein spots can be identified by peptide-mass fingerprinting using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Transcriptomics or transcriptional profiling uses DNA arrays to identify the level specific mRNA species at the genomic scale. From these emerging scientific fields it is clear that genomes are an continuing source of information.

To understand the tolerance mechanisms in *Pseudomonadacea*, bacterial genomes are an excellent source and future research in this field will definitely benefit from the exploitation of these genome sequences. To date genome sequencing projects have been started for 5 Pseudomonads [Table 7.2]. Of the 5 projects the genome of *P. aeruginosa* PAO1 has been published [136], the genome of *P. fluorescence* Pf0-1 is being annotated, and the other genome sequencing projects are still in progress.

Strain	Nucleotides sequenced (in Kb)	Number of Open reading frames found in sequenced genome
P. aeruginosa PAO-1	6264	5565
P. fluorescence Pf0-1	6528	5800
P. putida KT2240	1033	812
P. syringae DC3000	455	503
P. stutzeri	274	213

TABLE 7.2. Genome sequencing projects of Pseudomonads.

To demonstrate the use of genome sequences in discovering new genes we used the *P. aeruginosa* PAO1 genome sequence as an example to look for RND-type transporters. As described in Chapter 2 four RND-type of efflux mechanism are

know in *P. aeruginosa* involved in multiple drug and organic solvent efflux. Using the SrpB amino acid sequence we were able to find an additional 8 RND-type of transporters based of amino acid identity. One of these efflux mechanisms was very similar to the heavy metal transporter CzcABC from *Alcaligenes eutrophus* [93]. Seven of the RND-type of efflux mechanisms had no known function. Even in the not yet finished genome of *P. fluorescence* Pf0-1 we were able to find 10 proteins homologous to SrpABC [data not shown].

Transporter (type of protein and size in amino acids)						Identity of RND protein to SrpB in %	Substrates transported
MFP		RND		OMP			
SrpA	382	SrpB	1049	SrpC	470	100.0	organic solvents
MexA	383	MexB	1046	OprM	385	64.4	drugs, organic solvents
MexC	387	MexD	1043	OprJ	387	47.3	drugs, organic solvents
AmrA	396	AmrB	1045	–	–	46.7	drugs
MexE	414	MexF	1062	OprN	472	37.7	drugs, organic solvents
PA3523	353	PA3522	1053	PA3521	491	35.5	ND
PA4206	370	PA4207	1029	PA4208	487	29.5	ND
PA4374	376	PA4375	1018	–	–	28.0	ND
PA2528	426	PA2527	1043	PA2525	498	25.8	ND
		PA2526	1036			25.9	
PA1435	385	PA1436	1036	–	–	24.3	ND
CzcB	484	CzcA	1051	CzcC	428	21.1	divalent metal cation
PA0156	383					20.9	ND
PA0157	356	PA0158	1015	–	–		
PA3677	367	PA3676	1025	–	–	19.9	ND

TABLE 7.3. Comparison of SrpABC and transporter homologues found in the *P. aeruginosa* PAO1 genome sequence. Abbreviations: MFP, membrane fusion protein; RND, inner membrane (transporter) protein; OMP, outer membrane protein; ND, not determined.

SOLVENT TOLERANCE IN BIOTECHNOLOGY

The application of organic solvent-tolerant bacteria in biotechnology is an area with considerable opportunities. The uncovering of solvent tolerance mechanisms and the continuing expansion of isolated solvent-tolerant strains will greatly enhance the number of applications of solvent tolerance in biotechnology. Several possible applications of these interesting solvent-tolerant strains will be discussed.

Solvent-tolerant bacteria as cell factories

Organic solvent-tolerant bacteria can be used as cell factories for whole cell biotransformations. Solvent-tolerant production strains allow the use of two-liquid solvent-water systems. These systems have been studied in order to cope in production process with the accumulation of products that are toxic to non-tolerant bacteria [125]. The enhancement of production has been demonstrated in the formation of various mono- and diepoxides [127, 154]. Interestingly, recombinant *E. coli* strains have been used in two-liquid solvent-water systems reaching product concentrations up to 10 g/l [157]. However, the range of solvents that these non-tolerant microorganisms can withstand is limited in these systems. Therefore, the

application of a solvent–tolerant production strain introduces a new degree of freedom. It can tolerate organic solvents that are considerably more hydrophobic, therefore, increasing extraction efficiency of many important hydrophobic fine chemicals. This has recently been demonstrated for the production of the model substrate 3–methylcatechol in a genetically modified *P. putida* S12 strain using octanol as an extracting organic phase [45, 46, 149].

Solvent–stable enzymes

A second application of solvent–tolerant bacteria is the use of these strains as the source for solvent–stable enzymes. It has been demonstrated that an organic solvent–tolerant strain produced solvent–stable proteases and lipases [97, 98]. With the increasing diversity of solvent–tolerant strains such as *Pseudomonas*, *Flavobacterium*, *Rhodococcus*, *Alcaligenes* and even *Bacillus* [see Table 2.1], the spectrum of solvent–stable enzymes is concomitantly increasing.

Environmental biotechnology

Another application of solvent–tolerant microorganisms might be in the field of environmental biotechnology. High emission peaks of organic solvents in wastegas and wastewater treatment facilities are monitored very closely to prevent the toxic effects of the pollutants. Solvent–tolerant bacteria are better equipped to withstand these toxic chemicals. Therefore, bioremediation processes may become more stable by applying solvent–tolerant bacteria. Solvent–tolerant bacteria are able to degrade a variety of pollutants such as toluene, xylene, styrene and polychlorinated biphenyls [33, 99, 116]. Moreover, it has been demonstrated that the catabolic potential of solvent–tolerant bacteria is flexible, also in the presence of high concentrations of organic solvents [84, 116].

Transfer of solvent tolerance genes

Transfer of organic solvent tolerance genes to non–tolerant strains seems not to be an attractive application of solvent tolerance in biotechnology. As outlined in Chapter 2, the complexity of solvent tolerance limits the level of solvent tolerance by transfer of one single tolerance mechanism. This was demonstrated in Table 3.2 by transferring the solvent efflux system SrpABC to a solvent–sensitive *P. putida* strain. This strain became more tolerant to toluene but we were not able to accomplish tolerance to an organic phase of toluene.

REFERENCES

1. **Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Aono, R.** 1998. Improvement of organic solvent tolerance level of *Escherichia coli* by overexpression of stress–responsive genes. Extremophiles **2:**239–248.
3. **Aono, R., and H. Kobayashi.** 1997. Cell surface properties of organic solvent–tolerant mutants of *Escherichia coli* K12. Appl. Environ. Microbiol. **63:**3637–3642.
4. **Aono, R., K. Aibe, A. Inoue, and K. Horikoshi.** 1991. Preparation of organic solvent–tolerant mutants from *Escherichia coli* K12. Agri. Biol. Chem. **55:**1935–1938.
5. **Aono, R., M. Ito, A. Inoue, K. and Horikoshi.** 1992. Isolation of novel toluene–tolerant strain *Pseudomonas aeruginosa*. Biosci. Biotechnol. Biochem. **56:**145–146.
6. **Aono, R., M. Kobayashi, H. Nakajima, and H. Kobayashi.** 1995. A close correlation between improvement of organic solvent tolerance levels and alteration of resistance towards low levels of multiple antibiotics in *Escherichia coli*. Biosci. Biotechnol. Biochem. **59:**213–218.
7. **Aono, R., T. Negishi, and H. Nakajima.** 1994. Cloning of organic solvent tolerance gene *ostA* that determines *n*–hexane tolerance level in *Escherichia coli*. Appl. Environ. Microbiol. **60:**4624–4626.
8. **Arora, S.K., B.W. Ritchings, E.C. Almira, S. Lory, and R. Ramphal.** 1997. Transcriptional activator, FleQ, regulates mucin adhesion and flagellar gene expression in *Pseudomonas aeruginosa* in a cascade manner. J. Bacteriol. **179:**5574–5581.
9. **Asako, H., H. Nakajima, K. Kobayashi, M. Kobayashi, and R. Aono.** 1997. Organic solvent tolerance and antibiotic resistance increased by overexpression of *marA* in *Escherichia coli*. Appl. Environ. Microbiol. **63:**1428–1433.
10. **Asako, H., K. Kobayashi, and R. Aono.** 1999. Organic solvent tolerance of *Escherichia coli* is independent of OmpF levels in the membrane. Appl. Environ. Microbiol. **65:**294–296.
11. **Ausebel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (ed.).** 1991. Current Protocols in Molecular Biology. Greene Publishing Associates, New York.
12. **Birnboim, H.C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nuc. Acids Res. **7:**1513–1523.
13. **Cartwright, C.P., J.R. Juroszek, M.J. Beavan, F.M. S. Ruby, S.M.F. de Morais, and A.H. Rose.** 1986. Ethanol dissipates the proton motive force across the plasma membrane of *Saccharomyces cerevisiae*. J. Gen. Microbiol. **132:**369–377.
14. **Chen, Q.** 1996. Growth of *Pseudomonas oleovorans* in two liquid phase media. Effects of organic solvents and *alk* gene expression on the membrane. PhD thesis, University of Groningen, The Netherlands.
15. **Cruden, D.L., J.H. Wolfram, R.D. Rogers, and D.T. Gibson.** 1992. Physiological properties of a *Pseudomonas* strain which grows with *p*–xylene in a two–phase (organic–aqueous) medium. Appl. Environ. Microbiol. **58:**2723–2729.
16. **Davidson, P.M., and A.L. Branden.** 1981. Antimicrobial activity of non–halogenated phenolic compounds. J. Food Prot. **44:**623–632.
17. **de Bont, J.A.M.** 1998. Solvent–tolerant bacteria in biocatalysis. Trends Biotechnol. **16:**493–499.

18. **Dennis, J.J., and G.J. Zylstra.** 1998. Plasposons: modulair self-cloning minitransposons derivatives for rapid genetic analysis of Gram-negative bacterial genomes. Appl. Environ. Microbiol. **64:**2710–2715.
19. **Dennis, J.J., and P.A. Sokol.** 1995. Electrotransformation of *Pseudomonas*. Methods Mol. Biol. **47:**125–133.
20. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nuc. Acids Res. **12:**387–395.
21. **Diefenbach, R., and H. Keweloh.** 1994. Synthesis of *trans* saturated fatty acids in *Pseudomonas putida* P8 by direct isomerization of the double bond of lipids. Arch. Microbiol. **162:**120–125.
22. **Dinh, T., I.T. Paulsen, and M.H. Saier, Jr.** 1994. A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria. J. Bacteriol. **176:**3825–3831.
23. **Ditta, G., S. Stanfield, D. Corbin, and D.R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
24. **Ditty, J.L., A.C. Grimm, and C.S. Harwood.** 1998. Identification of a chemotaxis gene region from *Pseudomonas putida*. FEMS Microbiol. Lett. **159:**267–273.
25. **Duque, E., A. Segura, G. Mosqueda, and J.L. Ramos.** 2001. Global and cognate regulators control expression of the organic solvent efflux pumps TtgABC and TtgDEF of *Pseudomonas putida*. Mol. Microbiol. **39:**1100–1106.
26. **Ferrante, A.A., J. Augliera, K. Lewis, and A.M. Klibanov.** 1995. Cloning of an organic solvent-resistance gene in *Escherichia coli*: the unexpected role of alkylhydroperoxide reductase. Proc. Natl. Acad. Sci. USA **92:**7617–7621.
27. **Fukumori, F., H. Hirayama, H. Takami, A. Inoue, and K. Horikoshi.** 1998. Isolation and transposon mutagenesis of a *Pseudomonas putida* KT2442 toluene-resistant variant: involvement of an efflux system in solvent tolerance. Extremophiles **2:**395–400.
28. **Gotoh, N., H. Tsujimoto, K. Poole, J. Yamagishi, and T. Nishino.** 1995. The outer membrane protein OprM of *Pseudomonas aeruginosa* is encoded by *oprK* of the *mexA–mexB–oprK* multidrug resistance operon. Antimicrob. Agents Chemother. **39:**2567–2569.
29. **Guan, L., M. Ehrmann, H. Yoneyma, and T. Nakae.** 1999. Membrane topology of the xenobiotic-exporing subunit, MexB, of the MeAB-OprM extrusion pump in *Pseudomonas aeruginosa*. J. Biol. Chem. **274:**10517–10522.
30. **Guckert, J.B., M.A. Hood, D.C. White.** 1986. Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increase in the *cis/trans* ratio and proportions of cyclopropyl fatty acids. Appl. Environ. Microbiol. **52:**794–801.
31. **Harrop, A.J., M.D. Hocknull, and M.D. Lilly.** 1989. Biotransformation in organic solvents: a difference between gram-positive and gram-negative bacteria. Biotechnol. Lett. **11:**807–810.
32. **Hartmans, S., J.P. Smits, M.J. van der Werf, F. Volkering, and J.A.M. de Bont.** 1989. Metabolism of styrene oxide and 2-phenylethanol in the styrene degrading *Xanthobacter* strain 124X. Appl. Environ. Microbiol. **55:**2850–2855.
33. **Hartmans, S., M.J. van der Werf, and J.A.M. de Bont.** 1990. Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. Appl. Environ. Microbiol. **56:**1347–1351.
34. **Heipieper, H.-J., and J.A.M. de Bont.** 1994. Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes. Appl. Environ. Microbiol. **60:**4440–4444.
35. **Heipieper, H.-J., B. Loffeld, H. Keweloh, and J.A.M. de Bont.** 1995. The *cis/trans* isomerisation of unsaturated fatty acids in *Pseudomonas putida* S12: an indicator for environmental stress due to organic compounds. Chemosphere **30:**1041–1051.
36. **Heipieper, H.-J., F.J. Weber, J. Sikkema, H. Keweloh, and J.A.M. de Bont.** 1994. Mechanisms behind resistance of whole cells to toxic organic solvents. Trends Biotechnol. **12:**409–415.
37. **Heipieper, H.-J., G. Meulenbeld, Q. van Oirschot, and J.A.M. de Bont.** 1996. Effect of environmental factors on the *cis/trans* ratio of unsaturated fatty acids in *Pseudomonas putida* S12. Appl. Environ. Microbiol. **62:**2773–2777.

38. **Heipieper, H.-J., H. Keweloh, and H.J. Rehm.** 1991. Influence of phenols on growth and membrane permeability of free and immobilized *Escherichia coli*. Appl. Environ. Microbiol. **57:**1213–1217.
39. **Heipieper, H.-J., R. Diefenbach, and H. Keweloh.** 1992. Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. Appl. Environ. Microbiol. **58:**1847–1852.
40. **Hirayama, H., H. Takami, A. Inoue, and K. Horikoshi.** 1998. Isolation and characterization of toluene-sensitive mutants from *Pseudomonas putida* IH-2000. FEMS Microbiol. Lett. **169:**219–225.
41. **Holtwick, R., F. Meinhart, and H. Keweloh.** 1997. *cis-trans* isomerization of unsaturated fatty acids: cloning and sequencing of the *cti* gene from *Pseudomonas* P8. Appl. Environ. Microbiol. **63:**4292–4297.
42. **Holtwick, R., H. Keweloh, and F. Meinhart.** 1999. *cis/trans* Isomerization of unsaturated fatty acids of *Pseudomonas putida* P8: evidence for a heme protein of the cytochrome c type. Appl. Environ. Microbiol. **65:**2644–2649.
43. **Homma, M., D.J. DeRosier, and R.M. Macnab.** 1990. Flagellar hook and hook-associated proteins of *Salmonella typhimurium* and their relationship to other axial components of the flagellum. J. Mol. Biol. **213:**819–832.
44. **Hughes, K.T., and K. Mathee.** 1998. The anti-sigma factors. Annu. Rev. Microbiol. **52:**231–286.
45. **Husken, L.E., M.C. Dalm, J. Tramper, J. Wery, J.A.M. de Bont, and R. Beeftink.** 2001. Integrated bioproduction and extraction of 3-methylcatechol. J. Biotechnol. **88:**11–19.
46. **Husken, L.E., R. Beeftink, J.A.M. de Bont, and J. Wery.** 2001. High-rate 3-methylcatechol production in *Pseudomonas putida* strains by means of a novel expression system. Appl. Microbiol. Biotechnol. **55:**571–577.
47. **Ikura, Y., Y. Yoshida, and T. Kudo.** 1997. Physiological properties of two *Pseudomonas mendocina* strains which assimilate styrene in a two-phase (solvent-aqueous) system under static culture conditions. J. Ferment. Bioeng. **83:**604–607.
48. **Ingram, L.O.** 1977. Changes in the lipid composition of *Escherichia coli* resulting from growth with organic solvents and with food additives. Appl. Environ. Microbiol. **33:**1233–1236.
49. **Inoue, A., and K. Horikoshi.** 1989. A *Pseudomonas* thrives in high concentration of toluene. Nature **338:**264–266.
50. **Inoue, A., and K. Horikoshi.** 1991. Estimation of solvent-tolerance of bacteria by the solvent parameter log *P*. J. Ferment. Bioeng. **71:**194–196.
51. **Inoue, A., M. Yahamoto, and K. Horikoshi.** 1991. *Pseudomonas putida* which can grow in the presence of toluene. Appl. Environ. Microbiol. **57:**1560–1562.
52. **Inouye, S., M. Yamada, A. Nakazawa, and T. Nakazawa.** 1989. Cloning and sequence analysis of the *ntrA* (*rpoN*) gene of *Pseudomonas putida*. Gene **85:**145–152.
53. **Ishomoto, K.S., and S. Lory.** 1989. Formation of pili in *Pseudomonas aeruginosa* requires the alternative σ factor (RpoN) of RNA polymerase. Proc. Natl. Acad. Sci. USA **86:**1954–1957.
54. **Isken, S.** 2000. Physiology of solvent tolerance in *Pseudomonas putida* S12. PhD Thesis Wageningen University, The Netherlands.
55. **Isken, S., and J.A.M. de Bont.** 1996. Active efflux of toluene in a solvent-resistant bacterium. J. Bacteriol. **178:**6056–6058.
56. **Isken, S., and J.A.M. de Bont.** 1998. Bacteria tolerant to organic solvents. Extremophiles **2:**229–238.
57. **Isken, S., and J.A.M. de Bont.** 1998. Isolation of several solvent-tolerant *Bacillus* strains from bank-soil. Poster presented at the International congress on Extremophiles '98, Yokohama, Japan.
58. **Isken, S., P.M.A.C. Santos, and J.A.M. de Bont.** 1997. Effect of solvent adaptation on the antibiotic resistance in *Pseudomonas putida* S12. Appl. Microbiol. Biotechnol. **48:**642–647.
59. **Jackson, R.W., and J.A. de Moss.** 1965. Effects of toluene on *Escherichia coli*. J. Bacteriol. **90:**1420–1425.

60. **Keweloh, H., and H.-J. Heipieper.** 1996. Trans unsaturated fatty acids in bacteria. Lipids **31**:129–137.
61. **Kieboom, J., and J.A.M. de Bont.** 2001. Identification and molecular characterization of an efflux system involved in *Pseudomonas putida* S12 multidrug resistance. Microbiology **147**:43–51.
62. **Kieboom, J., and J.A.M. de Bont.** 2000. Mechanisms of organic solvent tolerance in bacteria, p. 393–402. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial Stress Responses, American Society for Microbiology, Washington D.C.
63. **Kieboom, J., J.J. Dennis, G.J. Zylstra, and J.A.M. de Bont.** 1998. Active efflux of organic solvents in *Pseudomonas putida* S12 is induced by solvents. J. Bacteriol. **180**:6769–6772.
64. **Kieboom, J., J.J. Dennis, J.A.M. de Bont, and G.J. Zylstra.** 1998. Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J. Biol. Chem. **273**:85–91.
65. **Kieboom, J., R. Bruinenberg, I. Keizer-Gunnink, and J.A.M. de Bont.** 2001. Transposon mutations in the flagella biosynthetic pathway of the solvent-tolerant *Pseudomonas putida* S12 result in a decreased expression of solvent efflux genes. FEMS Microbiol. Lett. **198**:117–122.
66. **Kim, E., and G.J. Zylstra.** 1995. Molecular and biochemical characterization of two meta-cleavage dioxygenases involved in biphenyl and m-xylene degradation by *Beijerinckia* sp. strain B1. J. Bacteriol. **177**:3095–3103.
67. **Kim, K., L. Lee, K. Lee, and D. Lim.** 1998. Isolation and characterization of toluene-sensitive mutants from the toluene-resistant bacterium *Pseudomonas putida* GM73. J. Bacteriol. 180:3692–3696.
68. **Kohler, T., M. Michea-Hamzehpour, U. Henze, N. Gotoh, L.K. Curty, and J.-C. Pechere.** 1997. Characterization of the MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol. Microbiol. **23**:345–354.
69. **Komatsu, T., K. Moriya, and K. Horikoshi.** 1994. Preparation of organic solvent-tolerant mutants from *Pseudomonas aeruginosa* strain PAO1161. Biosci. Biotech. Biochem. **58**:1754–1755.
70. **Kovach, M.E., R.W. Phillips, P.H. Elzer, R.M. Roop II, and K.M. Peterson.** 1994. pBBR1MCS: A broad-host-range cloning vector. Biotechniques **16**:801–802.
71. **Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss.** 1989. Expression of σ^{54} (*ntrA*)-dependent genes is probably united by a common mechanism. Microbiol. Rev. **53**:367–376.
72. **Laane, C., S. Boeren, K. Vos, and C. Veeger.** 1986. Rules for optimization of biocatalysis in organic solvents. Biotech. Bioeng. **30**:81–87.
73. **Lennox, E.S.** 1955. Virol. **1**:190–206.
74. **Li, L., T. Komatsu, A. Inoue, and K. Horikoshi.** 1995. A toluene-tolerant mutant of *Pseudomonas aeruginosa* lacking the outer membrane protein F. Biosci. Biotech. Biochem. **59**:2358–2359.
75. **Li, X.-Z., and K. Poole.** 1999. Organic solvent-tolerant mutants of *Pseudomonas aeruginosa* display multiple antibiotic resistance. Can. J. Microbiol. **45**:18–22.
76. **Li, X.-Z., D. Ma, D.M. Livermore, and H. Nikaido.** 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as a contributing factor to beta-lactam resistance. Antimicrob. Agents Chemother. **38**:1742–1752.
77. **Li, X.-Z., H. Nikaido, and K. Poole.** 1995. Role of MexA-MexB-OprM in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39**:1948–1953.
78. **Li, X.-Z., L. Zhang, and K. Poole.** 1998. Role of the multidrug efflux systems of *Pseudomonas aeruginosa* in organic solvent resistance. J. Bacteriol. **180**:2987–2991.
79. **Loffeld, B., and H. Keweloh.** 1996. *cis/trans* isomerization of unsaturated fatty acids as possible control mechanism of membrane fluidity in *Pseudomonas putida* P8. Lipids **31**:811–815.
80. **Ma, D., D.N. Cook, M. Alberti, N.G. Pon, H. Nikaido, and J.E. Hearst.** 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16**:45–55.

81. **Ma, D., D.N. Cook, M. Alberti, N.G. Pon, H. Nikaido, and J.E. Hearst.** 1993. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. J. Bacteriol. **175**:6299–6313.
82. **Ma, D., M. Alberti, C. Lynch, H. Nikaido, and J.E. Hearst.** 1996. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. Mol. Microbiol. **19**:101–112.
83. **Macnab, R.M.** 1992. Genetics and biogenesis of bacterial flagella. Annu. Rev. Genet. **26**:131–158.
84. **Marconi, A.M., J. Kieboom, and J.A.M. de Bont.** 1997. Improving the catabolic functions in the toluene–resistant strain *Pseudomonas putida* S12. Biotechnol. Lett. **19**:603–606.
85. **Masuda, N., E. Sakagawa, and S. Ohya.** 1995. Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39**:645–649.
86. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
87. **Moriya, K., and K. Horikoshi.** 1993. A benzene–tolerant bacterium utilizing sulfur compounds isolated from deep sea. J. Ferment. Bioeng. **76**:397–399.
88. **Moriya, K., and K. Horikoshi.** 1993. Isolation of a benzene–tolerant bacterium and its hydrocarbon degradation. J. Ferment. Bioeng. **76**:168–173.
89. **Mosqueda, G., and J.L. Ramos.** 2000. A set of genes encoding a second toluene efflux system in *Pseudomonas putida* DOT–T1E is linked to the *tod* genes for toluene metabolism. J. Bacteriol. **182**:937–43.
90. **Naglak, T.J., D.J. Hettwer, and H.Y. Wang.** 1990. Chemical permeabilization of cells for intracellular product release, p. 177–205. In: Asenjo J.A. (ed.) Separation Processes in Biotechnology. Dekker, New York, N.Y.
91. **Nakajima, H., K. Kobayashi, M. Kobayashi, H. Asako, and R. Aono.** 1995. Overexpression of the *robA* gene increases organic solvent tolerance and multiple antibiotic and heavy metal ion resistance in *Escherichia coli*. Appl. Environ. Microbiol. **61**:2302–2307.
92. **Nakajima, H., M. Kobayashi, T. Negishi, and R. Aono.** 1995. *SoxRS* gene increased the level of organic solvent tolerance in *Escherichia coli*. Biosci. Biotechnol. Biochem. 59:1323–1325.
93. **Nies, D.H., A. Nies, L. Chu, and S. Silver.** 1989. Expression and nucleotide sequence of a plasmid–determined divalent cation efflux system from *Alcaligenes eutrophus*. Proc. Natl. Acad. Sci. **86**:7351–7355.
94. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: role of permeability barriers and active efflux. Science (Washington, D.C.), **64**:382–388.
95. **Nikaido, H.** 1996. Multidrug efflux pumps of Gram–negative bacteria. J. Bacteriol. **178**:5853–5859.
96. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49**:1–32.
97. **Ogino, H., K. Miyamoto, and H. Ishikawa.** 1994. Organic solvent–tolerant bacterium which secretes an organic solvent–stable lipolytic enzyme. Appl. Environ. Microbiol. **60**:3884–3886.
98. **Ogino, H., K. Yasui, T. Shiotani, T. Ishihara, and H. Ishikawa.** 1995. Organic solvent–tolerant bacterium which secretes an organic solvent–stable proteolytic enzyme. Appl. Environ. Microbiol. **61**:4258–4262.
99. **Ohta, Y., M. Maeda, T. Kudo, and K. Horikoshi.** 1996. Isolation and characterization of solvent–tolerant bacteria which can degrade biphenyl/polychlorinated biphenyls. J. Gen. Appl. Microbiol. **42**:349–354.
100. **Okuyama, H., A. Ueno, D. Enari, N. Morita, and T. Kusana.** 1998. Purification and characterization of 9–hexadecenoic acid *cis–trans* isomerase from *Pseudomonas* sp. strain E–3. Arch. Microbiol. **169**:29–35.
101. **Okuyama, H., D. Enari, A. Shibahara, K. Yamamoto, and N. Morita.** 1996. Identification of activities that catalyze the *cis–trans* isomerization of the double bond of a mono–unsaturated fatty acid in *Pseudomonas* sp. E–3. Arch. Microbiol. **165**:415–417.

102. **Okuyama, H., S. Sasaki, S. Higashi, and N. Murata.** 1990. A *trans*–unsaturated fatty acid in a psychrophilic bacterium, *Vibrio* sp. strain ABE–1. J. Bacteriol. **172**:3515–3518.
103. **Osborne, S.J., J. Leaver, M.K. Turner, and P. Dunnill.** 1990. Correlation of biocatalytic activity in an organic–aqueous two–phase system with solvent concentration in the cell membrane. Enzyme Microb. Technol. **12**:281–291.
104. **Paje, M.L.F., B.A. Neilan, and I. Couperwhite.** 1997. A *Rhodococcus* species that thrives on medium saturated with liquid benzene. Microbiology (Reading) **143**:2975–2981.
105. **Palleroni, N.J.** 1984. Genus I. *Pseudomonas*, p. 141–199. *In* N.R. Krieg and J.G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins Co., Baltimore.
106. **Pandza,S., M. Baetens, C.H. Park, T. Au, M. Keyhan, and A. Matin.** 2000. The G–protein FlhF has a role in polar flagellar placement and general stress response induction in *Pseudomonas putida*. Mol. Microbiol. **36**:414–423.
107. **Paulsen, I.T., M.H. Brown, and R.A. Skurray.** 1996. Proton–dependent multidrug efflux systems. Microbiol. Rev. **60**:575–608.
108. **Pedrotta, V., and B. Witholt.** 1999. Isolation and characterization of the *cis–trans*–unsaturated fatty acid isomerase of *Pseudomonas oleovorans* Gpo12. J. Bacteriol. **181**:3256–3261.
109. **Pinkart, H.C., and D.C. White.** 1997. Phospholipid biosynthesis and solvent tolerance in *Pseudomonas putida* strains. J. Bacteriol. **179**:4219–4226.
110. **Pinkart, H.C., J.W. Wolfram, R. Rogers, and D.C. White.** 1996. Cell envelope changes in solvent–tolerant and solvent–sensitive *Pseudomonas putida* strains following exposure to *o*–xylene. Appl. Environ. Microbiol. **62**:1129–1132.
111. **Poole, K., D.E. Heinrichs, and S. Neshat.** 1993. Cloning and sequence analysis of an EnvCD homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in the secretion of the siderophore pyoverdine. Mol. Microbiol. **10**:529–544.
112. **Poole, K., K. Krebes, C. McNally, and S. Neshat.** 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. **175**:7363–7372.
113. **Poole, K., K. Tetro, Q. Zhao, S. Neshat, D.E. Heinrichs, and N. Bianco.** 1996. Expression of the multidrug resistance operon *mexA–mexB–oprM* in *Pseudomonas aeruginosa*: *mexR* encodes a regulator of operon expression. Antimicrob. Agents Chemother. **40**:2021–2028.
114. **Poole, K., N. Gotoh, H. Tsujimoto, Q. Zhao, A. Wada, T. Yamasaki, S. Neshat, J.–I. Yamagishi, X.–Z. Li, and T. Nishino.** 1996. Overexpression of the *mexC–mexD–OprJ* efflux operon in *nfxB*–type multidrug–resistant strains of *Pseudomonas aeruginosa*. Mol. Microbiol. **21**:713–724.
115. **Ramos, J.L., E. Duque, J.J. Rodriguez–Herva, P. Godoy, A. Haïdour, F. Reyes, and A. Fernandez–Barrero.** 1997. Mechanisms for solvent tolerance in bacteria. J. Biol. Chem. **272**:3887–3890.
116. **Ramos, J.L., E. Duque, M.J. Huertas, and A. Haïdour.** 1995. Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. J. Bacteriol. **177**:3911–3916.
117. **Ramos, J.L., E. Duque, P. Godoy, and A. Segura.** 1998. Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT–T1E. J. Bacteriol. **180**:3323–3329.
118. **Rekker, R.F., and H.M. de Kort.** 1979. The hydrophobic fragmental constant; an extention to a 1000 data point set. Eur. J. Med. Chem. **14**:479–488.
119. **Rodriguez–Herva, J.J., and J.L. Ramos.** 1996. Characterization of an OprL null mutant of *Pseudomonas putida*. J. Bacteriol. **178**:5836–5840.
120. **Rodriguez–Herva, J.J., M.–I. Ramos–Gonzales, and J.L. Ramos.** 1996. The *Pseudomonas putida* peptidoglycan–associated outer membrane lipoprotein is involved in maintenance of the integrity of the cell envelope. J. Bacteriol. **178**:1699–1706.
121. **Rojas, A., E. Duque, G. Mosqueda, G. Golden, A. Hurtado, J.L. Ramos, and A. Segura.** 2001. Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT–T1E. J. Bacteriol. **183**:3967–3973.

122. **Rothmel, R.K., D.L. Shinabarger, M.R. Parsek, T.L. Aldrich, and A.M. Chakrabarty.** 1991. Functional analysis of the *Pseudomonas putida* regulatory protein CatR: transcriptional studies and determination of the CatR DNA-binding site by hydroxyl-radical footprinting. J. Bacteriol. **173:**4717–4724.
123. **Segura, A, E. Duque, A. Hurtado, and J.L. Ramos.** 2001. Mutations in genes involved in the flagellar export apparatus of the solvent-tolerant *Pseudomonas putida* DOT-T1E strain impair motility and lead to hypersensitivity to toluene shocks. J. Bacteriol. **183:**4127–4133.
124. **Saier, M.H., Jr., R. Tam, A. Reizer, and J. Reizer.** 1994. Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. Mol. Microbiol. **11:**841–847.
125. **Salter, G.J., and D.B. Kell.** Solvent selection for whole cell biotransformation in organic media. Crit. Rev. Biotechnol. **15:**139–177.
126. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
127. **Schwartz, D.R., and C.J. MacCoy.** 1977. Epoxidation of 1,7-octadiene by *Pseudomonas oleovorans*: fermentation in the presence of cyclohexane. Appl. Environ. Microbiol. **34:**47–49.
128. **Shima, H., T. Kudo, and K. Horikoshi.** 1991. Isolation of toluene-resistant mutants from *Pseudomonas putida* PpG1 (ATCC 17453). Agric. Biol. Chem. **55:**1197–1199.
129. **Sikkema, J., B. Poolman, W.N. Konings, and J.A.M. de Bont.** 1992. Effects of the membrane action of tetralin on the functional and structural properties of artificial and bacterial membranes. J. Bacteriol. **174:**2986–2992.
130. **Sikkema, J., J.A.M. de Bont, and B. Poolman.** 1994. Interactions of cyclic hydrocarbons with biological membranes. J. Biol. Chem. **269:**8022–8028.
131. **Sikkema, J., J.A.M. de Bont, and B. Poolman.** 1995. Mechanisms of membrane toxicity of cyclic hydrocarbons. Microbiol. Rev. **59:**201–222.
132. **Smet de, M.J., J. Kingma, and B. Witholt.** 1978. The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of Escherichia coli. Biochim. Biophys. Acta **506:**64–80.
133. **Smith, P.K., R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, and D.C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.
134. **Spangenberg, C., T. Heuer, C. Buerger, and B. Tuemmler.** 1996. Genetic diversity of flagellins of *Pseudomonas aeruginosa*. FEBS Lett. **396:**213–217.
135. **Stanier, R.Y., N.J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159–271.
136. **Stover, C.K., X.Q. Pham, A.L. Erwin, S.D. Mizoguchi, P. Warrener, M.J. Hickey, F.S. Brinkman, W.O. Hufnagle, D. J. Kowalik, M. Lagrou, R.L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L.L. Brody, S.N. Coulter, K.R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G.K. Wong, Z. Wu, and I.T. Paulsen.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406:**959–64.
137. **Sunnarborg, A., D. Klumpp, T. Chung, and D.C. LaPorte.** 1990. Regulation of the glyoxylate bypass operon: cloning and characterization of *iclR*. J. Bacteriol. **172:**2642–2649.
138. **Totten, P.A., J.C. Lara, and S. Lory.** 1990. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J. Bact. **172:**389–396.
139. **Trevisan, V.** 1889. I generi e le specie delle Batteriacee. Zanaboni and Gabuzzi, Milan p. 1–35.
140. **Tsubata, T., and R. Kurane.** 1996. Selection of an *n*-heptanol-resistant bacterium from an organic solvent-sensitive bacterium and characterization of its fatty acids. Can. J. Microbiol. **42:**642–646.
141. **Vermuë, M., J. Sikkema, A. Verheul, R. Bakker, and J. Tramper.** 1993. Toxicity of homologous series of organic solvents for the gram-positive *Arthobacter* and *Norcardia* sp. and the gram-negative *Acinetobacter* and *Pseudomonas* sp. Biotechnol. Bioeng. **42:**747–758.

142. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA **76:**615–619.
143. **Vogler, A.P., M. Homma, V.M. Irikura, and R.M. Macnab.** 1991. *Salmonella typhimurium* mutants defective in flagellar filament regrowth and sequence similarity of FliI to FOF1, vacuolar, and archaebacterial ATPase subunits. J. Bacteriol. **173:**3564–3572.
144. **Weber, F.J., and J.A.M. de Bont.** 1996. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. Biochim. Biophys. Acta **1286:**225–245.
145. **Weber, F.J., L.P. Ooijkaas, R.M.W. Schemen, S. Hartmans, and J.A.M. de Bont.** 1993. Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. Appl. Environ. Microbiol. **59:**3502–3504.
146. **Weber, F.J., S. Isken, and J.A.M. de Bont.** 1994. *Cis/trans* isomerization of fatty acids as a defence mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. Microbiol. **140:**2013–2017.
147. **Wery, J., B. Hidayat, J. Kieboom, and J.A.M. de Bont.** 2001. An insertion sequence prepares *Pseudomonas putida* S12 for severe solvent stress. J. Biol. Chem. **276:**5700–5706.
148. **Wery, J., and G.J. Zylstra.** Unpublished Results.
149. **Wery, J., D.I. Mendes da Silva, and J.A.M. de Bont.** 2000. A genetically modified solvent-tolerant bacterium for optimized production of a toxic fine chemical. Appl. Microbiol. Biotechnol. **54:**180–5.
150. **West, S.E., H.P. Schweizer, C. Dall, A.K. Sample, and L.J. Runyen-Janecky.** 1994. Construction of improved *Escherichia–Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene **148:**81–86.
151. **Westbrock-Wadman, S., D.R. Sherman, M.J. Hickey, S.N. Coulter, Y.Q. Zhu, P. Warrener, L.Y. Nguyen, R.M. Shawar, K.R. Folger, and C.K. Stover.** 1999. Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. Antimicrob. Agents Chemother. **43:**2975–83.
152. **White, D.G., J.D. Goldman, B. Demple, and S.B. Levy.** 1997. Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA*, *soxS*, or *robA* in *Escherichia coli*. J. Bacteriol. **179:**6122–6126.
153. **Winstanley, C, J.A. Morgan, R.W. Pickup and J.R. Saunders.** 1994. Molecular cloning of two *Pseudomonas* flagellin genes and basal body structural genes. Microbiology **140:**2019–2031.
154. **Witholt, B., M.J. de Smet, J. Kingma, J.B. van Beilen, M. kok, R.G. Lageveen, and G. Eggink.** 1990. Bioconversions of aliphatic compounds by *Pseudomonas oleovorans* in multiphase bioreactors; background and economic potential. Trend Biotechnol. **8:**46–52.
155. **Woldringh, C.L.** 1973. Effects of toluene and phenylethyl alcohol on the ultrastructure of *Escherichia coli*. J. Bacteriol. **114:**1359–1361.
156. **Woodruff, W.A., and R.E.W. Hancock.** 1989. *Pseudomonas aeruginosa* outer membrane protein F: structural role and relationship to the *Escherichia coli* OmpA protein. J. Bacteriol. **171:**3304–3309.
157. **Wubbolts, M.G., O. Favre-Bulle, and B. Witholt.** 1986. Biosynthesis of synthons in two-liquid-phase media. Biotechnol. Bioeng. **52:**301–308.
158. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
159. **Zylstra, G. J., R.H. Olsen, and D.P. Ballou.** 1989. Cloning, expression, and regulation of the *Pseudomonas cepacia* protocatechuate 3,4-dioxygenase genes. J. Bacteriol. **171:**5907–5914.
160. **Zylstra, G.J.** 1993. Molecular analysis of aromatic hydrocarbon degradation. *In* Molecular Environmental Biology (S.J. Garte, ed.), p. 83–115, Lewis Publishers, Boca Raton, FL.

SUMMARY

The aim of the research presented in this thesis was to study the molecular mechanisms of organic solvent tolerance in *Pseudomonas putida* S12. This bacterium is capable of growth at saturated solvent concentrations, which are lethal to normal bacteria. Organic solvent–tolerant bacteria have potential advantages in either the remediation of highly polluted waste streams or biocatalytic applications for the production of specialty chemicals. The use of these bacteria in biocatalysis would allow the introduction of an organic phase to dissolve water–insoluble substrates or to remove toxic products.

As a first step in the identification of genes involved in solvent tolerance, toluene–sensitive transposon mutants of *P. putida* S12 were generated. As described in Chapter 3, we were able to isolate the genes involved in toluene efflux using the toluene–sensitive strain *P. putida* JK1. The deduced amino acid sequences encoded by the *srpABC* genes isolated were highly homologous to proteins involved in proton–dependent efflux. Transfer of the genes for the toluene efflux pump to a normally toluene–sensitive *P. putida* strain resulted in the acquisition of toluene tolerance. From these results we conclude that organic solvent efflux is the key factor in solvent tolerance.

In Chapter 4 it was found that the induction of the membrane associated efflux system SrpABC of *P. putida* S12 is inducible. Using a reporter vector, containing the *srp* promoter, it was determined that aromatic and aliphatic solvents and alcohols were capable of inducing the transcription the *srpABC* genes. However, antibiotics, heavy metals and general stress conditions (pH, temperature, NaCl, and organic acids) did not induce *srp* transcription. From the results presented in Chapter 4 we conclude that SrpABC–mediated efflux of organic solvents is solely induced by solvent stress.

The high levels of antibiotic resistance of *P. putida* S12 and the relationship between solvent tolerance and antibiotic resistance triggered us to study multidrug resistance in this strain. In analogy to the results presented in Chapter 3 the first step in the identification of genes involved in multidrug resistance was to generate transposon mutants of *P. putida* S12. In Chapter 5 we describe the isolation the *arp* genes involved in chloramphenicol efflux, using the isolated chloramphenicol–sensitive *P. putida* strains CM1 and CM2. Moreover, the ArpABC efflux system was

involved in the resistance towards tetracycline, carbenicillin, streptomycin, erythromycin, and novobiocin. Surprisingly, the deduced amino acid sequences encoded by the isolated *arpABC* genes were highly homologous to proteins involved in proton–dependent efflux of organic solvents. By constructing an *arp–srp* double mutant it was concluded that *arpABC* was not involved in efflux of organic solvents.

In Chapter 6 octanol–sensitive mutants of *P. putida* S12 were isolated, which were interrupted in genes for the flagella biosynthetic pathway. These mutants were nonmotile and the formation of the flagellum was totally impaired. The expression of the SrpABC efflux pump in the nonmotile mutants was decreased, possibly due to general regulatory mechanisms.

Several genes involved in multidrug resistance and solvent tolerance in *P. putida* S12 have been isolated and characterized. It would now be interesting to investigate the complex regulation of these systems and to identify new genes using the mutants described in this thesis.

SAMENVATTING

Het doel van het onderzoek, zoals beschreven in dit proefschrift, was het bestuderen van de moleculaire mechanismen van tolerantie voor oplosmiddelen in *Pseudomonas putida* S12. Deze bacterie is in staat te groeien in de aanwezigheid van verzadigde concentraties oplosmiddel. Deze hoeveelheden oplosmiddel is voor 'normale' bacteriën dodelijk, maar oplosmiddel tolerante bacteriën kunnen dit overleven. Een mogelijke toepassing van deze bacteriën zou gezocht moeten worden in de reiniging van zwaar verontreinigde afvalstromen of in de biotechnologische productie van chemicaliën. Het gebruik van deze organismen kan leiden tot stabielere systemen. Ook kan een organische fase geïntroduceerd worden waarmee onoplosbare substraten opgelost of toxische producten geëxtraheerd kunnen worden.

De eerste stap in de identificatie van genen die bij oplosmiddel tolerantie betrokken zijn, was het maken van oplosmiddel gevoelige mutanten van *P. putida* S12. Door het isoleren van de tolueen gevoelige stam *P. putida* JK1 konden de genen voor het transport van deze verbinding gekarakteriseerd worden. Dit staat beschreven in Hoofdstuk 3. De afgeleide aminozuurvolgorde van het geïsoleerde *srpABC* operon was sterk overeenkomstig met eiwitten betrokken bij protonen afhankelijk transport. Het overbrengen van deze genen naar een tolueen gevoelige *P. putida* stam leidde tot tolerantie voor tolueen. Uit deze resultaten kan geconcludeerd worden dat het transport van organische oplosmiddelen een sleutelrol speelt in de tolerantie voor oplosmiddelen.

In Hoofdstuk 4 is aangetoond dat het membraan–gebonden transport systeem SrpABC in *P. putida* induceerbaar is. Een studie naar de *srp* promotor toonde aan dat aromatisch en alifatische oplosmiddelen in staat zijn om transcriptie van *srpABC* te induceren. Echter, antibiotica, zware metalen en algemene stress condities (zoals pH, temperatuur, NaCl en organische zuren) induceerden *srp* transcriptie niet. Uit de in Hoofdstuk 4 beschreven resultaten kan geconcludeerd worden dat transport van organische oplosmiddelen door SrpABC uitsluitend geïnduceerd wordt door oplosmiddelen.

De hoge tolerantie voor antibiotica in *P. putida* S12 en de aangetoonde relatie tussen deze tolerantie en oplosmiddel–tolerantie gaf voldoende aanleiding om antibiotica–tolerantie in deze stam te onderzoeken. De eerste stap in de

identificatie van genen voor antibiotica–tolerantie was het maken van antibiotica gevoelige mutanten. Hiertoe is een methode analoog aan die uit Hoofdstuk 3 gebruikt. Met behulp van de twee geïsoleerde mutanten, *P. putida* CM1 en CM2, konden we de *arpABC* genen voor chlooramphenicol–tolerantie isoleren. Dit is beschreven in Hoofdstuk 5. Naast chlooramphenicol–tolerantie blijkt het ArpABC efflux systeem betrokken te zijn bij tolerantie tegen tetracycline, carbeniciline, streptomycine, erythromycine en novobiocine. Het is opvallend dat de afgeleide aminozuurvolgorde van *arpABC* grote overeenkomst vertoont met eiwitten die betrokken zijn bij het protonen afhankelijk transport van organische oplosmiddelen. Ondanks deze homologie is met behulp van een *arp–srp* dubbel–mutant aangetoond dat ArpABC niet betrokken is bij transport van organische oplosmiddelen.

De in Hoofdstuk 6 beschreven transposon–mutanten zijn gevoelig voor octanol en blijken gemuteerd te zijn in de genen voor de biosynthese van zweepstaarten. Deze mutanten zijn niet beweeglijk en ze zijn niet in staat zweepstaarten te vormen. De expressie van het SrpABC transport systeem bleek in de niet beweeglijke mutanten verlaagd te zijn. De conclusie van dit hoofdstuk is dat transposon–inserties in de genen voor de biosynthese–route van zweepstaarten tot een verminderde expressie van SrpABC leidt, waarschijnlijk door algemene regulatie–mechanismen.

Zoals in dit proefschrift beschreven is, zijn uit *P. putida* S12 enkele genen geïsoleerd en gekarakteriseerd, die een belangrijke basis vormen voor tolerantie voor oplosmiddelen en antibiotica in deze stam. Het onderzoeken van de complexe regulatie systemen in en het identificeren van nieuwe genen aan de hand van de beschreven mutanten zal een uitdaging zijn.

NAWOORD

Houten, 28 januari 2002

Op een warme zomerdag in 1994 werd ik totaal onverwacht opgebeld door Jan de Bont met de vraag of ik interesse had in een promotiebaan bij de vakgroep Industriële Microbiologie. Ik was er op dat moment nog niet uit wat ik na mijn studie wilde doen. Het feit dat ik op stage was, het bloedheet was en mijn interesse op dat moment meer uitging naar een ijskoud biertje op een van de gezellige terrasjes in Ceské Budejovice, bemoeilijkte een rationele keuze dan ook ten zeerste. Terug in Nederland en na een goed gesprek met Jan werd ik enthousiast en ben ik nog voor het einde van mijn studie begonnen als Assistent in Opleiding.

Het onderzoek naar de biologische productie van N-gesubstitueerde catecholen bracht niet wat we gehoopt hadden. Het was dan ook na twee jaar dat er besloten werd het over een andere boeg te gooien. Eind 1996 ging ik voor 6 maanden naar de Verenigde Staten om een oplosmiddeltolerante bacterie, *Pseudomonas putida* S12, nitrocatechol te laten produceren. Om de toegevoegde waarde van een oplosmiddeltolerante bacterie te demonstreren, wilden we de productie hiervan vergelijken met die van een oplosmiddel-gevoelige mutant. Niet de productie van 3-nitrocatechol maar de isolatie van deze mutant betekende echter een onverwachte doorbraak in het onderzoek.

Het resultaat van deze keuzes ligt nu voor u. Het proefschrift is af en de tijd is gekomen om enkele mensen te bedanken. Jan, allereerst wil ik jou bedanken voor je steun en de mogelijkheid die je me heb geboden om het onderzoek af te ronden. Jouw nimmer aflatende belangstelling en enthousiasme voor het onderzoek naar oplosmiddeltolerantie hebben mij erg geholpen bij de totstandkoming van dit proefschrift. Tevens wil ik iedereen bedanken die heeft bijgedragen aan mijn proefschrift en aan de goede werksfeer op IM. In het bijzonder wil ik mijn VMT labgenoten bedanken. Sjaak, Arie, Harald, Jan W., Jan V. en Hans, bedankt voor de gezellige werksfeer op het VMT lab en jullie betrokkenheid bij mijn onderzoek. Ik heb onze vakinhoudelijke discussies naast nuttig ook leuk gevonden. Vooral de 'gat' theorie was erg verhelderend.

Furthermore, I would like to thank Gerben Zylstra and Jon Dennis for their contribution to my thesis. The discovery of the *srp* genes was a major breakthrough

and was a new impulse in my research. Gerben, thank you for giving me the opportunity to work in your laboratory. I enjoyed it very much and I had a great time at Rutgers. Jon, thank you for teaching me the principles of prokaryotic genetics. Without your help my thesis wouldn't be the same.

Tenslotte wil ik Wim en Sigrid bedanken voor hun steun en het feit dat zij mij de mogelijkheid hebben geboden om het tot hier te schoppen. Mascha, ik kan mij nog momenten genoeg bedenken dat ik niet te genieten was omdat een experiment mislukt was en ik weer met een sacherijnige kop voor de TV zat. Bedankt voor je steun en dat je er gewoon voor mij was.

Momenteel raast er een zware storm door Nederland en de eerste zandzakken zijn op het NOS-journaal gesignaleerd. Storm en zandzakken, beter kan ik de weg naar de voltooiing van een proefschrift niet verwoorden. Gelukkig is het soms ook zomer.

Jasper

CURRICULUM VITAE

Jasper Kieboom werd op 18 augustus 1970 te Utrecht geboren. In 1989 haalde hij zijn VWO diploma aan de openbare scholengemeenschap De Randijk te Nieuwegein. In hetzelfde jaar begon hij met de studie Milieuhygiëne, oriëntatie Milieutechnologie, aan de toenmalige Landbouwuniversiteit te Wageningen. In 1994 rondde hij deze studie af, met als afstudeervakken Milieutechnologie en Industriële Microbiologie en een stage bij het Institute of Landscape Ecology te Ceské Budejovice in Tsjechië. Van oktober 1994 tot mei 2000 werkte hij als Assistent in Opleiding en toegevoegd onderzoeker bij de sectie Industriële Microbiologie van Wageningen Universiteit, waar hij het in dit proefschrift beschreven onderzoek verrichtte. Tijdens dit onderzoek verbleef hij een half jaar bij het Center for Agricultural and Molecular Biology van de Rutgers State University of New Jersey, onder begeleiding van dr. G.J. Zylstra. Na een korte periode als Postdoc bij de vakgroep Moleculaire Celfysiologie van de Vrije Universiteit Amsterdam, werkt hij sinds augustus 2001 bij de vakgroep Levensmiddelenhygiëne en –microbiologie van de Wageningen Universiteit.

LIST OF PUBLICATIONS

Wery, J., B. Hidayat, **J. Kieboom**, and J.A.M. de Bont. 2001. An insertion sequence prepares *Pseudomonas putida* S12 for severe solvent stress. J. Biol. Chem. **276**:5700–5706.

Kieboom, J., and J.A.M. de Bont. 2001. Identification and molecular characterization of an efflux system involved in *Pseudomonas putida* S12 multidrug resistance. Microbiology **147**:43–51.

Kieboom, J., R. Bruinenberg, I. Keizer–Gunnink, and J.A.M. de Bont. 2001. Transposon mutations in the flagella biosynthetic pathway of the solvent–tolerant *Pseudomonas putida* S12 result in a decreased expression of solvent efflux genes. FEMS Microbiol. Lett. **198**:117–122.

Kieboom, J. H. van den Brink, J. Frankena, and J.A.M. de Bont 2001. Production of 3–nitrocatechol by oxygenases–containing bacteria: optimization of the nitrobenzene biotransformation by *Nocardia* S3. Appl. Microbiol. Biotechnol. **55**:290–295.

Kieboom, J., and J.A.M. de Bont. 2000. Mechanisms of organic solvent tolerance in bacteria, p. 393–402. *In* G. Storz and R. Hengge–Aronis (ed.), Bacterial Stress Responses, American Society for Microbiology, Washington D.C.

Kieboom, J., J.J. Dennis, G.J. Zylstra, and J.A.M. de Bont. 1998. Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. J. Bacteriol. **180**:6769–6772.

Kieboom, J., J.J. Dennis, J.A.M. de Bont, and G.J. Zylstra. 1997. Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J. Biol. Chem. **273**:85–91.

Marconi, A.M., **J. Kieboom**, and J.A.M. de Bont. 1997. Improving the catabolic functions in the toluene–resistant strain *Pseudomonas putida* S12. Biotechnol. Lett. **19**:603–606.

Reij, M.W., **J. Kieboom**, J.A.M. de Bont, and S. Hartmans. 1995. Continuous degradation of trichloroethylene by *Xanthobacter* sp. strain Py2 during growth on propene. Appl. Environ. Microbiol. **61**:2936–2942.