Systems analysis of solvent tolerance mechanisms in *Pseudomonas putida* S12

Importance of energy metabolism and functional identification of the TrgI regulator

Rita Volkers

Systems analysis of solvent tolerance mechanisms in *Pseudomonas putida* S12

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Part 1: Organic solvents and bacteria

The toxicity of hydrophobic organic solvents

Bacterial membranes form a barrier between the cell and its environment. Without such a barrier, no life would exist. Inside the membranes, a stable micro-environment, that is different from the outside environment, is established and maintained. These different environments implicate the existence of gradients across the membranes, by which, amongst others, energy from the combustion of hydrocarbons can be harnessed. This electrochemical potential is maintained across the plasma membrane and is also called proton motive force (PMF). In the PMF, protons are transported from the inside to the outside of the membrane and electrons travel in the opposite direction. Membranes also regulate what enters and exits the cell by facilitating passive and active transport of substances such as nutrients or waste products. Proteins embedded in the membranes can function as molecular signals that allow cells to communicate with each other. Receptor proteins on the other hand receive signals from other cells or from the environment.

The toxicity of hydrophobic organic solvents is mainly caused by their accumulation in bacterial membranes, by which they disturb the integrity of the membranes and negatively influence membrane functions (Figure 1.1). The hydrophobicity of an organic solvent is expressed as the logarithm of the partition coefficient of the solvent in a mixture of *n*-octanol and water, or log $P_{o/w}$. For bacteria, organic solvents with a log $P_{o/w}$ between 1 and 5 have the highest toxicity (101). Examples are toluene (log $P_{o/w}$ of 1.4) and benzene (log $P_{o/w}$ of 2).

The accumulation of solvent molecules in a membrane lowers its rigidity and increases its fluidity and permeability (125). This will result in an increased rate of cell lysis and leakage of macromolecules from the cell to its environment. Also, a passive flux of protons across the membrane will take place, dissipating the PMF and lowering the energy status of the cell (uncoupling effect) (125). Additionally, the functioning of proteins and enzymes embedded in the membranes is negatively affected by the organic solvent molecules as properties such as membrane fluidity are altered (125). In the past years, several reviews describing the main mechanisms of solvent tolerance have been published (22, 53, 60, 67, 125). The main mechanisms will be described shortly in this chapter, where relevant.

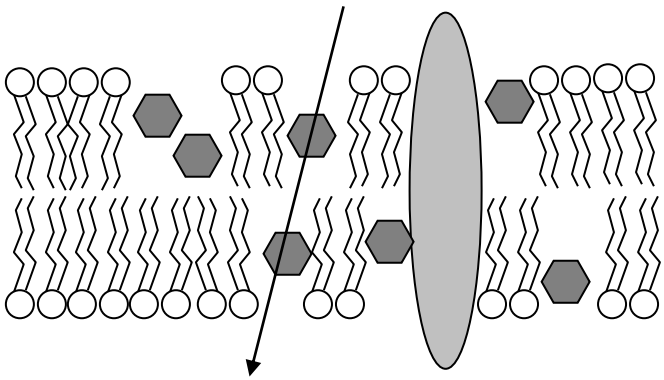

Figure 1.1 Schematic representation of solvent molecules accumulating in a bacterial membrane. The *arrow* represents the passive flux of protons; *dark grey*: solvent molecules; *light grey*: membrane embedded protein.

Solvent tolerant bacteria

In 1989, the isolation of an extremely solvent-tolerant micro-organism was reported for the first time. Inoue and Horikoshi (58) isolated a *Pseudomonas putida* strain (IH-2000) that was able to grow in a two-phase toluene-water system, without degrading toluene. After this publication, several other extremely tolerant strains were reported. Most of these strains are Gram-negative and many of them are *Pseudomonas* species. Several other strains of *P. putida* are tolerant to toluene, for example Idaho (21), DOT-T1E (109) and GM62 and GM73 (70). Also two *P. aeruginosa* strains (ST-001 (3) and LST-03 (100)), were reported to be tolerant to toluene as well as a *Sphingomonas aromaticivorans* strain (B0695) (35). As for Gram-positive bacteria, solvent-tolerant strains of *Bacillus* (86, 91), *Rhodococcus* (102, 111), *Arthrobacter* (63) and *Staphylococcus* (31, 156) have been reported. Recent findings of extremely solvent tolerant bacteria are the Gram-negative strain *Pseudomonas* sp. BCNU 171, which tolerates 60 M toluene (19), and the Gram-positive *Deinococcus geothermalis* T27 that tolerates a broad range of solvents at the comparatively high temperature of 45 ºC (72).

Pseudomonas putida S12

The isolation of the styrene degrading bacterium *Pseudomonas putida* S12 was reported in 1990 (45). This strain is able to grow in a second phase of toluene and in 14 mM benzene, without degrading these solvents. Research

into the remarkable solvent tolerance properties of *P. putida* S12 initially focused on the fatty acid composition of the membranes (50, 52). One of the effects of solvents on bacterial membranes is an increase in fluidity. Cells of *P. putida* S12 grown in the presence of toluene take diverse actions to counteract this effect. It was shown by Heipieper *et al.* (50) that the ratio of *trans* to *cis* unsaturated fatty acids increased at increasing concentrations of toluene. At the same time the degree of saturation of the saturated fatty acids decreased. Also a decrease in the ratio of C_{18} to C_{16} fatty acids was observed. In later years, the properties of the enzyme responsible for the *cis/trans* isomerisation, Cti, as well as the molecular mechanism of the cis-trans isomerisation were extensively studied (51, 54, 144).

Shortly after the first investigations on the fatty acid composition, it was discovered that *P. putida* S12 is able to actively extrude solvent molecules of the cell by an energy-dependent efflux system (59). The transport system was shown to be a tripartite efflux pump of the RND family and it was named Srp, for solvent resistance pump (68). The three genes *srpABC* that together encode the pump were transferred to a different strain of *P. putida* in which they effectuated a significant increase in toluene tolerance (68). The energy-dependency of SrpABC was confirmed by the yield decrease in toluene exposed cells by Isken *et al.* (62) who showed that the biomass yield on glucose decreased with increasing concentrations of toluene up to 3 mM. At higher concentrations, the yield was affected to the same extent as at 3 mM toluene. It was hypothesised that not only the energy consumption of Srp contributed to the yield decrease, but also the uncoupling effect of the solvent molecules embedded in the membrane (62). Conditions and chemicals that induce the promoter of *srpABC* were studied by Kieboom *et al.* (69). It was established that only lipophilic aromatic and aliphatic solvents and alcohols induced the promoter. Environmental stresses like pH, temperature and NaCl did not induce expression of *srpABC*, nor did heavy metals or antibiotics. Antibiotics did not only fail to induce *srpABC* expression, they were also found to be no substrates for the pump (61).

In further studies on the solvent tolerance mechanisms of *P. putida* S12, two other interesting phenomena were uncovered. An insertion sequence was found to be associated with constitutive expression of *srpABC* in cells that had survived a 1-% toluene shock. This insertion sequence, IS*S12*, interrupted *srpS*, one of the regulatory genes of *srpABC*. The interruption caused constitutive expression of the solvent extrusion pump which enabled the cells to survive the sudden toluene shock (147). Recently (127), another insertion sequence was identified in *P. putida* S12. This insertion sequence, IS*Ppu21*, was also found inserted in *srpS* in cells that survived a 20 % toluene shock

exerting a similar effect as IS*S12* (75). Furthermore, a relationship between flagella and solvent tolerance was established. In a transposon-mutagenesis experiment, several solvent sensitive mutants were isolated (65). Many of these mutants were non-motile and appeared to have the transposon inserted in different flagellar genes. The expression of *srpABC* was decreased in these mutants compared to wildtype cells suggesting a direct relationship between the disruption of flagellar genes and the expression level of the solvent extrusion pump.

In the year 2000 a study was conducted to investigate whether toxic fine chemical production could be improved by using the solvent tolerant *P. putida* S12 as a production host (148). The production of 3-methylcatechol increased two-fold using a two-liquid medium-octanol system compared to a single liquid phase system. This study was the start of a series of studies into the biological production of several toxic fine chemicals, from cheap and renewable carbon sources (97, 98, 138, 139, 151).

The availability of the genome sequence of the related strain *P. putida* KT2440 made it possible to design a DNA microarray that was successfully used to analyse gene expression in *P. putida* S12 (5). Very recently, the genome of *P. putida* S12 itself was sequenced (Ruijssenaars and de Winde, manuscript in preparation). A surprising finding was the occurrence of a megaplasmid, on which the Srp pump genes *srpSRABC* were located in addition to other genes that are possibly involved in resistance to chemical aggression. Thus, the solvent tolerant phenotype may be transmissible from one cell to the other by conjugation. A microarray was designed based on the genome sequence of *P. putida* S12, which made it possible to study the response of S12 to organic solvent more accurately and into even more detail.

Part 2: Transcriptomics and proteomics

Transcriptomics and proteomics in solvent tolerance research

The mechanisms of solvent tolerance that were first described before approximately the end of the twentieth century have been elucidated by a traditional reductionist approach. Although useful for identifying individual solvent tolerance mechanisms, this approach does not provide insight into global cellular responses related to solvent stress and the interactive dynamics of solvent tolerance mechanisms upon solvent exposure. Systems-level analysis techniques such as transcriptomics and proteomics are very promising tools for elucidating these aspects of solvent tolerance mechanisms. They can show the global response of cells exposed to toxic organic solvents as well as provide more detailed insight into the established solvent tolerance mechanisms and their interactions. Furthermore, new mechanisms may be uncovered. In the next paragraph, the results of several recent transcriptomics and proteomics studies (Table 1.1) on mechanisms of solvent tolerance are discussed. First, a short description of the techniques used in these studies is presented here.

In the transcriptomics studies that are described in this chapter, two different types of microarrays were used, spotted microarrays (27, 37, 47) and oligonucleotide microarrays (34). Spotted microarrays consist of cDNA sequences or PCR products contact-spotted or ink jet-deposited onto glass slides (83). Oligonucleotide microarrays consist of oligonucleotides synthesised directly onto the glass slides (83). A disadvantage of the very popular spotted arrays is a high background, batch variability and differences in spot morphology. However, the cDNA hybridised to the arrays can be labeled with one of each two cyanine dyes cy3 and cy5, so two samples can be hybridised simultaneously onto one array. Oligonucleotide arrays are a more reliable but also more expensive alternative. The oligonucleotide array system marketed by Affymetrix will be described into more detail below as this system was employed throughout the work described in this thesis. Each gene or transcript on the array is represented by 11-20 probes, which cover different regions of the gene (83). Moreover, each oligo is accompanied by a 'mismatch' counterpart containing a point mutation. This design is used to distinguish background, noise and cross-hybridisation from perfect hybridisation (83). Batch variability and differences in spot morphology no longer exist. Streptavidin-based detection is used to detect the hybridised biotin-labeled cDNA, allowing for measurement of absolute gene expression levels. In view of their high reliability, accuracy and reproducibility, the oligonucleotide microarray system

described above was used to perform the transcriptomics analyses described in this thesis (see Chapters 3, 4 and 5).

The proteomics techniques that are applied in the studies discussed in this chapter are all based on two-dimensional acryl amide gels. The differences between these gel-based methods are mainly found in the protein detection system. To make the protein spots visible, the gels can be stained post-electrophoresis, but the proteins can also be labeled pre-electrophoresis. Post-electrophoresis stains used in the studies that are described in this chapter are colloidal Coomassie (41, 104, 116, 119, 130), silver stain (7, 123, 130) and Sypro Ruby, a fluorescent dye (24).

Post-electrophoresis stains allow the analysis of only a single proteome per gel. To overcome gel-to-gel differences, analytical triplicates should be run. When also biological replicates, ideally triplicates, are included, an experiment in which two conditions are compared consists of eighteen gels. The detection limit of these post-electrophoresis staining procedures lies between 1 ng (silver stain and Sypro Ruby) and 8 ng (colloidal Coomassie). The linear dynamic range of silver stain is reported to be one or two orders of magnitude, of colloidal Coomassie it is between one and three orders of magnitude and the linear dynamic range of Sypro Ruby is around three orders of magnitude (89).

Both the detection limit and linear dynamic range can be increased and the amount of gels can be decreased by using 2D Difference In Gel Electrophoresis (2D-DIGE (84)), a pre-electrophoresis labeling technique. Two studies described in this chapter use 2D-DIGE (136, 154). Using this technique, the proteins are labeled with one of three fluorescent cyanine dyes Cy2, Cy3 or Cy5. One of the dyes is used to label an internal standard and the other two to label a proteome. The three of them are then run simultaneously on the same gel. Because of the use of an internal standard, analytical triplicates are no longer required. An experiment in which two conditions are compared thus consists of only three gels. The detection limit of 2D-DIGE can be as low as 0.1 ng and the linear dynamic range lies between three and five orders of magnitude (89). This, together with its great reproducibility and thus statistically robust results, and the decrease in amount of gels, makes 2D-DIGE the preferred technique for gel-based proteomics studies (81, 149). The proteomics studies described in Chapters 2 and 5 were performed using 2D-DIGE.

Insights into mechanisms of solvent tolerance from transcriptomics and proteomics analyses – an overview

In the past decade many studies have been published that described the –omics analysis of microorganisms exposed to organic solvents. Most of

these studies concerned either microorganisms that produced or degraded solvents, like for example *Clostridium acetobutylicum* producing butanol (2), or *Nitrosomonas europaea* degrading toluene and benzene (107). This thesis focuses on the solvent tolerance mechanisms of *Pseudomonas putida* S12, that produces nor degrades the investigated organic solvents benzene and toluene. Therefore, this overview will be mostly restricted to studies that focused on the effects of aromatic solvents on similar microorganisms, *i.e.*, Gram-negative, non-solvent degrading, non-solventogenic bacteria. Some studies on solvent degraders and non-aromatic alkanes were included because of their generic informative value on mechanisms of solvent tolerance. Studies investigating only part of the proteome or transcriptome, or that concerned mutant strains, were excluded. The selected –omics studies are briefly described in Table 1.1. The –omics responses are classified by regions of the cell or functional groups of genes and proteins that could be related to organic solvent tolerance.

Table 1.1 Studies that are described and discussed in this chapter.

Ref.	Strain	P/T[a]	Solvent[b] (logP)[c]	Culturing/sampling methods
	Gram-negative bacteria			
(116)	*Alcanivorax borkumensis* SK2	P	Hexadecane (8.8)	Batch, sampling in early exponential phase.
(136)	*"Aromatoleum aromaticum"* EbN1	P	Toluene (2.5) Phenol (1.5) *p*-cresol (1.9)* Ethylbenzene (2.9)*	Batch, samples taken either during growth with solvents (timepoint unknown) or 45, 250 or 600 minutes after shock with solvents.
(154)	*"Aromatoleum aromaticum"* EbN1	P	Benzoate (1.9) Benzaldehyde (1.7)* *p*-cresol (1.9)* Phenol (1.5) *o*-amino-benzoate (1.5)*	Batch, adapted cells were sampled in exponential phase.
(24)	*Burkholderia xenovorans* LB400	P	Biphenyl (3.7)*	Batch, sampling in exponential phase and transition phase between the exp. and stationary phase. The results were added up.

Continued on next page

Table 1.1 *Continued*

(47)	*Escherichia coli* JA300 *Escherichia coli* OST3410	T	Hexane (3.5)	30 minutes of hexane exposure in batch in exponential phase. Parent JA300 and tolerant mutant OST3410 were exposed to hexane and both compared to JA300 without hexane.
(34)	*Pseudomonas aeruginosa* PAO1	T	Pentachlorophenol (4.8)*	Chemostat with increasing level of PCP, sampling at 6.5, 13 and 26 hours after start of PCP feed.
(123)	*Pseudomonas putida* DOT-T1E	P	Toluene (2.5)	Batch, sampling in exponential phase.
(41)	*Pseudomonas putida* KT2440	P	Phenol (1.5)	Batch, sampling in exponential phase.
(119)	*Pseudomonas putida* KT2440	P	Phenol (1.5)	One hour phenol in batch, sampling in exponential phase.
(7)	*Pseudomonas putida* KT2440	P	2,4-dichlorophenol (3.0)*	Batch, not clear when samples were taken.
(27)	*Pseudomonas putida* KT2440 (pWW0)	T	Toluene (2.5) *o*-Xylene (3.1)	15 minutes of toluene or *o*-xylene exposure in batch in exponential phase.
(120)	*Pseudomonas* sp. M1	P	Phenol (1.5)	Two hours of phenol exposure in batch in exponential phase.
	Gram-positive bacteria			
(104)	*Acinetobacter radioresistens* S13	P	Phenol. (1.5)	Only membrane proteome. Batch, sampling in late exponential phase.
(130)	*Bacillus subtilis* 168	P, T	Phenol (1.5)	10 minutes of phenol exposure in batch in exponential phase.
	Yeast			
(37)	*Saccharomyces cerevisiae* S288C	T	1-Octanol (2.9) Pentane (3.0)	2 hours of 1-octanol or pentane exposure in batch in exponential phase.

a) P = proteomics, T = transcriptomics
b) Only toxic organic solvents relevant for this review are mentioned, other stresses or solvents investigated in these studies are not discussed.
c) LogP is the partition coefficient of a solvent in an octanol/water system, values taken from Laane *et al.* (75), except for the solvents marked with (*) of which logP was calculated with the free online cheminformatics service at www.molinspiration.com/cgi-bin/properties.

The outer cell structure

Non-polar organic solvents are lipophilic and therefore accumulate in bacterial membranes. The outer cell structure of Gram-negative bacteria is comprised of the lipopolysaccharide layer, the outer membrane, the periplasmic

space, the peptidoglycan layer and the inner membrane (Figure 1.2). The outer layers form barriers that may prevent entry of solvents whereas the cytoplasmic membrane is the most important target site for solvents. Therefore, extensive expression responses may be expected of genes and proteins associated with the outer cell structure upon solvent exposure.

Fatty acids

In cells exposed to solvents, changes in the ratio of saturated and unsaturated fatty acids can be found. Whether the ratio decreases or increases depends on the solvent: polar water-miscible solvents cause an increase in the content of unsaturated fatty acids, whereas more lipophilic solvents that still are water soluble but not completely miscible cause an increase in the content of saturated fatty acids (125). Synthesis of saturated fatty acids can only be

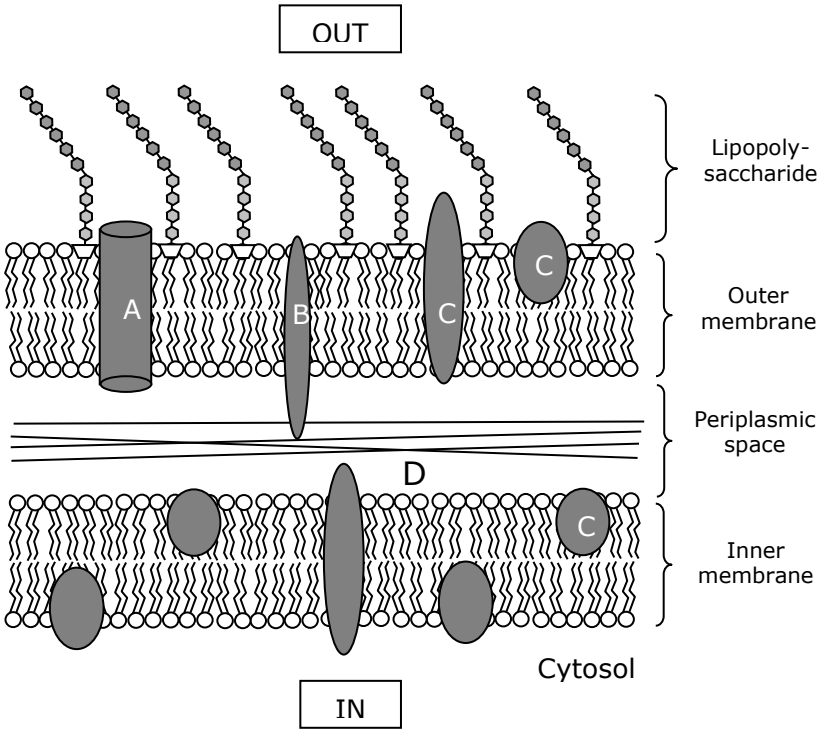

Figure 1.2 Schematic representation of the cell wall of Gram-negative bacteria. A) porin; B) murein lipoprotein; C) membrane protein; D) peptidoglycan.

accomplished by *de novo* synthesis (60). Unsaturated fatty acids can either be synthesised *de novo* (16) or can be converted from saturated fatty acids by fatty acid desaturase (159). In several studies into the effects of the polar solvent phenol, genes involved in the synthesis of unsaturated fatty acids were up-regulated (27, 41, 130). The up-regulation of one of these proteins, FabB, in *Alkanivorax borkumensis* under hexadecane stress (116) however, was unexpected because this solvent is non-polar. The authors explain this phenomenon by a possible accumulation of AlkB alkane hydroxylase protein in the inner membrane of cells grown on alkanes. AlkB protein perturbs the membrane structure and unsaturated fatty acids are needed to maintain membrane fluidity and integrity. This also explains the up-regulation of fatty acid desaturase in the same organism. Another interesting finding was the up-regulation of Cls, cardiolipin synthase, in *A. borkumensis* (116). Cardiolipin was reported to decrease membrane permeability to organic solvents (143) and, in *P. putida*, the corresponding gene, *cls*, is constitutively expressed (10).

The up-regulation of diverse genes or proteins involved in fatty acid metabolism in *P. putida* S12 and KT2440 (27, 41), *A. borkumensis* (116) and "*Aromatoleum aromaticum*" EbN1 (136), suggest a higher fatty acid turn-over rate. In strain EbN1 however, also two genes related to fatty acid biosynthesis were down-regulated (136).

Lipopolysaccharides and peptidoglycan

The lipopolysaccharide (LPS) layer is important in solvent tolerance because it determines the hydrophobicity of the cells' exterior, in conjunction with the other cell wall components. There is evidence that bacterial cells with a higher outer cell-hydrophobicity are more sensitive to organic solvents than cells with a lower hydrophobicity (4, 71). In several studies of bacteria cultured in the presence of organic solvents, genes and proteins of the LPS biosynthesis pathway were differentially expressed, suggesting an elevated activity, probably resulting in a more hydrophobic cell exterior (7, 27, 104, 119, 154).

No evidence has been found so far that the peptidoglycan layer of the cell wall is influenced by organic solvents. However, it is discussed here shortly because it is part of the outer cell structure and differential expression of several peptidoglycan biosynthesis genes has been observed in *P. putida* KT2440 when exposed to phenol (119), toluene or *o*-xylene (27). The homolog of one of the genes that was down-regulated under toluene stress in *P. putida* KT2440, *mpl* (27), was shown to cause large oval-to-spherical cells and lysis when over-expressed in *E. coli* (87). Although the exact role of the *mpl* gene is unclear, the observed down-regulation in *P. putida* KT2440 upon solvent

exposure is in agreement with the decreased membrane stability apparently associated with the over-expression of this gene.

Flagella and pili

The existence of a relationship between flagella and solvent tolerance has been established in numerous studies. Kieboom *et al.* (65) isolated solvent sensitive transposon mutants of *P. putida* S12 that were not motile because several flagella-related genes were interrupted by the transposon. The authors also found a decreased activity of the *srp* promoter in these mutants resulting in a decreased expression of the solvent efflux pump genes *srpABC*. It was hypothesised that the regulatory mechanism of the *srp* operon was somehow interlinked with the regulatory mechanism of the flagella biosynthetic pathway. Amongst the organisms described here, differential expression of flagella-related genes was only found in *P. putida* KT2440 (27, 41) and *E. coli* (47). Most of the flagellar genes that responded to solvent exposure were down-regulated; these genes were furthermore different from the genes found by Kieboom *et al.* (65) to be associated with solvent tolerance. Since *P. putida* KT2440 does not harbour the *srp* operon, another connection between solvent tolerance and down-regulation of flagellar systems must exist. Possibly, solvent molecules are prevented from entering the cell through or alongside the anchor point or the basal body rings in the membrane, or via the transport system that translocates flagellar parts across the membrane. Both of these systems form 'holes' in the cytoplasmic membrane through which solvents may enter the cell. Alternatively, abandoning or decreasing motility may save energy to compensate the uncoupling effect brought about by organic solvents. Moreover, regulatory effects could occur as solvent-associated responses and the flagellar system may share common regulators. Although it is evident that a relationship between flagella and solvent tolerance exists, the exact nature remains to be elucidated.

Pili-related genes and proteins were found to be down-regulated in two studies (27, 116). Pili can have different functions in bacterial cells; they are involved in attachment to other cells but they also play a role in motility (17, 99). The expression of pili genes can be influenced by many environmental factors, such as temperature, pH and osmolarity (73), although the influence of organic solvents was unknown until the publication of the two abovementioned studies. The differential expression of pilus-related genes and proteins could be a side-effect caused by the differential expression of other genes. However, a direct relationship with the effects of solvents on the outer cell structure cannot be excluded.

The –omics analyses of various organisms reconfirmed that there is an effect of organic solvents on the outer cell structure, especially on the fatty acid content. The –omics techniques also revealed several new genes and proteins possibly involved in solvent tolerance. No relationship between peptidoglycan associated genes and solvents was known before the start of the –omics research. This also holds for pili-related genes and proteins. In view of earlier findings, the differential expression of flagellar genes was expected. However, the novel flagellar genes identified in the –omics analyses were different from those earlier identified as being associated with solvent tolerance, in both nature and expression behaviour. Thus, little additional insights were obtained into the relationship between flagella and solvent tolerance.

Pumps, porins and transporters

Microorganisms harbour a wide variety of pumps, porins and transporters embedded in their membranes. The changes that occur in the (outer) membranes of cells exposed to organic solvents influence the functioning of enzymes, and thus also transporters, embedded in or bound to them (125). When solvent molecules accumulate in the membrane, it can easily be envisaged that the interactions between the membrane lipids and the enzymes change. Also the water layer surrounding and stabilising the parts of the enzymes that are at the outside of the cells is distorted by the addition of organic solvents to the medium (75). In view of these considerations, changes in the expression of pump, porin and transport genes and proteins are expected.

The most important types of differentially expressed transporters are the solvent efflux pumps and the multidrug efflux pumps. In several strains of *Pseudomonas putida* solvent efflux pumps were identified that extrude organic solvent molecules from the cell and thus form an excellent solvent resistance mechanism. In *P. putida* S12, SrpABC was identified as a toluene efflux pump (68). This pump is homologous, but not identical, to TtgGHI found in *P. putida* DOT-T1E, which also harbours the solvent pumps TtgABC and TtgDEF (113). Genes encoding TtgABC were also identified in the genome sequence of *P. putida* KT2440 (94). In *P. putida* KT2440 (pWW0) grown in the presence of *o*-xylene *ttgC* and the regulatory gene *ttgR* were found to be up-regulated, and when cultured in the presence of toluene *ttgACR* were up-regulated (27). This response is opposite to the response of *P. putida* DOT-T1E, in which *ttgABC* is expressed to high levels in cells growing in the absence of toluene but lower levels in cells growing with toluene (28). In this strain however, two other solvent efflux pumps are present the expression or regulation of which might influence the regulation of *ttgABC*. In *P. aeruginosa* the MexAB-OprM transporter is responsible for efflux of solvents and antibiotics (80). When

exposed to pentachlorophenol, the genes coding for this system were up-regulated, as expected (34).

In organisms exposed to toxic organic solvents, also multidrug efflux pumps were found to be up-regulated. Most of these multidrug transporters are uncharacterised in that their substrates are unknown. It cannot be excluded that at least some of these efflux pumps also pump organic solvents out of the cell, as is the case for MexAB-OprM. In addition to this system, two other genes coding for a multidrug resistance protein and a drug efflux transporter were up-regulated in *P. aeruginosa* exposed to pentachlorophenol. The other strains in which uncharacterised or incompletely characterised multidrug efflux pumps were found to be up-regulated are *P. putida* KT2440 (pWW0) (27), *A. borkumensis* SK2 (116) and *E. coli* OST3410 (47). In *Pseudomonas* sp. M1 and *P. putida* KT2440 exposed to phenol TolC was up-regulated (119, 120). TolC is important for solvent tolerance in *E. coli* as a part of different efflux pumps (108) and may have the same function in *Pseudomonas* sp. M1 and *P. putida* KT2440.

In addition to solvent and multidrug efflux pumps, a wealth of other transporters was differentially expressed in many of the organisms when exposed to solvents. The number of differentially expressed transporters in response to solvent addition, their substrate molecules and the directionality of the differential expression vary greatly between or even within studies. This diversity can be attributed to the variety of organisms, solvents and culturing methods: different organisms harbour different transporters and each environment requires other types of transporters. At the same time, all transporters reside in the same solvent-affected membrane and likely suffer similar effects from the change in environment. Thus, generic expression changes may be expected at the level of transport-associated genes, in addition to specific effects for particular types of transporters.

Energy-associated genes and proteins

Solvent molecules entering the membrane cause leakage of protons and, thus, cause dissipation of the proton motive force. Moreover, solvent efflux pumps are energy consuming. It may therefore be expected that genes and proteins related to the energy household of solvent-exposed cells are differentially expressed compared to non-solvent exposed cells. In all studies described here, except that on *Acinetobacter radioresistens* S13 (104), differential expression of energy-associated genes or proteins was found. Most of these were up-regulated and comprised of genes or proteins associated with the energy-status of the cells, being involved in oxidative phosphorylation, the citric acid cycle, and carbon utilisation. However, in the study of Dominguez-

Cuevas *et al.* (27) on *P. putida* KT2440 exposed to toluene or xylene, the fraction of down-regulated energy-associated genes was significantly larger. As these genes belong to the same functional groups as the up-regulated genes in the other studies, the results seem to be contradictory. This effect can probably be attributed to the time frame of solvent exposure, being only 15 min instead of 30 min to 26 h. In this case, mostly an initial stress response was investigated whereas in the other studies the cells had reached a more solvent-adapted state.

Although energy-related genes and proteins commonly respond in a similar manner to solvent exposure, the function of the responding genes and proteins is highly diverse as illustrated by the effect of phenol on *P. putida* KT2440 (41, 119), *Pseudomonas* sp. M1 (120), *B. subtilis* (130) and *"A. aromaticum"* EbN1 (136). In both *P. putida* KT2440 studies the citric acid cycle protein aconitate hydratase AcnB was up-regulated, whereas an ATP synthase protein was up-regulated in one study (119) and down-regulated in the other (41). In *Pseudomonas* sp. M1 two proteins of ATP synthase were up-regulated (120) and in the other studies no such protein was differentially expressed. The up-regulated genes and proteins involved in carbon catabolism and storage range from transaldolase in *P. putida* KT2440 (119) to glucose dehydrogenase in *B. subtilis* (130) and acetoacetyl-CoA reductase, glutamine synthetase and poly(3-hydroxyalkanoate)synthase in *"A. aromaticum"* EbN1 exposed to a mixture of phenol and *p*-cresol (136). This latter finding is peculiar because the synthesis of storage materials like polyhydroxyalkanoate (PHA) conflicts with energy production that would be expected to prevail under solvent-stressed conditions.

The diversity in responses shows that each organism responds in its own unique way to the energy-depleting effect of organic solvents. However, the different growth conditions and duration of solvent-exposure makes is difficult to compare the studies and will have an intrinsic effect as well. Standardised experiments are necessary to improve the comparability between studies and to investigate the effect of experimental conditions into depth.

Stress responses

When microorganisms encounter sub-optimal environmental conditions, a stress reaction starts to take place. The heat shock response is the classical example; upon sudden exposure to high temperatures, cells quickly synthesise a set of proteins to protect them against the devastating effects of the high temperature. Toxic organic solvents do not cause such a distinct and specific stress response. However, genes or proteins that are generally regarded

as stress-response genes are frequently differentially expressed upon addition of organic solvents.

Heat shock proteins have often been found to be differentially expressed after addition of solvents (7, 24, 27, 34, 37, 41, 47, 119, 120, 123, 130, 136). Chaperonin subunits GroES, GroEL, and/or DnaJK were up-regulated in *P. putida* KT2440 (27, 41), *P. putida* DOT-T1E (123), *Burkholderia xenovorans* LB400 (24) and *B. subtilis* 168 (130) whereas these were down-regulated in another study on *P. putida* KT2440 (7) and in *P. aeruginosa* PAO1 (34). Other differentially expressed heat shock proteins range from HtpG that was up-regulated in all *P. putida* KT2440 studies except (7) to HSP12 that was up-regulated in *Saccharomyces cerevisiae* S288C exposed to octanol, and down-regulated when exposed to pentane (37). The differential expression of heat shock proteins in cells exposed to organic solvents suggest that similarities exist between heat stress and solvent stress. The disruption of cellular membranes and subsequent release of membrane-bound proteins by organic solvents causes misfolding of these proteins, as do high temperatures. It is possible that the misfolding of proteins under solvent stress induce a heat shock response.

In several studies (7, 24, 27, 41, 47, 119, 120, 130, 136) genes and proteins were up-regulated that suggest that the cells experience oxidative stress. Examples are hydroperoxidase (47), alkyl hydroperoxide reductases (24) and oxidoreductases (130). Dominguez *et al.* (27) monitored hydrogen peroxide production in *P. putida* KT2440 after addition of toluene, xylene or 3-methylbenzoate. They found a consistent increase in hydrogen peroxide production and it was hypothesised that the impairment of electron transfer after addition of solvents may lead to the generation of active oxygen species in the respiratory chain.

In addition to members of the two groups described above, also other stress-response genes and proteins were found to be differentially expressed, *e.g.*, DNA repair genes (27), antibiotic resistance proteins and a phage shock operon (47), as well as a cold acclimation gene (34). It is possible that the cells experience a situation that resembles the type of stress that these genes would normally respond to. The differential expression of these genes also may be a secondary effect caused by the differential expression of regulators involved in the expression of genes with a more obvious relation to solvent tolerance.

Part III: Outline of this thesis

In the preceding part of this introductory chapter, the current knowledge about solvent tolerance in bacteria was described, especially the knowledge that was gained with proteomics and transcriptomics. In the research described in this thesis, both techniques were used to investigate the mechanisms of benzene- and toluene-tolerance of *P. putida* S12. Information about these mechanisms is important for the development of two-phase water-organic solvent fermentations, that could be used for the production of fine chemicals by microorganisms. These systems allow for efficient product removal during fermentation and hence a higher production yield. The organic solvents used however, are often toxic to bacteria, as are the (fine) chemicals that are produced. To successfully use these systems, a highly solvent tolerant bacterium like *Pseudomonas putida* S12 is mandatory. Insight into the mechanisms of solvent tolerance will greatly assist process control and optimisation of the bioproduction of fine chemicals.

The development of whole genome- and whole proteome analysis techniques enabled a systems-level study of the response of *P. putida* S12 to toxic organic solvents. This thesis starts with a 2D-DIGE proteomics study of *P. putida* S12 cultured in the presence of 3 (sub-lethal) and 5 mM (lethal to non-solvent tolerant bacteria) toluene (**Chapter 2**). The use of chemostats for culturing ensured constant growth conditions, to allow for a comparison between presence and absence of toluene without interference of growth rate-related effects. Notable differences in abundance of proteins involved in the energy metabolism were observed, *e.g.* several proteins of the citric acid cycle had an increased abundance in the presence of toluene.

In **Chapter 3**, chemostat-cultured toluene exposed *P. putida* S12 was analysed at the transcriptome level. In addition to the confirmation that the energy metabolism is indeed important in solvent-exposed *P. putida* S12, this transcriptome analysis resulted in another important finding. A hypothetical protein of which the abundance was shown to decrease in the presence of toluene in the proteomics study also showed decreased corresponding mRNA levels. The hypothetical protein was renamed TrgI and knock-out- and over-expression mutants of the corresponding gene were constructed. In Chapter 3, the physiological analysis of these mutants is described and a hypothesis about the function of *trgI* is formulated.

The assumed function of *trgI* is further investigated in **Chapter 4**, in which *P. putida* S12 and the knock-out mutant *P. putida* S12ΔTrgI were

subjected to sudden exposure to 5 mM toluene. This experiment was carried out in exponentially growing batch cultures that were not adapted to the presence of toluene. The transcriptional response of both strains was monitored during the first 30 min of exposure, providing interesting leads to the function of *trgI* as well as a fascinating view into the early response of *P. putida* S12 to toluene. To our knowledge, this is the first experiment in which such an early response to an organic solvent has been purposely investigated.

In **Chapter 5** an alternative way to examine the mechanisms of solvent tolerance of *P. putida* S12 is described. A mutant with improved benzene tolerance was obtained by exposing the wildtype to increasing concentrations of benzene, thus performing laboratory-scale evolutionary selection. This mutant was subsequently cultured in chemostats in the presence and absence of benzene and its proteome and transcriptome were analysed and compared to the wildtype. This analysis, together with the results of the previous chapters, clearly showed that the flexibility of the energy-generating systems is an essential aspect of solvent tolerance that was not recognized as such before the –omics era. This is discussed in **Chapter 6**.

Chemostat-based proteomics analysis of toluene-affected *Pseudomonas putida* S12

The aim of this study was to assess the cellular response of the solvent-tolerant *Pseudomonas putida* S12 to toluene as the single effector. Proteomics analysis (2-Dimensional Difference-In-Gel-Electrophoresis) was used to assess the response of *P. putida* S12 cultured in chemostats. This approach ensures constant growth conditions, both in the presence and absence of toluene. A considerable negative effect of toluene on the cell yield was found. The need for energy in the defense against toluene was reflected by differentially expressed proteins for cell energy management. In toluene-stressed cells the balance between proton motive force (PMF) enforcing and dissipating systems was shifted. NAD(P)H generating systems were up-regulated whereas the major proton driven system, ATP synthase, was down-regulated. Other differentially expressed proteins were identified: outer membrane proteins; transport proteins; stress related proteins and translation-related proteins. In addition, a protein with no assigned function was found. This study yielded a more detailed view of the effect of toluene on the intracellular energy management of *P. putida* S12 and several novel leads have been obtained for further targeted investigations.

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Introduction

Solvent-tolerant bacteria are quite exceptional in their ability to withstand a wide range of toxic organic solvents in concentrations that are lethal to most micro-organisms (60, 67, 108). These solvents easily accumulate in lipid membranes of living cells, thus causing an increase of membrane fluidity and a decrease of bilayer stability (125, 145). The proton motive force (PMF) is negatively affected by solvent exposure, due to passive flux of protons across the membrane. Furthermore, the interaction between membrane-embedded proteins and lipid molecules alters (125). Three types of membrane-associated solvent tolerance mechanisms have been elucidated: (i) cytoplasmic membrane changes, (ii) outer membrane changes, (iii) active efflux of organic solvents (55, 60, 67, 108, 110, 145). Solvent-tolerant Pseudomonads have all three modes of solvent tolerance, but it is probable that other mechanisms are active and that there are cross-relations between the pathways in these mechanisms.

Proteome and transcriptome analyses allow the study of global cellular response to solvents like toluene. These techniques offer a tool for uncovering new mechanisms of solvent tolerance, as well as establishing their interrelation and cellular regulation. The proteomics approach has recently been followed for *Pseudomonas putida* in studying the cellular response of strain KT2440 to several toxic compounds. The work of Hallsworth *et al.* (41) on the effect of chaotropic compounds, demonstrated up-regulation of proteins involved in stabilization of biological macromolecules and membrane structures. Phenol-induced stress in strain KT2440, as studied by Santos *et al.* (119), revealed up-regulation of 68 and down-regulation of 13 specific proteins. In their solvent-tolerant DOT-T1E strain, Segura *et al.* (123) observed new solvent-related proteins. The general picture that emerged from these three studies is that proteomic analysis indeed provides a handle to study adaptational changes in *P. putida* as affected by toxic compounds.

Proteomics stress-related studies in bacteria, including those mentioned above, usually have been carried out with batch-grown cells (41, 74, 118, 119, 123). In batch cultures, the environment of the cell changes continuously and dramatically (56).

In this study we aimed to further uncover the cellular responses of *P. putida* S12 (45) to toluene as the single effector. We analyzed the proteome of toluene-affected S12 in chemostats. Strain S12 is used as a platform for the production of aromatics (97, 151). By studying the cellular response to product accumulation under defined bioreactor conditions, leads to bacterial

counteraction of product toxicity may be obtained. Strain S12 does not metabolize toluene, and consequently observed effects are not correlated to proteins involved in toluene degradation. It was found previously that different nutrient limitations have a distinct effect on gene expression (11). Obviously, only proteins differentially expressed in the presence of toluene in both media are truly involved in a response linked to toluene stress. Consequently, we chose to monitor the effect of toluene under conditions of either nitrogen or carbon limitation.

Results and discussion

P. putida S12 was cultured in chemostats in mineral glucose medium either in the presence or absence of 5 mM of toluene. This compound has been used previously as a model to studysolvent tolerance in *P. putida* (62, 68, 123, 147). A concentration of 5 mM of toluene is near water saturation and it is well above the threshold that triggers the adaptational responses of *P. putida* S12 to toluene (69). Carbon- or nitrogen limited steady state conditions were obtained by variation of the glucose/NH_4Cl ratio in the mineral medium. *P. putida* utilizes glucose via extracellular conversion into keto-gluconate, via gluconate. In the carbon-limited culture, all glucose was consumed and no residual gluconate or keto-gluconate remained (Table 2.1). In the nitrogen-limited culture, indeed all nitrogen was consumed. Under toluene stress, the biomass yields decreased by 45% and 56% in the N-limited- and C-limited medium, respectively (Table 2.1). Previously, Isken *et al.* (62) reported a comparable decrease in cell yield of continuously cultured *P. putida* S12 in the presence of supersaturating amounts of toluene under C-limited conditions. These observations imply that the energy supply of the cells is severely compromised in the presence of toluene.

Two phenomena that lower the energy status of the cell could account for these effects. Firstly, the presence of toxic levels of toluene severely perturbs the membrane, bringing about the passive flux of protons across the membrane (18, 32, 76, 124), thus dissipating the PMF (18, 124). Secondly, toluene induces expression of the solvent-resistance pump SrpABC (68, 69), which is a proton-dependent solvent-efflux system (59, 62). These two effects result in a drain of energized protons causing the compromised energy supply.

In order to gain insight in the effect of toluene on the cellular response, the proteomes of *P. putida* S12 grown in chemostats under the four different

conditions mentioned above (presence/absence of toluene and N/C-limitation) were analyzed by two-dimensional Difference-In-Gel-Electrophoresis (2D-DIGE) (Figure 2.1). This technique was used to accurately and reliably determine differential protein expression levels (84). This procedure yielded 80 protein spots of interest, from which we were able to identify 21 different proteinsby mass spectrometry (Figure 2.1). Except for the tellurium resistance protein TerZ, all proteins showed highest similarity with proteins from the sequenced strain *P. putida* KT2440 (NCBI access no. NC_002947). The establishment of protein identity allowed a more detailed consideration of the various processes of solvent resistance.

Table 2.1 Cell dry weight (CDW), amount of glucose consumed, gluconate and keto-gluconate produced and yield of *P. putida* S12 during continuous culturing in N-limited- and C-limited mineral medium, in the absence and presence of 5 mM toluene. Values are the means of two independent experiments.

	Culture conditions[a]			
	N-limited mineral medium		C-limited mineral medium	
	0 mM toluene	5 mM toluene	0 mM toluene	5 mM toluene
CDW ($g \cdot l^{-1}$)	0.65	0.37	0.74	0.33
Glucose consumed ($g \cdot l^{-1}$)	4.24	4.87	1.6	1.6
Yield (g protein $\cdot g^{-1}$ glucose)	0.09	0.05	0.28	0.12
Gluconate produced (g)	0.28	0.41	0	0
Keto-gluconate produced (g)	2.81	2.90	0	0

a) Mineral salts medium (MM) (44) with NH_4Cl as the nitrogen source and glucose as the carbon source was used as a standard medium. MM with a carbon limitation contained 10 mM glucose and 30 mM NH_4Cl (C:N = 2:1), MM with a nitrogen limitation contained 44 mM glucose and 4 mM NH_4Cl (C:N = 66:1). Continuous culturing was done in chemostats (BioFloIIc, New Brunswick Scientific) with a working volume of 1.0 litre, pH 7.0, 350 rpm, 30°C, 5 l/h oxygen and a dilution rate of 0.2 h^{-1} in duplicate. Toluene was added separately from the medium with a KD Scientific syringe pump (Applikon). Samples were taken at steady state, which was reached after 5 volume changes.

The need for maintaining the PMF in the presence of toluene was perfectly reflected by the differential expression of energy management related proteins, some of which have not been found in relation with (solvent-) stress before (Table 2.2). Five enzymes of the TCA cycle, most of which are involved in NADH producing steps, were up-regulated in the presence of toluene (Figure 2.2). Other differentially expressed proteins, GuaB and FabG, for which no obvious relationship with toluene stress exists, were also categorized in this

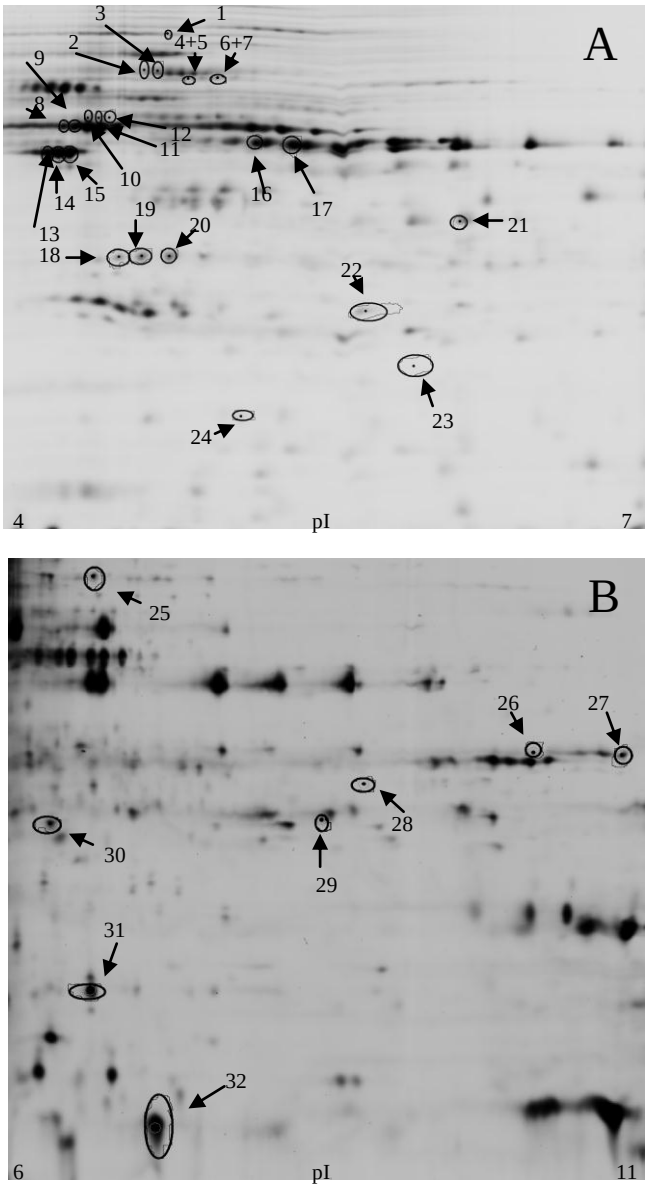

Figure 2.1 2D-DIGE gels of the cytosolic proteome of continuously cultured *P. putida* S12. A) pI range 4-7 B) pI range 6-11. The gels were scanned on a Typhoon 9400 Imager at 100 µm resolution. Image-analysis was done using the DeCyder™ software, versions 5.01 and 6.0 (all GE Healthcare). Encircled spots were up- or down-regulated in the presence of 5 mM toluene vs. the absence of toluene with an average ratio of 1.2 or higher and 0.8 or lower and a Student's T-test *p*-value of 0.03 or below in a duplicate experiment. These spots were analyzed with MS. Triplicate samples of each chemostat were included in the 2D-DIGE analyses. The experiment (culturing and proteomics analysis) was done in duplicate.

group. The up-regulated protein GuaB catalyses the reaction from IMP (inosine-5-monophosphate) to XMP (xanthosine-5-monophosphate) and generates NADH in this process (48). The down-regulated protein FabG is part of the fatty acid biosynthesis pathway and consumes NADPH. Thus, the

observed differential expression of both proteins fits well in the cells' strategy tokeep the cellular NAD(P)H levels sufficiently high under toluene stress. AtpF, a protein that is part of the F_0 subunit of ATP synthase, was found to be dramatically down-regulated in the presence of toluene. As the production of ATP mainly is a proton-gradient driven process, it competes with the solvent pump and with proton leakage for the PMF. Therefore, several new aspects of energy management in *P. putida* S12 upon solvent stress have been uncovered.

Table 2.2 Differentially expressed proteins in *P. putida* S12 in the presence of 5 mM toluene vs. the absence of toluene.

Spot no.	Protein[a]	Accession no.	Ratio±SD[b]	N/C/D[c]
	Energy household related proteins			
26	Succinate dehydrogenase, iron-sulphur protein, SdhB	NP_746307	1.89	N
21	Succinyl-CoA synthetase, α-subunit, SucD	NP_746302	1.88±0.37	C, (123)[d]
1	Isocitrate dehydrogenase, NADP-dependent, monomeric-type	NP_746142	1.6±0.13	N
4-7	Fumarate hydratase, class II, FumC-1	NP_743105	1.52±0.15	N
4-7	2-oxoglutarate dehydrogenase, lipoamide dehydrogenase component, LpdG	NP_746304	1.52±0.15	C, (123)
24	ATP synthase F0, B subunit, AtpF	NP_747517	0.14±0.04	C, (42)[e]; (119)[e]
25	Inosine-5-monophosphate dehydrogenase, GuaB	NP_743192	1.61±0.18	N
28	3-oxoacyl-(acyl-carrier-protein)-reductase, FabG	NP_744068	0.72±0.09	N
	Outer membrane proteins			
23	Outer membrane protein H1, OprH	NP_743345	12.01±2.82	C, (42)
13-15	Outer membrane protein, OprF	NP_744239	0.66±0.13	C, (42)
	Transport proteins			
18-20	Amino acid ABC transporter, periplasmic amino acid-binding protein	NP_742449	0.57±0.06	N
16, 17	Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein, BraC	NP_743302	0.33±0.13	D, (119)
	Stress related proteins			
2-7	Chaperonin, 60 kDa, GroEL	NP_743520	1.71±0.08	C, (42); (123)
32	Cold shock domain family protein	NP_743146	0.67±0.16	N
31	Organic hydroperoxide resistance protein, Ohr	NP_744014	0.65±0.12	N
22	Tellurium resistance protein, TerZ	NP_790779	0.57±0.05	N
	Translation related proteins			
8-12	Elongation factor Tu-B (EF-Tu-B), TufB	Q88QN7	0.56±0.13	D, (123)
27	Ribosomal protein L1, RplA	NP_742610	1.38	C, (42)[f]

Continued on next page

Table 2.2 *Continued*

31	Ribosome-binding factor A, RbfA (cold – shock)	AAN70283	0.65±0.12	N
29	Ribosome recycling factor, Frr	NP_743751	0.48±0.07	N
	Hypothetical protein			
30	Hypothetical protein PP3611	NP_745747	0.34±0.11	N

a) Protein identification was done by matrix-assisted laser desorption ionisation (MALDI) mass spectrometry and micro-liquid chromatography electrospray tandem mass spectrometry (μLC-ESI MS/MS). Trypsin digest (46) from selected spots were first subjected to MALDI MS, with peptide map matching by MASCOT (103) to the NCBI non-redundant database (search limitation: Bacteria). When the MASCOT probability based match factor was larger than (>53 was significant, $p<0.05$, when compared with random matching probability), no further MS experiments were done. In those cases where MALDI MS did not produce reliable results, samples were subjected to μLC-ESI MS/MS, to obtain amino acid sequence information from observed digest peptides. The sequence information thus obtained provides unequivocal information on the protein identity.
b) Ratio's are the average of all spots per protein in both experiments.
c) New (N), Confirmatory (C), Discrepant (D) compared to proteomics results in relevant literature, references in parentheses.
d) Succinyl-CoA synthetase, ß-subunit (SucC)
e) ATP synthase delta chain (AtpH). [f] Ribosomal protein RpsA

Among the outer membrane proteins (OMPs), OprF was down-regulated in the presence of toluene. OprF is a major membrane protein in *P. aeruginosa*, where it has a non-specific porin function (43). Li *et al.* (79) showed that an *oprF*- mutant of *P. aeruginosa* was toluene-tolerant, and therefore it was proposed that toluene enters the cell through the OprF channel in wild-type cells. Our proteomics study supports this hypothesis for another Pseudomonad, with the observation that *P. putida* S12 appears competent to shut down such solvent channels upon toluene stress. The OMP OprH showed the most dramatic up-regulation, by a factor of 12, of all proteins analyzed OprH has a function in positive support of membrane stabilization (6). It was previously found to play a role in resistance to the antibiotics polymyxin B and gentamicin and also to EDTA under Mg^{2+}-deficiency (6). The above results strongly indicate that *P. putida* S12 counteracts toluene stress by preventing influx through the OprF channel and by increasing membrane stabilization by the incorporation of higher levels of OprH.

The observed up-regulation of GroESL relates well to proteomics results of other studies with solvent-stressed *P. putida* strains (123). Overexpression of *groESL* in *Clostridium acetobutylicum* (135), in *Lactococcus lactis* and in *Lactobacillus paracasei* (25) also led to increased solvent-tolerance. Other differentially expressed proteins were hydroperoxide resistance protein Ohr, tellurium resistance protein TerZ, amino acid transporters, a hypothetical protein and several translation related proteins

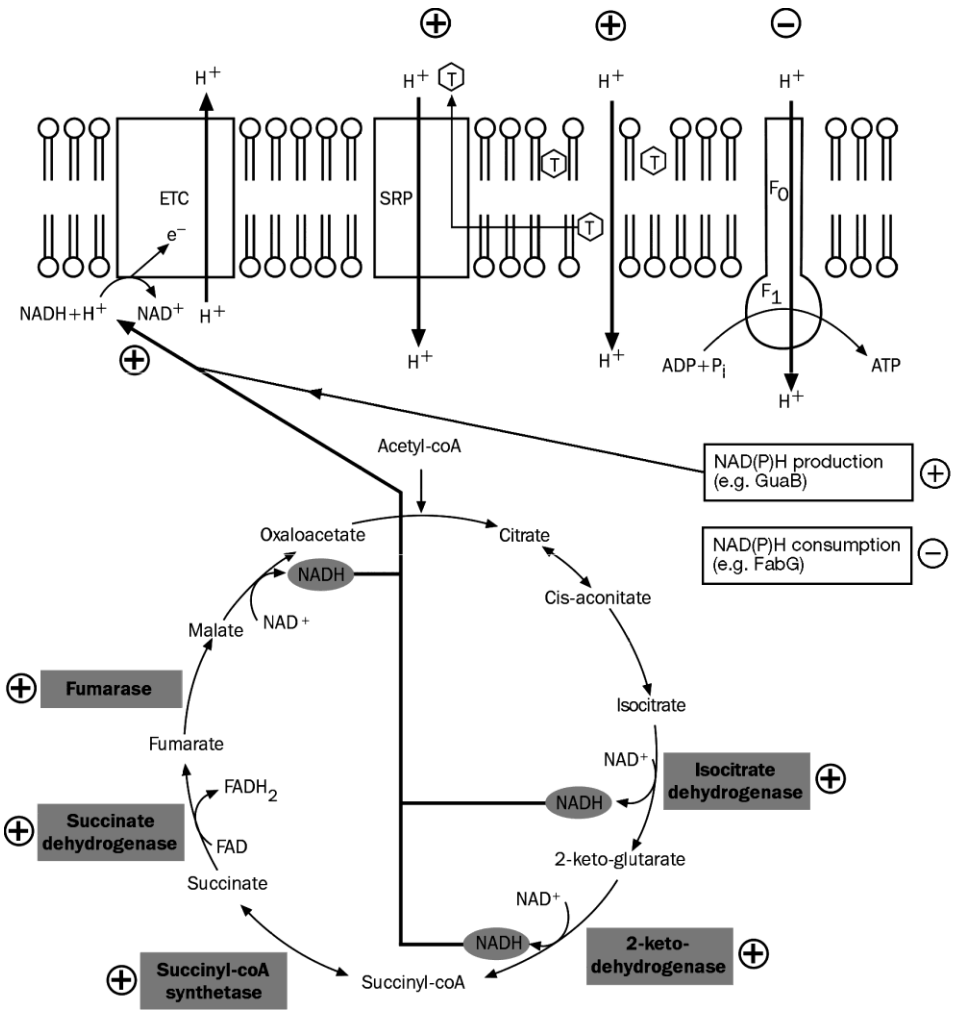

Figure 2.2 Schematic representation of the interplay between energy-producing and -consuming processes in *P. putida* S12 in the presence of toluene as derived from this proteomics study. The accumulation of toluene (T) in the membrane increases leakage of protons across the membrane. The solvent resistance pump (SRP) exports toluene from the cell and it is proton driven. Both processes cause a dissipation of the proton motive force, which requires up-regulation of NADH production. This is reflected by up-regulation of the TCA-cycle enzymes in the grey rectangles and down-regulation of NAD(P)H consuming systems in *P. putida* S12 in the presence of toluene. Simultaneously, the proton-consuming ATP synthase is down-regulated (this study).

(Table 2.2). In the current state of knowledge it is difficult to connect these proteins to the toluene stress response in *P. putida* S12.

Interestingly, the present study also yielded some results that appear to conflict with findings by others (Table 2.2). Toluene-affected *P. putida* DOT-T1E grown in shake flasks had upregulated the elongation factor Tu (123), whereas we found that this protein was down-regulated in the presence of toluene. Two cold-shock proteins (a cold shock domain family protein and RbfA) were down-regulated in *P. putida* S12, where others found evidence of solvent-triggered up-regulation of other cold shock proteins (123). These apparent conflicts suggest that expression of Tu-B and the two cold-shock proteins is not necessarily determined by toluene stress only.

In conclusion, this study focused exclusively on the effect of the single effector toluene. By using chemostat culturing under various growth conditions, relevant constraints for this investigation were provided. New results, as well as confirmation of some earlier results were obtained. Also some discrepancies with previous studies were found.

TrgI, toluene repressed gene I, a novel gene involved in toluene-tolerance in *Pseudomonas putida* S12

Pseudomonas putida S12 is well known for its remarkable solvent tolerance. Transcriptomics analysis of this bacterium grown in toluene-containing chemostats revealed the differential expression of 253 genes. As expected, the genes encoding one of the major solvent tolerance mechanisms, the solvent efflux pump SrpABC and its regulatory genes *srpRS* were heavily up-regulated. The increased energy demand brought about by toluene stress was also reflected in transcriptional changes: genes involved in sugar storage were down-regulated whereas genes involved in energy generation such as isocitrate dehydrogenase and NADH dehydrogenases, were up-regulated in the presence of toluene. Several flagella-related genes were up-regulated and a large group of transport genes were down-regulated. In addition, a novel *Pseudomonas*-specific gene was identified to be involved in toluene tolerance of *P. putida* S12. This toluene repressed gene, *trgI*, was heavily down-regulated immediately upon toluene exposure in batch cultures. The relationship of *trgI* with solvent tolerance was confirmed by the increased resistance to toluene shock and toluene induced lysis of *trgI* knock-out mutants. We propose that down-regulation of *trgI* plays a role in the first line of defence against solvents.

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Introduction

The solvent-tolerant bacterium *Pseudomonas putida* S12 is quite exceptional in its ability to withstand a wide range of toxic organic solvents such as toluene and benzene, in concentrations that are lethal to most microorganisms (60, 67, 108). Organic solvents accumulate in lipid membranes of living cells, causing an increase of the membrane fluidity and a decrease of bilayer stability (125, 145). A major adverse effect of solvent accumulation is the dissipation of the proton motive force (PMF) by a passive flux of protons across the cell membrane. Also the functioning of membrane-embedded proteins is compromised as their interaction with the membrane lipids is affected (125). Three types of membrane-associated solvent tolerance mechanisms have been elucidated (50, 60, 67, 108, 110, 145): (i) cytoplasmic membrane changes, (ii) outer membrane changes, and (iii) active efflux of organic solvents. All three modes of solvent tolerance have been found in solvent-tolerant Pseudomonads, but it is likely that additional mechanisms exist. Moreover, several cross-relations may exist between these protective mechanisms.

Recently, we reported the proteomics analysis of toluene-affected chemostat-grown *P. putida* S12 (142). This and another recent proteomics study on *P. putida* DOT-T1E (123) yielded valuable information with regard to the toluene response at the protein level, *e.g.*, the observation that a number of tricarboxylic acid (TCA) cycle enzymes were upregulated. In addition to these proteomics studies, a number of transcriptomics studies have been carried out on several organisms exposed to different organic solvents (37, 47, 85). In yeast, the genes involved in isooctane tolerance were found to be constitutively expressed rather than being induced by the solvent (85). Fujita et al. (37) mainly confirmed previous insights into the tolerance of yeast to straight-chain alcohols and hydrocarbons, like *e.g.* the involvement of genes associated with cell growth inhibition and morphological changes. In *E. coli* six genes related to hexane tolerance were identified with transcriptomics and tested in overexpression mutants (47). Only overexpression of MarA led to increased solvent tolerance, which confirmed earlier observations that this gene is involved in solvent tolerance. Dominguez-Cuevas et al. (27) assessed the transcriptional response of *P. putida* KT2440[pWW0] to a shock treatment with toluene, *o*-xylene and 3-methylbenzoate in batch cultures. They concluded that this toluene-degrading strain responds to toluene as a stressor rather than a nutrient. Several recent studies furthermore combine transcriptomics and proteomics analyses. In most cases, the two –omics technologies were used

supplementarily to each other yielding a panoramic view of the responses of the organism to various conditions (14, 15, 38, 92).

The aim of the present study was to gain further insight into the solvent tolerance mechanisms of *P. putida* S12, in addition to results from our previous proteomics study (142). Our finding that the hypothetical protein PP3611 was down-regulated (142) was confirmed at the transcriptional level. We furthermore established the relationship between PP3611, renamed TrgI, and toluene tolerance and present evidence that the down-regulation of TrgI is part of the first line of defence against toluene in *P. putida* S12.

Results

Toluene exposure has a modest effect on global gene expression as compared to nutrient limitation

Different nutrient limitations have a dramatic effect on global gene expression as has been shown for *e.g.*, *Saccharomyces cerevisiae* (11, 155) and *E. coli* (57). This phenomenon should be considered when studying the effects of a chemical effector such as toluene at the transcriptome level. Therefore, we chose to monitor the effect of 0, 3 and 5 mM toluene on the transcriptome of *P. putida* S12 both in nitrogen and carbon limited chemostat cultures. This setup allowed for the identification of transcriptional responses specific to toluene exposure irrespective of the nutrient limitation as the noise caused by nutrient limitations can be filtered out from the data. Five mM of toluene is near water saturation and 3 mM of toluene is just above the threshold that triggers the adaptational responses of the organism to toluene (69). The transcriptomes were analysed using custom Affymetrix high-density microarrays (5).

The effect of the culture conditions on global gene expression was determined by Principal Component Analysis (PCA) (Figure 3.1A). All genes on the array were included in the calculation, and the values are the average of two microarrays. The plot shows that the distance between the "N-limited" microarrays and the "C-limited" microarrays was much larger than the distances between the microarrays from the cultures with and without toluene. This confirmed that the effect of different nutrient limitations on global gene expression is larger than the effect of the presence of toluene. The residual concentration of glucose and nitrogen in the steady state cultures of *P. putida* S12 was similar to the previous study (142), the decrease in the biomass yield in the presence of toluene (data not shown) was comparable to earlier findings

(62). The biomass yield in the presence of 3 mM toluene was equal to the biomass yield in the presence of 5 mM toluene.

The genes that were differentially expressed in response to toluene, independent of the nutrient limitation, were identified using a 2-way ANOVA test (Tables 3.1 and 3.2 and Materials and methods). The conditions used in the calculation were 'nutrient limitation' and 'toluene concentration'. The group of genes that were differentially expressed because of variation in the nutrient limitation comprised of 571 genes, whereas the group of genes that was differentially expressed because of variation in the toluene concentration contained 253 genes (Figure 3.1B). The genes differentially expressed as a result of a nutrient limitation-dependent response on toluene formed a group of

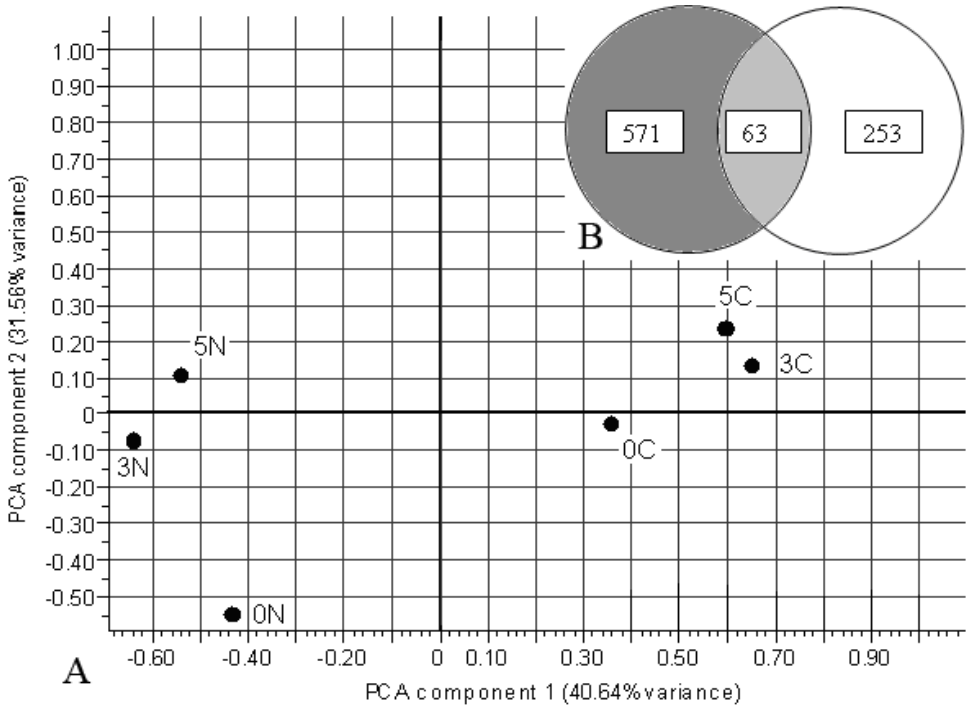

Figure 3.1 (A) Principle component analysis (PCA) of global gene expression profiles on the following conditions: mineral salts medium with carbon limitation (C); mineral salts medium with nitrogen limitation (N); 0 mM toluene, 3 mM toluene, 5 mM toluene (0, 3, 5). Values are the average of two microarrays). (B) Venn diagram of 2-way ANOVA results. Dark gray: genes differentially expressed because of nutrient limitation; white: genes differentially expressed because of toluene concentration; light grey: genes differentially expressed because of interaction between nutrient limitation and toluene concentration.

63 genes. Thus, more than twice as many genes were differentially expressed as a result of nutrient limitation than as a result of toluene concentration. These results were in good agreement with the results from the PCA (Figure 3.1).

Identification of differentially expressed genes in the presence of toluene

A total of 253 genes were differentially expressed solely as a result of variation of the toluene concentration. In this group, 85 genes were up-regulated (34%) and 168 genes were down-regulated (66%). In Tables 3.1 and 3.2 on pages 45-49 the genes in the most relevant functional categories, based on a previously conducted proteomics experiment, are shown (142),. Transport genes are interesting because one of the solvent-tolerance mechanisms comprises the active efflux of solvent molecules. Because solvents have a high impact on the membrane and the energy-household, these categories are also shown. The complete list of genes is presented as supplementary material in the original publication (S1). Genes are sorted by predicted function and their probable relation with solvent tolerance.

The defence against solvents is highly energy demanding (62, 123, 142). The upregulation of several NADH dehydrogenase subunits in the presence of toluene is in agreement with this observation. In addition, a TCA cycle gene was up-regulated: isocitrate dehydrogenase, which was also found in our proteomic study (142). In contrast, two other TCA cycle genes were down-regulated: *fumC-II* and *acnA*. However, since the primary metabolism is regulated at various levels, this result must be looked upon cautiously. The upregulation of genes encoding several cytochrome *o* ubiquinol oxidase subunits can be interpreted as an energy-effect as well, although *cyoC* has also been suggested to play a role in cell surface hydrophobicity (71). The transcript levels of genes associated with the storage of sugars, for example encoding glycogen synthase, are down-regulated in the presence of toluene, both under N- and C-limitation. At the same time, the glucono- and 2-ketogluconate-kinases and -transporters were up-regulated. These observations are indicative of an increased rate of sugar consumption and decreased sugar storage under solvent stress.

Since solvents primarily affect the integrity of the cellular membranes, genes relating to membrane-associated functions and outer cell structures were envisaged to be more than averagely represented among the differentially expressed proteins in the toluene-challenged chemostats. As expected, the genes encoding the tripartite solvent efflux pump SrpABC were up-regulated, together with their regulatory genes *srpR* and *srpS*. SrpABC is the only RND-family transporter that was up-regulated. Three *ompA*-like genes, expectedly

encoding outer membrane proteins, were down-regulated. In addition, five flagella-associated genes were found to be up-regulated, whereas five pili-related genes were down-regulated. Two transglycosylases, N-acetylmuramoyl-L-amidase and a penicillin-binding protein, all involved in the biosynthesis of the peptidoglycan cell wall, were up-regulated under toluene stress.

Although toluene clearly is a stressor, only two general stress response genes were up-regulated, encoding HtpX, a heat shock protein and FlkB-1, peptidyl-prolyl cis-trans isomerase (FKBP-type). CspD, a cold shock protein, was down-regulated.

The *Pseudomonas*-specific hypothetical gene PP3611, renamed *trgI*, is down-regulated in the presence of toluene

The hypothetical gene corresponding to locus *PP3611* in the *P. putida* KT2440 genome was found to be down-regulated in this study, in agreement with our previously published proteomics analysis of *P. putida* S12 grown in the presence of toluene (142). Since the gene is repressed in response to toluene, we renamed it toluene repressed gene I, or *trgI*. Nucleotide sequencing confirmed that *trgI* in *P. putida* S12 was >98 % identical to *PP3611* of *P. putida* KT2440. Eight nucleotides differed, resulting in a single amino acid change in residue 61 (aspartate in strain S12 and glutamate in strain KT2440). Sequencing of the region around *trgI* showed that the genomic organization of *trgI* and its neighbouring genes in *P. putida* S12 is identical to *P. putida* KT2440 (data not shown). The genes directly upstream (a putative TonB-dependent receptor) and downstream (a hypothetical gene) of *trgI* are transcribed in the opposite direction. There are no indications that *trgI* is part of an operon.

TrgI is a relatively small protein consisting of 193 amino acids. Protein BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) indicated that TrgI is also present in *P. putida* F1 (99% identity), *P. putida* BIRD-1 (99%), *P. putida* GB-1 (90% identity) and *P. putida* W619 (87% identity). Also a relatively high percentage of identity with hypothetical proteins of other *Pseudomonas* species was found (*P. entomophila* L48 (83%), *P. fluorescens* PfO-1 (47%) and *P. fluorescens* Pf-5 (46%)). TrgI appears to be *Pseudomonas*-specific, the BLAST hits of proteins from other genera all scoring below 32% identity. SignalP in SMART (78, 121) (http://smart.embl-heidelberg.de) identified a signal peptide in the first 26 amino acids (MNPIRTLARAVTLATLASAASFTVQA) of TrgI, which may suggest that TrgI is a periplasmic protein. No conserved domains were detected in TrgI (http://www.ncbi.nlm.nih.gov/Structure/cdd/ wrpsb.cgi),

Table 3.1 Genes that are up-regulated in *P. putida* S12 in the presence of 3 mM and 5 mM toluene.

Locus tag[a]	Gene name	Description[e]	Ratio[c] 3 mM toluene / no toluene	Ratio[c] 5 mM toluene / no toluene	N/C/D[d]
	Energy				
PP0626	Ndh	NADH dehydrogenase	1.36	2	N
PP0812	CyoA	Cytochrome o ubiquinol oxidase, subunit II	1.97	2.05	N/D[f]
PP0813	CyoB	Cytochrome o ubiquinol oxidase, subunit I	2.1	2.16	N/D[f]
PP0814	CyoC	Cytochrome o ubiquinol oxidase, subunit III	2.01	1.94	N/D[f]
PP0815	CyoD	Cytochrome o ubiquinol oxidase, protein CyoD	1.93	2.05	N/D[f]
PP0816	CyoE	Protoheme IX farnesyltransferase	2.08	2.25	N/D[f]
PP3377		2-Ketogluconate transporter, putative	4.59	6.59	N
PP3378	KguK	2-Ketogluconate kinase	3.98	5.69	N
PP3416	GnuK	Gluconokinase	2.61	4.19	N
PP3417	GntP	Gluconate transporter	2.33	3.48	N
PP4012		Isocitrate dehydrogenase, NADP-dependent, monomeric-type	2.22	2.6	C[g]
PP4128	NuoK	NADH dehydrogenase I, K subunit	1.95	2.1	N
PP4129	NuoL	NADH dehydrogenase I, L subunit	1.62	1.92	N
PP4130	NuoM	NADH dehydrogenase I, M subunit	1.85	1.82	N
	Transport				
AF029405[b]	SrpB	*Pseudomonas putida* solvent transporter gene, inner membrane transporter protein	10.42	10.8	N
AF029405[b]	SrpA	*Pseudomonas putida* solvent transporter gene, periplasmic linker protein	13.35	13.6	N
AF029405[b]	SrpC	*Pseudomonas putida* solvent transporter gene, outer membrane channel protein	10.75	10.95	N
AF029405[b]	SrpR	*Pseudomonas putida* solvent transporter gene, regulatory gene	6.46	6.68	N
AF029405[b]	SrpS	*Pseudomonas putida* solvent transporter gene, regulatory gene	10.19	10.99	N
PP0113		ABC transporter, permease protein	1.53	1.48	N

Continued on next page

Table 3.1 *Continued*

PP1272		Multidrug efflux MFS membrane fusion protein, putative	2.36	2.76	N
PP1743		Sodium:solute symporter family protein	6.57	8.39	N
PP4881		Iron ABC transporter, periplasmic iron-binding protein, putative	1.69	2.27	N
PP5196		Iron ABC transporter, periplasmic iron-binding protein, putative	2.69	3.07	N
PP5307	ExbD	Ferric siderophore transport system, inner membrane protein ExbD	1.55	1.96	N
	Membrane				
PP1036		Periplasmic binding domain transglycosylase SLT domain fusion protein	1.46	1.58	N
PP1871	HtpX*	Heat shock protein HtpX	1.69	2.27	N
PP2244		Membrane protein, putative	1.46	1.59	N
PP4352	FlhB	Flagellar biosynthetic protein FlhB	1.49	1.43	N
PP4354	FliQ	Flagellar biosynthetic protein FliQ	1.81	2	N
PP4356	FliO	Flagellar assembly protein FliO	2.21	1.94	N
PP4367	FliH	Flagellar assembly protein FliH	1.53	1.3	N/D[f]
PP4368	FliG	Flagellar motor switch protein FliG	2	1.88	N
PP4805	MltB	Membrane-bound lytic murein transglycosylase B	2.1	2.03	N
PP4897		N-acetylmuramoyl-L-alanine amidase	1.55	1.94	N
PP5084		Penicillin-binding protein	1.18	1.74	N
	Other				
PP0684	FlkB-1*	Peptidyl-prolyl cis-trans isomerase, FKBP-type	1.4	1.98	N

*) General stress response gene
a) Locus tag based on genome of *P. putida* KT2440 (www.pseudomonas.com)
b) NCBI database no.
c) Ratio of expression in 3 mM or 5 mM toluene versus the absence of toluene
d) New (N), confirmatory (C), discrepant (D) compared with results in relevant literature
e) Description of genes based on locus tag description
f) Refers to (27)
g) Refers to (142)

Table 3.2 Genes that are down-regulated in *P. putida* S12 in the presence of 3 mM and 5 mM toluene.

Locus tag[a]	Gene name	Description[e]	Ratio[c] 3 mM toluene/no toluene	Ratio[c] 5 mM toluene/no toluene	N/C/D[d]
	Energy				
PP1755	FumC-II	Fumarate hydratase, class II	0.57	0.51	C[f]
PP2112	AcnA	Aconitate hydratase 1	0.61	0.47	N
PP4050	GlgA	Glycogen synthase	0.49	0.35	C[f]
PP4051		Alpha-amylase family protein	0.42	0.3	C[f]
PP4052	MalQ	4-Alpha-glucanotransferase	0.4	0.34	C[f]
PP4053		Glycosyl hydrolase, putative	0.5	0.4	C[f]
PP4055	GlgX	Glycogen operon protein GlgX	0.52	0.39	C[f]
PP4058	GlgB	1,4-Alpha-glucan branching enzyme	0.61	0.42	N
PP5007		Polyhydroxyalkanoate granule-associated protein GA2	0.6	0.49	C[f]
PP5041	GlgP	Glycogen phosphorylase	0.57	0.36	N
	Transport				
AF183959[b]	ArpB	*Pseudomonas putida* inner membrane transporter protein	0.49	0.47	N
AF183959[b]	ArpA	*Pseudomonas putida* periplasmic linker protein	0.52	0.44	N
AF183959[b]	ArpR	*Pseudomonas putida*	0.44	0.4	N
PP0147		Citrate transporter	0.56	0.58	N
PP0699		Transporter, LysE family	0.72	0.72	N
PP0803		Protein secretion ABC efflux system, membrane fusion protein	0.65	0.64	C[f]
PP0804		Protein secretion ABC efflux system, permease and ATP-binding protein	0.35	0.39	N
PP0805		Outer membrane efflux protein	0.33	0.32	N
PP0883		Porin, putative	0.41	0.33	N
PP0884		Dipeptide ABC transporter, periplasmic binding protein	0.3	0.23	N
PP0885		Dipeptide ABC transporter, periplasmic peptide-binding protein	0.36	0.27	C[f]

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Table 3.2 *Continued*

PP1416		Tricarboxylate transport protein TctA, putative	0.52	0.38	N
PP1417		Tricarboxylate transport protein TctB, putative	0.48	0.36	N
PP1418		Tricarboxylate transport protein TctC, putative	0.42	0.34	N
PP1419		Porin, putative	0.37	0.34	N
PP1724		ABC transporter, permease protein	0.67	0.35	C[f]
PP2092	NasA	Nitrate transporter	0.3	0.28	N
PP2094		Nitrate-binding protein NasS, putative	0.68	0.49	N
PP2195		Periplasmic polyamine-binding protein, putative	0.52	0.42	N
PP2264		Sugar ABC transporter, periplasmic sugar-binding protein, putative	0.46	0.4	N
PP2411		Major facilitator family transporter	0.71	0.75	N
PP3211		ABC transporter, ATP-binding protein	0.55	0.45	N
PP3213		ABC transporter, periplasmic binding component-related protein	0.82	0.59	N
PP3635		Sulfonate ABC transporter, permease protein, putative	0.5	0.36	N
PP3636		Sulfonate ABC transporter, periplasmic sulfonate-binding protein, putative	0.43	0.33	N
PP3637		Sulfonate ABC transporter, ATP-binding protein, putative	0.45	0.31	N
PP3940		Major facilitator family transporter	0.54	0.43	N
PP3954		Periplasmic binding protein, putative	0.4	0.3	N
PP4282		Aquaporin Z	0.48	0.43	N
PP4309		Transporter, NCS1 nucleoside transporter family	0.6	0.34	N
PP4653		Transporter, putative	0.7	0.46	N
PP5173		RND efflux transporter	0.34	0.29	N
PP5174		Efflux membrane fusion protein, RND family	0.53	0.45	N
PP5207		ABC transporter, ATP-binding protein permease protein, putative	0.67	0.53	N
PP5341		ABC transporter, periplasmic polyamine-binding protein, putative	0.51	0.4	N
	Membrane				
PP1121		OmpA family protein	0.47	0.32	C[f]
PP1122		OmpA family protein	0.57	0.39	C[f]

PP1408	PhaG	acyl-transferase	0.45	0.3	N
PP1502		OmpA family protein	0.51	0.46	N
PP2358		Type 1 pili subunit CsuA B protein, putative	0.42	0.39	C[f]
PP2359		Type 1 pili subunit CsuA B protein, putative	0.33	0.28	C[f]
PP2360		Type 1 pili subunit CsuA B protein, putative	0.28	0.21	C[f]
PP2361	CsuC	Type 1 pili usher pathway chaperone CsuC	0.39	0.31	N
PP4361	FliK	Flagellar hook-length control protein FliK	0.66	0.48	N
PP5081		Type IV pili biogenesis protein	0.76	0.67	N
	Other				
PP3611	TrgI	Toluene repressed gene	0.36	0.28	C[g]
PP4010	CspD*	Cold-shock protein CspD	0.34	0.35	N

*) General stress response gene
a) Locus tag based on genome of *P. putida* KT2440 (www.pseudomonas.com)
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c) Ratio of expression in 3 mM or 5 mM toluene versus the absence of toluene
d) New (N), confirmatory (C), discrepant (D) compared with results in relevant literature
e) Description of genes based on locus tag description
f) Refers to (27)
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but a BLASTP2 search (http://dove.embl-heidelberg.de/Blast2/) showed that a 71-amino acid stretch (residues 40-110) has 29 % identity with a transcription regulator of the MarR family. This family is involved in regulation of resistance to antibiotics, disinfectants and organic solvents, amongst other compounds. Many of the MarR-like regulators respond to aromatic compounds (1).

TrgI is immediately down-regulated after addition of toluene

Downregulation of *trgI* was observed in fully adapted, toluene-exposed steady state cultures. In order to establish the dynamic response of *trgI* to toluene, the expression profile of *trgI* was also studied in toluene-challenged batch cultures by qPCR analysis. In parallel, the expression profile of the solvent pump gene *srpB* was analysed. It has been shown in a previous study (69) that *srpB* is maximally expressed up to several hours after toluene exposure and can therefore be considered a late response. Toluene (5 mM) was

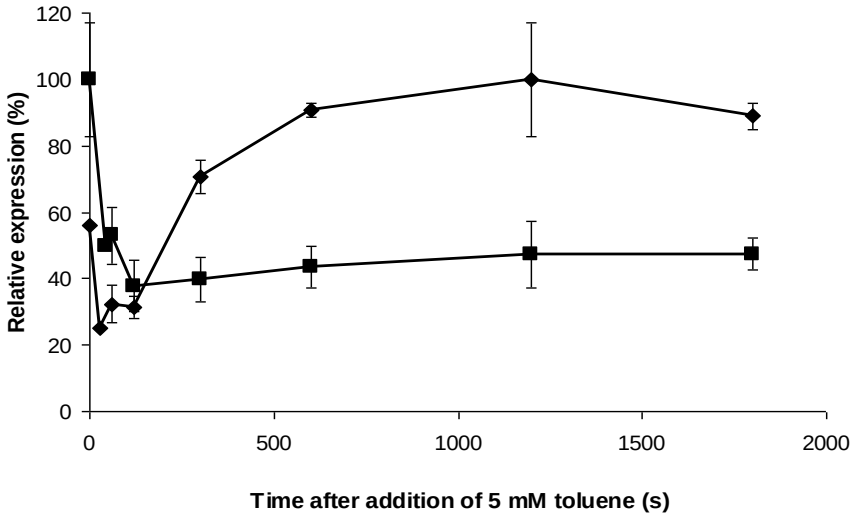

Figure 3.2 Relative expression in percentage of the maximum expression of: (■) *trgI* and (♦) *srpB* after addition of 5 mM toluene to an exponentially growing culture of *P. putida* S12, as determined by qPCR. Values are the average of duplicate experiments (error bars represent deviation from mean) and corrected for experimental variation using expression of KT2440 genes *plsB* (PP1520) and the putative *paaI* (PP3281) identified in *P. putida* S12. For the latter genes no change in expression as response to the presence/absence of toluene in glucose grown chemostat cultures of *P. putida* S12 was found using transcriptomics analysis. The expression of the genes was defined unchanged before and after addition of 5 mM toluene.

added to an exponentially growing culture of *P. putida* S12 and timed samples were drawn for mRNA isolation. It was observed that transcription of *trgI* rapidly declined and reached a minimum after only two minutes upon addition of toluene (Figure 3.2). At that time point, the transcription of *srpB* started to increase, reaching a maximum after twenty minutes.

Characterization of *trgI* knock-out and *trgI* overexpression mutants

To further investigate the role of *trgI* in solvent tolerance and its possible mode of action, we constructed a *trgI* overexpression (*P. putida* S12pTrgI) and a *trgI* knock-out mutant (*P. putida* S12ΔTrgI) by disrupting the gene with a *tetA* marker. The *trgI* knockout strain appeared to be much more resistant to toluene-induced lysis than wildtype S12 and the *trgI* overexpression mutant. Although able to thrive in LB in the presence of 3 mM toluene, the overexpression-mutant and wild-type S12 showed significant lysis whereas the *trgI*-knockout culture showed no cell lysis at all (Figure 3.3).

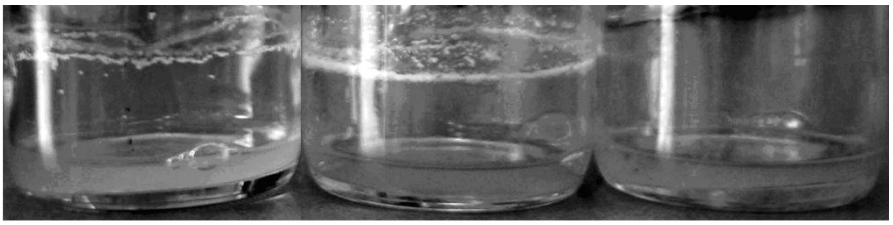

Figure 3.3 (A) *P. putida* S12, (B) *P. putida* S12ptrgI, and (C) *P. putida* S12ΔtrgI after 24 hours of culturing in the presence of 3 mM toluene. Debris of dead cells is deposited onto the wall of the bottles in the cultures of *P. putida* S12 and *P. putida* S12ptrgI.

The above mentioned strains were also subjected to a 30 minute 1 % (v/v) toluene shock. The cells were not pre-adapted to toluene prior to the shock because *trgI* expression rapidly decreases after addition of toluene, making adapted wildtype cells phenotypically indistinguishable from *trgI*-knockout cells as far as *trgI*-related responses are concerned. Survival frequency was determined by measuring colony forming units before and after the solvent shock treatment. The experiment was repeated six times, as the numbers obtained deviated significantly (not shown). Nonetheless, the survival frequency of S12ΔtrgI was consistently higher than that of S12, by a factor ranging from 3 to 8600. The survival frequency of S12 was in its turn higher than that of the *trgI*-overexpression mutant: the mutant showed no survival in

four of the six experiments, whereas in the other two the survival frequency was negligibly low. Control cultures with a *tetA*-insertion in *pyrR* (PP4997) or the gene encoding a class III aminotransferase (PP5182) did not show an increased tolerance to toluene shock or toluene-induced lysis. Thus, it can be concluded that the *tetA* gene used to disrupt the *trgI* gene does not contribute to the toluene tolerance of the *trgI*-knockout mutant.

Table 3.3 MIC's (mg/L; see Materials and Methods) after one day of exposure to various antibiotics of *P. putida* S12, *P. putida* S12ΔtrgI and *P. putida* S12ptrgI. Values are the average of two experiments. (nd) not determined because strain possesses tetracyclin or ß-lactam resistance gene.

Antibiotic	*P. putida* S12	*P. putida* S12ΔTrgI	*P. putida* S12pTrgI
Ampicillin	500	1000	nd
Carbenicillin	500	1000	nd
Piperacillin	125	750	nd
Chloramphenicol	500	62.5	375
Streptomycin	46.9	3.9	62.5
Tetracyclin	23.4	nd	31.3
Polymyxin B	3.9	3.9	3.9
Novobiocin	2000	1000	2000
Gentamycin	7.8	3.9	nd
Kanamycin	3.9	3.9	3.9

The improved lysis resistance and solvent shock tolerance of *P. putida* S12ΔTrgI suggests a relationship between the *trgI* deletion and altered properties of the outer cell structure. As several genes involved in the biosynthesis of the cell wall component peptidoglycan were found to be up-regulated in the presence of toluene, a relationship may exist between the improved lysis resistance of *P. putida* S12ΔTrgI and peptidoglycan synthesis. Therefore, growth of *P. putida* S12, *P. putida* S12pTrgI and *P. putida* S12ΔTrgI was investigated in the presence of β-lactam and other antibiotics (Table 3.3). *P. putida* S12ΔTrgI showed an increased initial level of resistance against the ß-lactams ampicillin, piperacillin and carbenicillin as compared to *P. putida* S12. It was less resistant to chloramphenicol and streptomycin. Only slight differences in resistance between the strains were observed for tetracycline, polymyxin B, novobiocin, gentamicin and kanamycin.

In addition to improved β-lactam resistance, the improved toluene stress tolerance of S12ΔTrgI coincided with other effects that may relate to the outer cell structure. Strain S12ΔTrgI lost the ability to grow in mineral salts medium on glucose or fructose. As the functioning of transport proteins (9) and

membrane protein topology (12) are known to be affected by membrane composition, the inability to utilize these sugars may be connected to changes in the outer cell structure preventing the sugars from being transported into the cells. The effect appears to be specific as the ability of S12ΔTrgI to utilize glycerol, succinate or decanol was not affected.

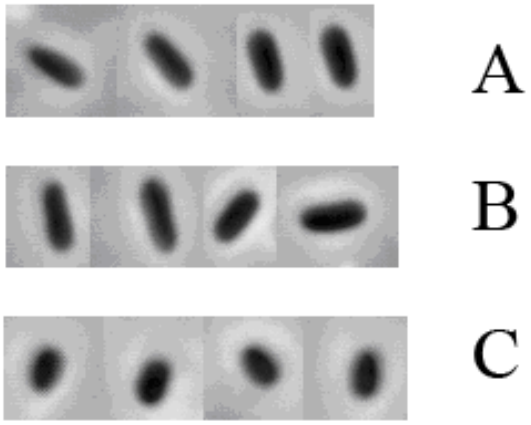

Figure 3.4 Typical cells from stationary-phase cultures in the absence of toluene of (A) *P. putida* S12, (B) *P. putida* S12ptrgI, (C) *P. putida* S12ΔtrgI. Phase-contrast, 100X magnification, Leitz Aristoplan microscope, Leica DC500 camera..

It was found that strain S12ΔTrgI could not be transformed with plasmids pJTTtrgI and pJNNtrgI(t) by electroporation or heat-shock transformation. These methods routinely yield ample transformants with wild-type S12, demonstrating that the cell envelope of S12ΔTrgI is difficult to penetrate. Also cell morphology appeared to be affected by the *trgI* deletion. Figure 3.4 shows that stationary-phase wild-type S12 and S12pTrgI have cell shapes quite different from S12ΔTrgI. Wild-type and S12pTrgI cells are stretched rods with a surface-to-volume ratio of 2.6, whereas the cells of the knock-out mutant are shorter rods with a surface-to-volume ratio of 1.8.

The above observations support the suggestion that the outer cell structure of S12ΔTrgI differs from that of unadapted wild-type S12, making the cells less permeable and more robust. Since a role of membrane lipids appears obvious, the membrane fatty acid composition was analysed of exponentially growing cells of S12ΔTrgI and wild-type S12 cultured in LB medium, either with or without 5 mM toluene. The fatty acid composition changed upon

addition of toluene as expected and reported previously (50): the saturation degree as well as the *trans : cis* ratio of unsaturated fatty acids increased whereas the C_{18} : C_{16} ratio decreased (not shown). However, no differences in fatty acid composition were observed between wildtype and *trgI* deletion mutant, either or not exposed to toluene.

Discussion

In a previous study, the solvent tolerance responses of *P. putida* S12 were studied on the proteomic level (142). In the present study, the transcriptional responses to toluene exposure have been investigated. The two studies should be regarded as complementary, as it has proven difficult to compare proteomics and transcriptomics directly (49), both for technical (e.g., pI range and solubility of proteins) and fundamental reasons (protein expression is not only regulated at the transcriptional level).

The transcriptional responses of *P. putida* S12 to toluene could be categorized into three types: i) responses that can be directly connected to (known) solvent tolerance mechanisms; ii) responses related to the increased energy demand brought about by the solvent; iii) responses of which the relationship with the presence of solvent is not immediately clear. From the viewpoint of global transcription responses, toluene stress has a relatively small impact as compared to the differential gene expression that is invoked by switching between carbon and nitrogen limitation. This is in agreement with earlier observations in *S. cerevisiae (11, 37)* and *E. coli* (57). The relatively weak effect on the global transcription level, however, is no measure of the seriousness of the impact that toluene may have on the physiological level. The effect of toluene on differential expression of general stress response genes is nevertheless relatively small. It should be noted that steady-state chemostat cultures were studied, which implies that the adaptational machinery to toluene is fully induced. Apparently, this machinery is of such a high efficiency that the stress sensed by the cells is below the trigger threshold of general stress responses. Segura et al. (123) found a heat-shock response in batch-cultured *P. putida* DOT-T1E, which was absent in chemostat-cultured *P. putida* S12. It may be argued that batch-cultured cells of *P. putida* DOT-T1E experience more or a different form of stress from toluene than chemostat cultured *P. putida* S12 cells, which are at steady state and, thus, fully adapted to the presence of a constant concentration of toluene.

Responses connected to toluene tolerance mechanisms

The up-regulation of the solvent pump *srpABC* and its presumed regulators *srpRS* is an expected response directly linked to a well-known toluene tolerance mechanism. The up-regulation of *srpRS* appears to contradict the finding by Wery *et al.* (147) that SrpS and SrpR are repressors of *srpABC*. However, if SrpS, in analogy to the *ttgGHI* regulator TtgV in *P. putida* DOT-T1E (37) (96% identical to SrpS), is released from its operator site by a toluene-trigger, transcription of both *srpABC* and *srpRS* may be initiated at the same time.

Also the up-regulation of genes involved in peptidoglycan biosynthesis (transglycosylase (PP1036), penicillin-binding protein (PP5084), N-acetylmuramoyl-L-alanine amidase (PP4897) and membrane-bound lytic murein transglycosylase B (*mltB*, PP4805)) (92), may be regarded as a specific toluene resistance response. The effect of toluene on cell morphology, i.e, an increased cell diameter under solvent stress (95), may invoke increased turnover of peptidoglycan which must be compensated by the upregulation of peptidoglycan synthesis. Also solvent-induced damage of the peptidoglycan layer may be a reason for the upregulation observed.

Responses relating to the energy status of the cell

The biomass yield was negatively affected by the presence of toluene as observed previously (62, 142): at 3 mM toluene, the biomass yield dropped by ~50% compared to cultures without toluene, but no change in biomass yield was observed between 3 and 5 mM toluene. Interestingly, the expression levels of the genes encoding the solvent resistance pump *srpABC* followed a reciprocal trend: *srpABC* levels were 10-fold increased in 3 mM compared to 0 mM toluene, and levels remained equally high at 3 and 5 mM toluene. Equal levels of *srpABC* mRNA are expected to yield equal levels of active pump, resulting in an equal metabolic burden brought about by the energy demand of SrpABC-mediated, PMF-driven toluene extrusion. This observation provides an indication that energy consumption by SrpABC is the major cause of the yield loss in the presence of toluene.

Numerous transcriptional responses were observed that are in agreement with an increased energy demand in the presence of toluene. The up-regulation of gluconate and 2-keto-gluconate kinases and transporters, via which glucose is chanelled to the Entner-Doudoroff pathway, is indicative of a higher glucose consumption rate. The inability of the *trgI*-knock-out strain to grow on glucose and fructose can be explained by *trgI* not being expressed at all in this strain, whereas in the wild-type it is only down-regulated to 35% of the non-stressed expression level in 3 mM toluene and to 28% in 5 mM toluene.

Also, changes in the outer cell structure of S12ΔtrgI may influence the functioning of the proteins involved in transport of glucose and fructose (9, 12).

In agreement to the up-regulation of the gluconate and 2-keto-gluconate kinases and transporters, several genes involved in sugar storage were down-regulated. A TCA cycle gene was up-regulated, as were genes for respiratory chain elements such as several subunits of NADH dehydrogenase. These responses are envisaged to enable the cells to compensate for the loss of energy invoked by toluene exposure.

Also, a large group of transport systems were down-regulated. Down-regulation of these transport systems may be related to further energy saving, as suggested in our recent proteomics analysis (142). Down-regulation of transporters may also decrease the number of possibilities for toluene molecules to enter the cell. The hypothesis of general energy saving may also apply to other down-regulated genes which have no direct relation to solvent stress. The overall group of down-regulated genes is larger than the up-regulated genes and the level of expression of most down-regulated genes is inversely proportional to the concentration of toluene.

Responses that have an indirect relationship with toluene tolerance

In the presence of toluene, genes encoding three flagellar structural proteins, a flagellar export component and a flagella basal body subunit were up-regulated. The gene coding for the flagellar hook-length protein was down-regulated. A relationship between flagella and solvent tolerance has been reported previously in solvent-tolerant *P. putida* strains (27, 65, 122). In each case, different flagella genes were found to influence solvent tolerance. Our findings confirm the existence of a link between flagella and solvent tolerance, but its nature remains to be elucidated and may be found at the regulatory level (65, 108). However, a direct relationship between flagellar systems and toluene tolerance could exist if the flagellar export system is able to export toluene molecules as well.

***TrgI*, a novel solvent-tolerance-related gene**

Both in the present transcriptomics and our previous proteomics study a strong down-regulation of the hypothetical gene *trgI* (*P. putida* KT2240-locus PP3611) was observed. In addition, it was demonstrated that *trgI* is downregulated immediately upon exposure to toluene. This observation explains the poor reproducibility of the toluene shock experiments. The momentaneous downregulation of *trgI* compromises the discrimination between the phenotypes of toluene-exposed wild-type S12 and the *trgI*-knockout strain. The extremely low survival frequency of the *trgI*

overexpression mutant stresses the importance of downregulation of the gene for solvent tolerance. The fast response of *trgI* suggests a role in initial solvent stress response. As toluene dissolves in the S12 membrane at a high rate reaching the maximum concentration within 10 minutes (95), mechanisms conferring toluene tolerance at a short time scale are a prerequisite for the cells to switch on the toluene extrusion pump SrpABC. This pump is crucial for survival in the presence of toluene, but *srp*-promoter driven LacZ expression has been shown not to start until over two hours after toluene exposure (68). The expression profile of *srpB* obtained in the present study confirmed that *srpABC* expression is a late response. The fast down-regulation of *trgI* suggests a role in such a first line of defence against toluene.
It was demonstrated that the *trgI*-knockout has an altered cell morphology and an altered level of resistance against antibiotics. In addition, the *trgI* knockout strain is incapable of utilizing glucose and fructose and is more resistant to toluene-induced lysis. These observations strongly suggest an effect of *trgI* on the outer cell structure. An influence of *trgI* on fatty acid composition is unlikely since the fatty acid composition of *P. putida* S12, either or not exposed to toluene, was not affected by the *trgI* deletion. Still, preliminary indications were found that the phospholipid headgroup composition may be affected by the *trgI* deletion, most notably resulting in an increased cardiolipin content (unpublished).

Conclusion

The present study presents important new and additional information concerning transcriptional responses of solvent tolerant *P. putida* to solvent exposure. Several new groups of differentially expressed genes have been revealed that have not been linked previously to toluene tolerance, such as the gluconate and 2-ketogluconate kinase and transporter genes and the genes involved in biosynthesis of the peptidoglycan layer. Importantly, a combined transcriptomic and proteomic approach (44, 123) revealed the interplay between specific responses (e.g., solvent resistance pump SrpABC) and global compensatory responses (e.g., TCA cycle), as well as previously unknown functions. The discovery of the new solvent tolerance gene *trgI* has provided an important new piece of information that will help to solve the puzzle of solvent tolerance.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are shown in Table 3.4. *Pseudomonas putida* S12 was originally isolated as a styrene utilising bacterium (44). The expression vectors pJTTtrgI and pJNNtrgI(t) were constructed as follows: oligonucleotides 5'-GCGGCGGCCGCGTCAGCGCGAGGTTTCG-3' (forward) and 5'-CGCGAATTCCTATGAACCCCATTCGTAC-3' (reverse) were designed based on the published sequence of *P. putida* KT2440 PP3611 (GenBank accession no. NC_002947) including restriction sites for cloning in the expression vectors pJTTmcs (98) and pJNNmcs(t) (97). The vectors and the PCR-amplified *trgI* were restricted with NotI and EcoRI. Ligation of *trgI* into the vectors yielded plasmids pJTTtrgI and pJNNtrgI(t). *P. putida* S12 was transformed with pJTTtrgI, resulting in *P. putida* S12pTrgI.

Table 3.4 Bacterial strains and plasmids used in this study.

Strains	Characteristics	Source or reference
P. putida S12	Wild type	(45)
P. putida S12pTrgI	*P. putida* S12 with plasmid pJTTtrgI, Gm^R	This study
P. putida S12ΔTrgI	*P. putida* S12 with interrupted *trgI*	This study
P. putida S12ΔpyrR	*P. putida* S12 with interrupted *pyrR*	This study
P. putida S12Δat	*P. putida* S12 with interrupted aminotransferase	This study
Plasmids		
pJTTmcs (formerly named pTac)	Expression vector with constitutively expressed *tac* promotor, Gm^R, Amp^R	(98)
pJNNmcs(t)	Expression vector with inducible promoter nagAa	(152)
pJQ200SK	Suicide vector, Gm^R	(106)
pJTTtrgI	Expression vector pJTT with *trgI*, Gm^R, Amp^R	This study
pJNNtrgI(t)	Expression vector pJNNmcs(t) with *trgI*, Gm^R	This study
pJQtrgI	pJQ200SK containing interrupted *trgI*	This study

The knockout mutant *P. putida* S12ΔTrgI was constructed as follows: oligonucleotides (Table 3.5) were designed on the published sequence of *P. putida* KT2440 (GenBank accession no. NC_002947) including restriction sites for cloning of *trgI,* in two parts and with flanking sequences (the fragments had a total length of 1 kb), in the suicide vector pJQ200SK (106). The tetracycline resistance gene *tetA* was cloned into the XbaI site of the resulting plasmid yielding pJQtrgI. After transforming pJQtrgI to *P. putida* S12 and subsequent

selection on tetracycline and gentamicin-containing medium, a mutant with the interrupted *trgI* gene stably integrated in the genome was obtained: *P. putida* S12ΔTrgI. The knock-out mutants *P. putida* S12ΔpyrR and *P. putida* S12Δat were constructed analogously to *P. putida* S12ΔTrgI (Table 3.5 for oligonucleotide sequences).

Table 3.5 Oligonucleotides used for construction of the knockout mutants *P. putida* S12ΔTrgI, *P. putida* S12ΔpyrR and *P. putida* S12Δat.

Strain	Nucleotide sequence (5' → 3')[a]	F/R[b]	Restr. enzyme
P. putida S12ΔTrgI	CGCGGATCCGCTGCACGCACCCATCC	F	BamHI
	CGCTCTAGAGAAAGCGACTGAAGAGTCC	R	XbaI
	CGCTCTAGACGTGTCACGTAGATGTCG	F	XbaI
	CGCGCGGCCGCCGACACGCTCGATGTTGG	R	NotI
P. putida S12ΔpyrR	CCGGGATCCGCTGACCACGTCACAGG	F	BamHI
	GGCTCTAGACCTTCGACCTCGAACGG	R	XbaI
	CCGTCTAGAAACTGTTCGATTACGGCCG	F	XbaI
	GGCGCGGCCGCGATGGCGTCAGGCTTGG	R	NotI
P. putida S12Δat	GCGGGATCCGATGTAGTCCGACCAGTTATAG	F	BamHI
	GCGTCTAGAAAGGTGAAGGAGATCCTCGCC	R	XbaI
	GCGGCGGCCGCGCCGCCAGCCTACTGTGTGG	R	NotI
	GCGTCTAGATCGGCGATGAAGGCCGCGAC	F	XbaI

a) The underlined bases form the restriction site.
b) (F) forward; (R) reverse.

Culture conditions

Mineral salts medium (MM) (44) was used as the standard culturing medium, with the following modifications: 37 mM K-phosphate, 1.5 mM Na_2SO_4 as the sulphur source, NH_4Cl as the nitrogen source and glucose as the carbon source. *P. putida* was cultured at 30ºC; *E. coli* at 37°C. For culturing under carbon limitation, 10 mM glucose and 30 mM NH_4Cl (C:N = 2:1) were added to MM. For culturing under nitrogen limitation MM contained 44 mM glucose and 4 mM NH_4Cl (C:N = 66:1). Luria-Bertani broth (LB) (117) was used with 1.5% (w/v) agar as a solid medium. Batch cultivation was carried out in 250-ml Erlenmeyer flasks containing 50 ml of liquid medium, placed on a horizontally shaking incubator. Batch cultivation with toluene was carried out in 250 ml airtight Boston bottles with Mininert valves (Alltech, Deerfield, Illinois, USA) containing 10 ml LB medium. Toluene shocks were applied to cells growing exponentially in LB medium (optical density at 600 nm of 0.8 – 1.0) by incubating for 30 minutes with a second phase of toluene (1% (v/v)).

The survival frequency was determined by counting the number of colony-forming units before and after the toluene shock. Dilutions of the cultures made in 0.9 % (w/v) saline were plated on agar plates by drop-plating. Per dilution, 5 drops of 10 µl were applied on the plates. After drying, the plates were incubated for 18 hours and the colonies were counted in the drops that contained separate colonies.

Continuous culturing in the presence and in the absence of toluene under nitrogen- and carbon-limitation was performed in chemostats (BioFloIIc, New Brunswick Scientific, Edison, New Jersey, USA) with a working volume of 1.0 litre, at pH 7.0, a stirring speed of 350 rpm, and a dilution rate of 0.2 h^{-1}. Pure oxygen was supplied at 5 ml/min. Toluene was added separately with a KD Scientific syringe pump (Applikon, Schiedam, The Netherlands) to final concentrations of 3 and 5 mM. The chemostats were inoculated with a 50-ml culture growing at mid-log growth rate. Samples for transcriptomics analysis and determination of glucose, gluconate, 2-keto-gluconate and ammonium concentrations were drawn at steady state, which was reached after 5 volume changes, as was confirmed by cell density measurements. After sampling the culture without toluene, toluene was added to 3 mM and samples were drawn after the next steady state had been reached. Subsequently, toluene was added to 5 mM and sampling was performed again at steady state. Culturing was performed in duplicate and each steady state was sampled once, resulting in 12 microarrays, two per toluene concentration.

Culturing in the presence of a concentration series of antibiotics was carried out in 96-wells plates. 10 µl of an exponentially growing culture was added to 90 µl of LB medium supplemented with antibiotic. Growth was assessed by visual inspection after 1 day, 2 days and 5 days. MIC (minimal inhibiting concentration) was defined as the antibiotic concentration at which no growth was observed.

Transcriptomics analysis

Sampling, mRNA isolation and cDNA preparation for transcriptomics analysis were performed as described previously (150). In brief, 1-ml culture samples were quenched in 1 ml ice-cold methanol, centrifuged and 1 ml RNAlater (Ambion, Foster City, California, USA) was applied to prevent RNA degradation. Pellets were flash frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated using the RNeasy Mini Purification Kit (Qiagen, Hilden, Germany). mRNA was isolated using the MICROB*Express* Bacterial mRNA Purification Kit (Ambion). Random priming, cDNA synthesis, purification and labelling were performed according to the microarray manufacturer's instructions (Affymetrix, Santa Clara, California, USA). The

custom high-density microarrays used were based on the genome of *P. putida* KT2440, which has 81.8% similarity on the gene level with *P. putida* S12, with additional probe sets based on known sequences of *P. putida* S12 and related strains (5, 150). Hybridisation was performed according to the manufacturer's instructions. Scanning of the microarrays was carried out by ServiceXS (Leiden, The Netherlands) on a high resolution Gene Chip Scanner 3000 7G system with autoloader (Affymetrix) using standard default analysis settings (filter: 570 nm; pixel size: 2,5 µm).

Data analysis

Microarray data were imported into the GeneSpring GX 7.3.1 software package (Agilent Technologies, Santa Clara, California, USA) using the GC RMA algorithm. After normalisation (signals below 0.01 were taken as 0.01; per chip: normalise to 50th percentile; per gene: normalise to median) of the data, probesets representing intergenic regions and control genes were removed, as well as non-changing genes (between 0.667- and 1.334-fold change). On the resulting set of 995 differentially expressed genes a 2-way ANOVA test was applied, including a Benjamini and Hochberg false discovery rate test with a *p* value cut-off of 0.03 in order to identify the genes that were differentially expressed exclusively as a consequence of the presence of toluene. The two conditions varied were toluene concentration (0, 3 and 5 mM) and nutrient limitation (nitrogen- and carbon-limitation). The genes that were designated as differentially expressed as a result of variation in the toluene concentration were divided into two groups, one with up-regulated genes and one with down-regulated genes.

Analytical methods

Cell densities were measured at 600 nm with a WPA CO800 Biowave Cell Density Meter (Biochrom, Cambridge, United Kingdom). Glucose, gluconate, 2-keto-gluconate and ammonium concentrations were determined as described previously (97). The biomass yield was determined by calculating the amount of protein produced per amount of glucose consumed. An optical density of 1 at 600 nm corresponds to a concentration of cell dry weight of 465 mg/l and 279 mg/l protein (data not shown).

qPCR-Analysis

Total RNA extractions were performed with the RNeasy kit, as described by the manufacturer (Qiagen). After DNase treatment, the RNA samples were treated with the RNeasy MiniElute (Qiagen) clean-up kit. RNA-concentrations were measured using an NanoDrop-1000 spectrophotometer

(Thermo Scientific, Waltham, Massachusetts, USA). All primers (Table 3.6) were purchased from Eurofins MWG Operon (Ebersberg, Germany).

Table 3.6 Primers used for qPCR analysis.

Target gene	Primer name	Nucleotidic sequence (5' → 3')	Amplicon size
trgI	PP3611_A_f	ATGTCACCACACAGGTAACG	79
	PP3611_A_r	ACATCCTTCTTCTCGTCAGG	
srpB	srpB_B_f	ATCTGCTGGAAACCGTAGAC	174
	srpB_A_r	ACATGACCAGGAAGACCAGT	
plsB	PP1520_A_f	GACTACCTGCTGCTGTCGTA	182
	PP1520_A_r	TGCAGGTACTCGTTGAACAC	
paaI	PP3281_B_f	CTGCACCATCGACTACCTG	198
	PP3281_B_r	CATTCATCTTGCGTCTCCTG	

Targets for q-RT-PCR and qPCR were the genes and corresponding mRNAs coding for *trgI*, *srpB* and putative *plsB* and *paa*. The latter two genes are housekeeping-genes used as a control to correct for mRNA losses during RNA-preparation and for experimental variations. They were selected after transcriptomics analysis under comparable experimental conditions in which constant gene expression was found. Specific primers (Table 3.6) were designed using Primer3-software (http://fokker.wi.mit.edu/primer3/input-040.htm; length max. 20 bases, G/C content 50-60%, T_m 55 - 60 °C). PCR-primer target sequences were chosen to achieve amplicon lengths of 75-200 bp. To predict possible amplicon secondary structures Mfold–software (http://www.bioinfo.rpi.edu/applications/ mfold/) was used. qPCR was performed with a spectrofluorimetric thermal cycler (iCycler thermal cycler equipped with optical module; BioRad, Hercules, California, USA) using iQ SYBR Green Supermix for qPCR or iScript One-Step RT-PCR Kit with SYBR Green for q-RT-PCR (BioRad) of total RNA samples in 96-well plates according to manufacturers' protocols ($T_{annealing}$: 58 °C). Ribonuclease inhibitor Superase·In (Ambion) was added at a concentration of 0,5 U/µl to all q-RT-PCR batches. Fluorescence of ds-DNA-bound SYBR Green was measured at the end of each polymerisation step. A denaturation curve (55 °C to 95 °C, 0,05 °C s^{-1}) specific for each amplified sequence was established at the end of the PCR reaction. The increment in fluorescence versus reaction cycle was plotted and the threshold cycle (C_T) was obtained by manual positioning of the threshold baseline at 100 relative fluorescence units. Calibration curves relating the C_T as function of log of copy number of target gene were established using 10-fold serial dilutions of *P. putida* S12 genomic DNA or plasmid DNA carrying either

a cloned *trgI-* or *srpB gene*, respectively. Standard curves spanned a range of 10 to 10^7 gene copies per µl of template DNA. Efficiencies of PCR amplifications were approximately 95%. All absolute quantifications were obtained using iCycler iQ real-time detection system software version 3.0 (BioRad). The C_T of individual *trgI* and *srpB* measurements were normalized by average factors (0,97 – 1,02) calculated from differences in copy numbers of transcripts of the housekeeping genes in different samples to a constant value.

The dynamic response of *Pseudomonas putida* S12 to the sudden addition of toluene and the potential role of the solvent tolerance gene *trgI*

In this study, the genome wide transcriptional response of *Pseudomonas putida* S12 to sudden toluene exposure was monitored for 30 min after toluene addition. Through this approach we obtained insight into the primary defence mechanisms operating against sudden solvent stress. Key findings indicated that during toluene exposure, high oxygen-affinity cytochrome *c* oxydase is specifically needed to provide for an adequate proton gradient. Concomitantly, the glyoxylate bypass route was upregulated, indicating a toluene stress-induced NADH surplus possibly caused by a disturbed redox balance. The response of a knock-out mutant of *trgI*, a recently identified, specifically toluene-repressed gene, was investigated in order to identify TrgI function. Remarkably, following the addition of toluene the number of differentially expressed genes in the *trgI*-mutant strain initially was much lower than in the wildtype strain. This suggested that deletion of *trgI* prepared the cells for a sudden addition of organic solvent. Differential expression was observed for many genes of highly diverse functions in *trgI*-mutant cells as compared to wildtype cells, before as well as after addition of toluene. This leads to the hypothesis that TrgI was not only involved in the regulation of solvent-elicited responses but in addition affected basal expression levels of large groups of genes.

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Introduction

Pseudomonas putida S12 is an exceptionally solvent-tolerant bacterium that thrives in the presence of organic solvents such as 1-octanol, toluene and benzene (58, 60, 140). Several mechanisms of solvent tolerance have been identified, the most important of which is the solvent extrusion pump SrpABC. This RND-type transporter actively removes solvent molecules from the cell at the expense of a proton gradient (62, 68).

Previously, we reported on the proteome and transcriptome-level responses of *P. putida* S12 to toluene (141, 142). Several expected responses were observed, *e.g.*, the induction of the solvent extrusion pump SrpABC and differential expression of membrane-associated and stress-response genes. A novel finding was the differential expression of energy-management systems, presumably in response to the energy requirement brought about by the solvent extrusion pump and the accumulation of solvent molecules in the cytoplasmic membrane. Moreover, a novel gene of solvent tolerance, *trgI*, was identified. The expression level of *trgI* was shown to decrease immediately and very rapidly after sudden addition of toluene. In steady-state chemostats, both the gene and its encoded protein were significantly down-regulated in the presence of 5 mM toluene (3.6, respectively, 2.9-fold) (141, 142). Deletion of *trgI* (141) resulted in a more rounded cell morphology and increased resistance to sudden toluene shocks. Although the precise function of *trgI* remained obscure, it was hypothesised that it is involved in the first line of defence against solvents (141).

The immediate down-regulation of *trgI* upon toluene exposure provoked the question how expression of other genes in *P. putida* S12 would react to the sudden addition of toluene. This would provide valuable insight into the cellular functions involved in the early adaptional response to organic solvents. Although in the (recent) past several -omics studies of solvent-exposed microorganisms have been published (for example (27, 41, 119, 136, 141, 142)), none of these involved a genome-wide monitoring of the early adaptational response. Instead, batch cultures were sampled at a single time-point, or steady-state chemostat cultures that were fully adapted to organic solvent were analysed. Therefore, we studied the global gene expression of *P. putida* S12 in the first 30 min after the addition of toluene. In addition to wild type *P. putida* S12, the *trgI* knock-out mutant *P. putida* S12ΔTrgI was investigated to shed more light onto the role of this gene in the early solvent tolerance response.

Results

The dynamic response of *P. putida* S12 to sudden toluene exposure - global analysis of transcriptomics data

The addition of toluene to exponentially growing *P. putida* S12 provoked an extensive change in the global transcriptional activity (Figure 4.1). Although this effect was observed both in the wildtype and the *trgI*-knockout strain, marked differences were observed in the timing and the total number of differentially expressed genes. In wildtype *P. putida* S12, a large number of genes were differentially expressed immediately after toluene addition, reaching a maximum after 5 min and showing a transient decrease after 10 min. In *P. putida* S12ΔtrgI, the expression response appeared to be delayed. The number of differentially expressed genes was initially much lower and did not reach its maximum until 10 min after toluene addition. The total number of differentially expressed genes, however, eventually exceeded that in the wildtype strain.

The number of toluene-responsive genes that were differentially expressed in both the wildtype strain and *P. putida* S12ΔTrgI followed a course that was remarkably similar to the overall number of differentially expressed genes in the *trgI*-knockout strain (Figure 4.1). These genes may respond to the -very rapid- downregulation of *trgI*. The peak in the transcriptional response of the wildtype strain 5 min after toluene addition may indicate a generic rearrangement response that is independent of TrgI.

The fold changes of the toluene-responsive genes in both wild type and the ΔtrgI strain were calculated for every possible combination of two time-points, *i.e.*, between 0 and 1 min, 0 and 2 min, 5 and 30 min, etc. These combinations represent all possible time-frames within the experiment. The toluene-responsive genes were furthermore classified by functional category to determine whether specific cellular functions were overrepresented within certain time frames. Functional classification was performed by the categories defined in the NCBI COG database (Clusters of Orthologous Groups of proteins; http://www.ncbi.nlm.nih.gov/COG/old/palox.cgi?fun=all) (131, 132)). Table 4.1 shows a summary of the results; a complete overview for both strains is presented in the Supplementary Results at the end of this chapter (Figure S4.1).

The overrepresentation of specific functional categories was most explicit in wildtype *P. putida* S12, for the genes that were upregulated immediately after addition of toluene. Among these genes, category C (Energy

production and conversion), E (Amino acid transport and metabolism), I (Lipid metabolism) and O (Posttranslational modification, protein turnover and chaperones) were significantly overrepresented. In *P. putida* S12ΔtrgI only genes of category O were overrepresented among the genes with this expression profile, which is consistent with the delayed global transcriptional response of the *trgI* deletion mutant.

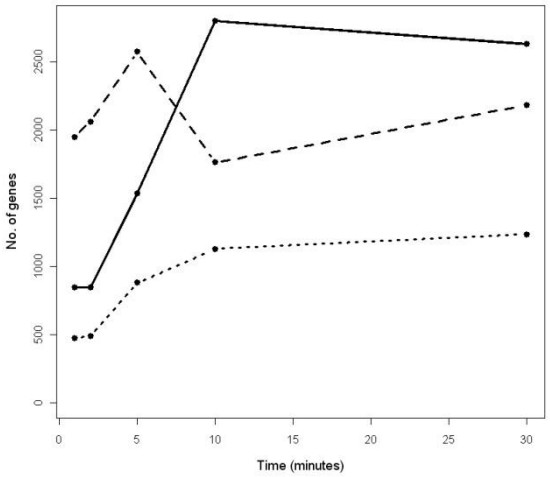


Figure 4.1 Summary of the transcriptomics results. Number of genes differentially expressed with an absolute log2(expression level at t = 0 / expression level at t= t) ≥0.5 in wildtype S12 (dashed line) and mutant S12ΔTrgI (solid line) at the indicated time points are shown. The dotted line shows the number of genes that is present in both comparisons. T = 0: no toluene present.

In the group of genes that were initially upregulated in response to toluene followed by downregulation, category T (signal transduction) was overrepresented in both wildtype *P. putida* S12 and the *trgI*-knockout strain. Category N (motility) was furthermore overrepresented in the wildtype strain. Category N was also overrepresented in the *trgI*-knockout strain, but rather among the genes that were only downregulated later after toluene addition. Other categories overrepresented in both wildtype *P. putida* S12 and the *trgI*-knockout strain among this group of genes that were downregulated later were J (translation) and H (coenzyme metabolism). Genes of category F (nucleotide transport and metabolism) were overrepresented in both strains among the

Table 4.1 Overrepresentation of COG groups per time-period in *P. putida* S12 and *P. putida* S12ΔTrgI.

COG groups showing described pattern			
Description of pattern	***P. putida* S12** [b]	***P. putida* S12ΔTrgI**[b]	**Example**[c,d]
High significance of overrepresentation among upregulated genes.	C,E,I,O (M,Q,V)[a]	O (V,P)[a]	8.7 8.2 6.8 2.2 0.6 8.5 2.3 2 0.7 0.1 4.6 1.5 2.8 0 0 3.1 1.2 0 0 0 2.2 0.5 0 0 0 0.2 0.3 0 0 0
Overrepresented genes are initially upregulated, later downregulated.	N,T	T	0 0 0 0.1 0 0 0 1 0.2 0.1 2.2 0.7 0.6 11 11 6.9 0.1 4.9 8.6 12 3.5 0.1 3.6 6.6 8.5 0 0 0.7 2.1 2.2
Overrepresented genes are downregulated later in time.	J,H	N,J,H	0.1 0 0 0.1 0.2 0 0 0 0.1 1 0.1 0.1 0.1 18 11 0.7 0.6 6.6 13 12 0.6 0.4 5.1 12 9.6 0.1 0.1 3.1 12 9.6
Overrepresented genes are downregulated shortly after addition of toluene.	F	F	0 0.2 0.2 0.1 0.1 0 0 0.1 0 0.2 0 0 0.3 0.7 0.9 0 1.1 0.9 0.6 1.1 0 1.1 0.4 1.5 1.2 5.2 5.5 1.7 4.3 4.7
Overrepresented genes respond slowly with downreg., later with upregulation.	K	-	0.7 0.2 1.1 3.6 2.3 0.1 0 0 0 0 0 0 0 0.4 0.1 0.6 0.5 3.5 3.2 1.6 0.6 3.5 2.4 2 1.7 0 0.1 0.6 3 0.6
Overrepresented genes are upregulated.	-	K	4.2 3.8 3.9 8.2 6.9 0.6 0.3 0.3 2 0 0.1 0.8 0.4 0.3 0 0 0.8 2.2 1.3 0.4 0.4 0 1.9 0.7 0.3 0.1 0 0.8 0.9 0.1
No significant overrepresentations	A,D,G,J,L, P,U,W	A,C,D,E,G, I,L,M,Q,U,W	-

a) Same pattern of overrepresentation, but with lower significances
b) Genes without COG (43 genes) and genes belonging to COG's S (Function unknown, 2292 genes) and R (General function prediction only, 596 genes) were not taken into account. Abbreviations (with total number of genes in brackets): A RNA processing and modification (16), C Energy production and conversion (260), D Cell division and chromosome partitioning (24), E Amino acid transport and metabolism (431), F Nucleotide transport and metabolism (90), G Carbohydrate transport and metabolism (164), H Coenzyme metabolism (131), I Lipid metabolism (166), J Translation, ribosomal structure and biogenesis (78), K Transcription (418), L DNA replication, recombination and repair (225), M Cell envelope biogenesis and outer membrane (214), N Cell motility (72), O Posttranslational modification, protein turnover and chaperones (117), P Inorganic ion transport and metabolism (246), *Continued on next page*

Table 4.1 *Continued* Q Secondary metabolites biosynthesis, transport and catabolism (123), T Signal transduction mechanisms (239), U Intracellular trafficking and secretion (56), V Defence mechanisms (159), W Extracellular structure (4).
c) Values in the figures are –log(*p*-value), values above the diagonal represent significance of overrepresentation of upregulated genes and below the diagonal downregulated genes are represented. On the x-axis the boxes represent (from left to right) time points 0, 1, 2, 5, 10 and 30 minutes, on the y-axis the boxes represent (from top to bottom) time points 30, 10, 5, 2, 1 and 0.
d) A figure of overrepresentation for each COG in both strains is shown in the Supplementary Results at the end of this chapter, Figure S4.1.

genes that were downregulated early after toluene addition. In the wildtype strain, category K (transcription) was overrepresented among the genes that were downregulated a few minutes after addition of toluene, followed by upregulation. In *P. putida* S12ΔTrgI, genes of this category were among the genes that were consistently upregulated after toluene addition. Thus, clear differences were visible between wildtype *P. putida* S12 and the *trgI* deletion mutant, not only in the global transcriptional response to toluene but also in the timing of expression of genes from different functional categories.

Effect of *trgI* deletion on the transcriptional response to toluene

The analysis of the global transcriptional response to toluene revealed clear differences between wildtype *P. putida* S12 and the *trgI*-knockout strain. To establish which genes were affected by the *trgI* deletion, a systematic classification based on expression behaviour was applied to each individual gene (Table 4.2). For this classification, the genes were divided into two groups at three different levels. First, a division was made between genes that did (Group I) or did not (Group II) show a response to toluene in the wildtype strain. Next, the genes were classified based on their intrinsic expression levels, *i.e.,* the expression level prior to toluene exposure. The intrinsic expression levels of the genes in Group A were similar in the wildtype and *trgI*-knockout strains; the genes in Group B showed an altered (higher or lower) intrinsic expression level in *P. putida* S12ΔTrgI. Finally, the response to toluene was considered: Group 1 contained the genes whose response to toluene was similar in the wildtype and *trgI*-knockout strain; Group 2 contained the genes that showed an altered response to toluene between the two strains. Classifying the genes in a hierarchical order based on these three criteria (toluene responsiveness in wildtype strain > intrinsic expression level > toluene response) yielded eight clusters of genes with similar expression behaviour in relation to the *trgI* deletion. Furthermore, a functional category (see above) was coupled to each individual gene to assess whether specific cellular functions were more affected than others by the *trgI* deletion (Table 4.2).

Table 4.2 Number of genes and overrepresented COGs per expression profile cluster in *P. putida* S12 and *P. putida* S12ΔTrgI.

Category	Intrinsic expression level in S12ΔTrgI versus S12[b]	Toluene response in S12ΔTrgI versus S12[c]	No. of genes (% of total)	Overrepresented COGs
Toluene responsive genes in S12 (Total no. of genes: 3999)[a]				
I-A-1	=	=	942 (15 %)	OHJFD (LA)
I-B-1	≠	=	414 (7 %)	C (IEKMN)
I-A-2	=	≠	1049 (17 %)	(ILPFAN)
I-B-2	≠	≠	1594 (26 %)	SQEC
Toluene non-responsive genes in S12 (Total no. of genes: 2165)				
II-A-1	=	=	345 (6 %)	O (D)
II-B-1	≠	=	58 (1 %)	O (EPW)
II-A-2	=	≠	1289 (21 %)	JU(SWnocog)
II-B-2	≠	≠	473 (8 %)	EG (VW)

a) (=) expression level or response are the same; (≠) expression level or response are different.
b) Intrinsic expression level is expression level at t = 0, immediately before addition of toluene. The expression level was defined as 'different' at a ratio of <-0.5 or >0.5.
c) Toluene response is response immediately after addition of toluene until t = 30 minutes. The expression level was defined as 'different' when the correlation between S12 and S12ΔTrgI <0.8.

Clusters I-A-1 and II-A-1 represent the genes that were unaffected by the *trgI* deletion, with regard to both the intrinsic expression level and the response to toluene. Surprisingly, these two clusters comprised only 1287 genes, *i.e.*, less than 20 % of the total genome. In these clusters, particularly COGs O (posttranslational modification, protein turnover and chaperones), H (coenzyme metabolism) and D (cell division and chromosome partitioning) were overrepresented. This observation suggests that these basic cell processes were not affected to a large extent by the *trgI*-deletion. Clusters I-B-1 and II-B-1, showing an unaltered response to toluene but an altered intrinsic expression level, also contained a small number of genes (<500). In cluster I-B-1, COG C (energy production and conversion) was overrepresented, indicating that the *trgI* deletion caused an intrinsic change in the energy metabolism. In cluster II-B-1, COG O was overrepresented, which suggests that *trgI* affects the transcription of chaperone-encoding genes.

The large majority of the genes that were affected by the *trgI*-deletion showed an aberrant toluene response. These genes are contained within clusters I-A-2, I-B-2, II-A-2 and II-B-2 and comprise a total of more than 4300 genes. Among these genes, a large number (> 1700) appeared to have gained toluene responsiveness due to the *trgI* deletion (cluster II-A-2 and II-B-2), but an even larger number (> 2600) appeared to have lost this property (clusters I-

A-2 and I-B-2). Many functional groups were overrepresented in these clusters, some of which (COGs C, I, W, G) can be linked to solvent tolerance. Others (COGs A, F, E) are rather associated with primary cellular processes.

Effect of toluene and deletion of *trgI* on genes and pathways associated with toluene tolerance

As indicated above, deletion of *trgI* affected the expression of a very large number of genes, which suggested that *trgI* exerted a high-level regulatory effect. This regulatory effect was strongly associated with the response to toluene. Although the *trgI*-affected genes covered many different functional groups, COGs C, E and I (energy, amino acid metabolism and lipid metabolism) appeared to be overrepresented (Tables 4.1 and 4.2). Since these functional categories can be rationally linked to solvent tolerance, the transcriptional response of genes and pathways within these COGs was studied in more detail, together with genes and pathways with an established role in solvent tolerance.

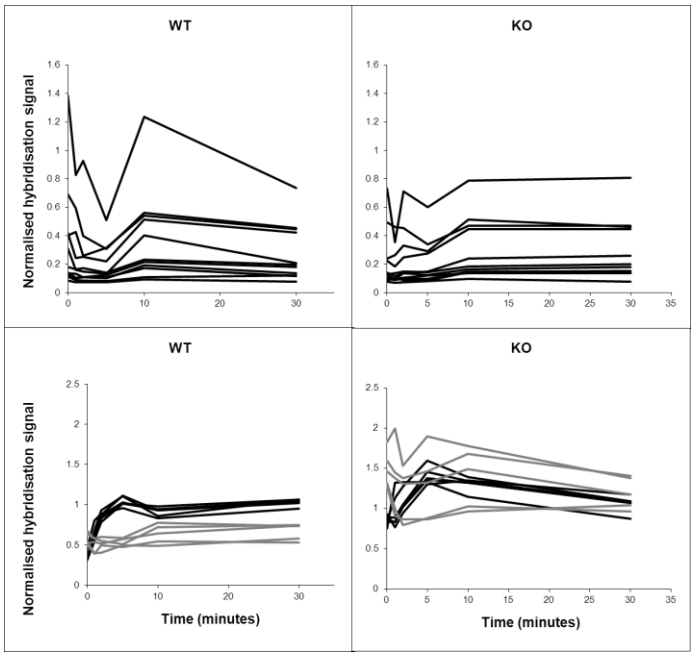


Figure 4.2 Normalised hybridisation signals of: Upper panels: cytochrome *c* oxidase aa3-type genes, Lower panels: cytochrome *c* oxidase cbb3-type genes. (black solid) cbb_3-1, (grey) cbb_3-2. (WT) *P. putida* S12; (KO) *P. putida* S12ΔTrgI.

Toluene-responsive genes involved in energy metabolism

The accumulation of organic solvents in the bacterial membrane puts a heavy burden on the cellular energy metabolism as a result of uncoupling and the energy requirement of the solvent extrusion pump. This was clearly reflected in the differential expression of many genes associated with cellular energy management systems, most notably cytochrome *c* oxidase-encoding genes (Figure 4.2). *P. putida* S12 harbours several different types of terminal cytochrome *c* oxidases: in addition to the high-oxygen affinity types *cbb*$_3$-1 and *cbb*$_3$-2 there is also the low affinity variant *aa*$_3$.

The genes encoding the *aa*$_3$-type were consistently expressed to a lower level than the genes encoding the *cbb*$_3$-type, irrespective of the presence of toluene or *trgI*. The two *cbb*$_3$ variants were expressed to different levels depending on the presence of toluene, suggesting a preference for the *cbb*$_3$-1 type over the *cbb*$_3$-2 type. In the *trgI* mutant, the intrinsic expression level of both *cbb*$_3$ type-encoding genes was higher than in the wildtype strain. In the

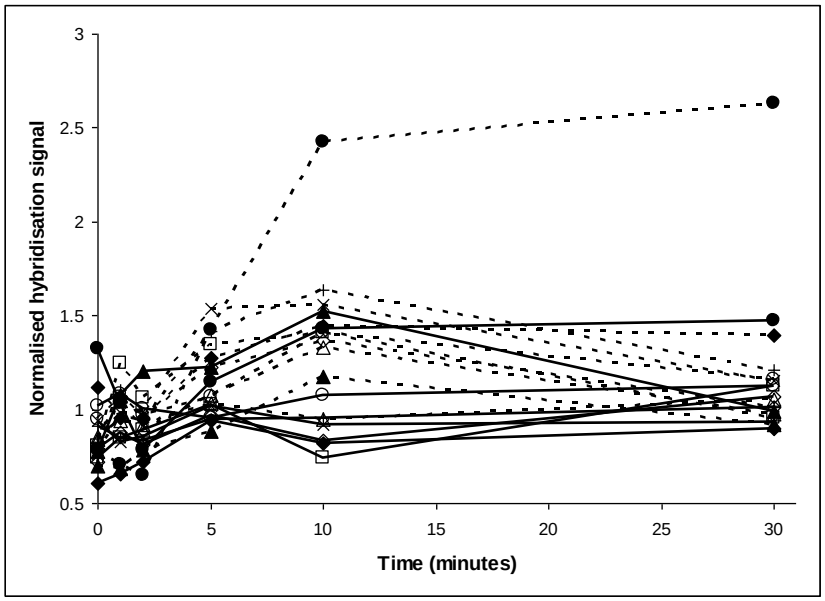


Figure 4.3 Normalised hybridisation signals of NADH dehydrogenase genes. Solid lines: *P. putida* S12; dotted lines: *P. putida* S12ΔTrgI. ● NADH dehydrogenase (RPPX05605), ♦ NADH dehydrogenase (RPPX00059), ▲ NADH-quinine oxidoreductases chain A, × NADH-quinone oxidoreductases chain B, + NADH-quinone oxidoreductases chain C, ○ NADH-quinone oxidoreductases chain E, Δ NADH-quinone oxidoreductases chain I, □ NADH-quinone oxidoreductases chain L.

presence of toluene, the expression level of the *cbb*$_3$1 genes increased even further and subsequently decreased to the wildtype level. The slightly variable expression levels of the *cbb*$_3$2 genes consistently exceeded those of wildtype *P. putida* S12 upon toluene exposure.

In addition to cytochrome *c* oxidase-encoding genes, several genes encoding subunits of the NADH dehydrogenase complex were expressed to a higher level in the *trgI*-knockout strain within 10 min after toluene exposure (Figure 4.3). After 30 min, the expression level of the genes encoding the quinone oxidoreductase-subunit had returned to the wildtype level whereas the genes coding for the dehydrogenase subunit remained at a high level for at least 30 min after toluene exposure.

Toluene-responsive genes involved in lipid metabolism

The accumulation of organic solvent molecules in the bacterial membrane leads to cell lysis, releasing membrane fatty acids into the medium. In *E. coli* free fatty acids induce fatty acid degradation via *fadR* (64). *P. putida* S12 does not harbour a *fadR* homologue, but the gene *psrA* (RPPX05955 + RPPX00992) is most likely responsible for the regulation of part of the fatty acid degradation pathway (64). In toluene-exposed *P. putida* S12 *psrA* as well as the genes of the fatty acid degradation pathway were up-regulated after addition of toluene (Supplementary Results at the end of this chapter, Figure S4.2, solid lines). In addition to fatty acid degradation, fatty acid synthesis may be expected to occur in solvent-exposed *P. putida* S12 since newly synthesized fatty acids are required to counteract the toluene-induced degradation effects (53). Differential expression of genes involved in fatty acid biosynthesis, however, was variable (Supplementary Results at the end of this chapter, Figure S4.3). In general, COG I was not overrepresented amongst the group of genes that was up-regulated after addition of toluene in S12ΔTrgI, while it was overrepresented in the wildtype (Table 4.1). Hence, the number of COG I genes that was highly up-regulated was relatively lower in the mutant than in the wildtype. It should be noted that the fatty acid biosynthetic genes are poorly annotated in the *P. putida* S12 genome (unpublished), which prevented a more detailed analysis of the responses of these genes to toluene.

Toluene-responsive genes involved in acetyl-CoA metabolism

The genes encoding isocitrate lyase and malate synthase were found to be up-regulated in response to toluene. This effect was observed in both wildtype *P. putida* S12 and the *trgI* mutant (Supplementary Results at the end of this chapter, Figure S4.4) although the level of up-regulation was higher in the latter. The encoded enzymes constitute the glyoxylate bypass, which

converts acetyl-CoA into succinate and malate for biosynthesis of, i.a. amino acids of the aspartate family. This pathway is normally active during growth on C_2-compounds and is controlled at the enzymatic level through NADH-mediated inhibition of isocitrate dehydrogenase (8). Induction of the glyoxylate shunt genes is indicative of the shunt being active and hence, an NADH surplus during toluene stress. Possibly, differential expression of respiratory chain components in response to toluene (as described above) leads to a disturbed redox balance that is counteracted by inducing the glyoxylate bypass.

Alternatively, the glyoxylate shunt may be co-regulated with the degradation of fatty acids, or with valine, leucine and isoleucine degradation. In these pathways, the main degradation product is acetyl-CoA which effectively represents a C_2 substrate (64). It should be noted that multiple genes involved in the degradation of these amino acids were up-regulated in response to toluene and, again, the level of up-regulation was significantly higher in the *trgI* mutant strain (Supplementary Results at the end of this chapter, Figure S4.5). These pathways may well have been induced by the relative abundance of valine and (iso-)leucine in the LB culture medium.

Toluene-responsive genes involved in solvent extrusion

Expression of *srpABC*, coding for the solvent extrusion pump SrpABC, and *srpRS*, the associated regulatory genes, increased immediately in wild type *P. putida* S12 after addition of toluene. This is in accordance with previous observations (141), but expression did not reach a maximum after 30 min of toluene exposure as reported earlier (69) (Figure 4.4). Expression of other loci encoding part of the putative toluene transport system Ttg2FEDC and the multidrug transporter ArpABC (RPPX02774-2777 and RPPX04100-4103) increased immediately after addition of toluene, reaching a maximum after 30 min of toluene exposure (Figure 4.4). All expression levels were similar in wildtype and the *ΔtrgI* strain prior to toluene exposure, but increased much faster in the *ΔtrgI* strain, to much higher levels. It is unclear to which extent Ttg2FEDC is involved in toluene tolerance, and ArpABC has been established to play a role in antibiotic resistance rather than solvent tolerance (66) . These observations suggest *trgI* to be involved in a more general response to stress conditions including, but not restricted to, solvent-associated stress.

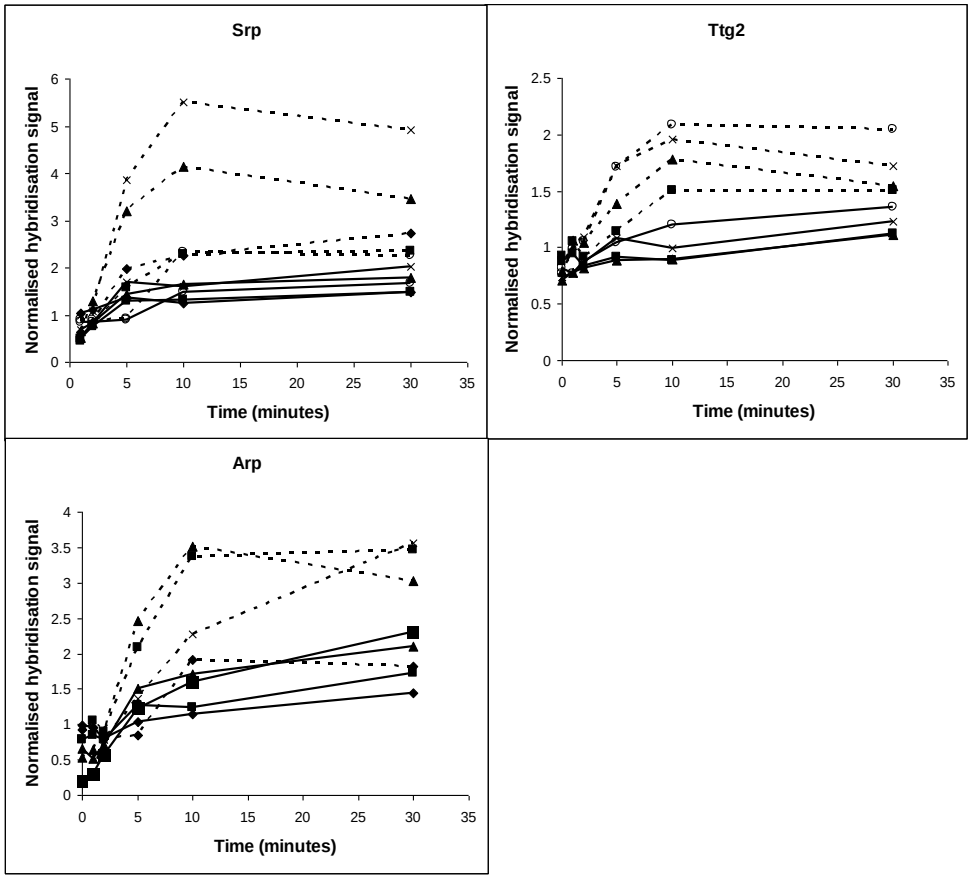


Figure 4.4 Normalised hybridisation signals of *srpRSABC* and *ttg2DC* and the genes coding for a toluene transport system permease and ATP-binding protein. Solid lines: *P. putida* S12; dotted lines: *P. putida* S12ΔTrgI. Upper left panel: ♦ RPPX00287 (*srpR*); ■ RPPX00288 (*srpS*); ▲ RPPX00289 (*srpA*); × average of RPPX00290 and RPPX00291 (*srpB*); ○ RPPX00292 (*srpC*). Upper right panel: ■ RPPX02774 (*ttg2D*); ▲ RPPX02775 (*ttg2C*); × RPPX02776 (toluene transport system permease); ○ RPPX02777 (toluene transport system ATP-binding protein). Lower right panel: ♦ RPPX04100 (*arpC*), ■ RPPX04101 (*arpB*), ▲ RPPX04102 (*arpA*), × RPPX04103 (*arpR*).

Effect of toluene and deletion of *trgI* on genes and pathways that are not directly associated with toluene tolerance

Differential expression of the arginine deiminase pathway in response to toluene

The ubiquitous arginine deiminase (ADI) pathway serves to generate energy from arginine fermentation under anoxic or carbon-limiting conditions (88). In the ADI pathway, arginine degradation via citrulline to ornithine yields

1 ATP per arginine molecule. Each ornithine generated is exported and exchanged for another arginine molecule via an arginine-ornithine specific antiporter. In wildtype *P. putida* S12, the genes encoding the enzymes for this pathway (arginine deiminase, ornithine carbamoyltransferase and carbamate kinase; RPPX04947-4949 and 2731-2732) and the arginine/ornithine antiporter (RPPX04945) were upregulated immediately after addition of toluene (Supplementary Results at the end of this chapter, Figure S4.6). In contrast, another associated arginine/ornithine antiporter gene (RPPX04946) appeared to be downregulated, together with *aotM*, *aotP* and *aotQ*, that constitute an arginine/ornithine importer. In *P. putida* S12ΔTrgI the expression of the ADI

pathway genes was considerably higher; already in the absence of toluene, the expression was similar to that of the wild type after 30 min of toluene exposure. Upon addition of toluene, the expression levels increased even further. The expression of the antiporter gene RPPX04945 decreased in the *trgI*-knockout mutant, after an initial up-regulation, to a level below that of the wildtype strain after 30 min of toluene exposure, as did expression of RPPX04946 and *aotM*, *aotP* and *aotQ*. Since the ADI pathway genes were upregulated where the antiporter genes were downregulated, it appears that the ADI pathway rather serves to accumulate intracellular ornithine than to generate energy under toluene-stressed conditions.

Differential expression of protein folding genes in response to toluene

In the periplasm, proteins can be folded oxidatively via disulphide bond formation. The enzymes involved in this process are *DsbAB*, which oxidizes unfolded proteins and *DsbCDG*, which isomerizes disulfide bonds in misfolded proteins (20). In *P. putida* S12, six loci have been annotated as part of this system, representing *dsbACDG* (RPPX02408, 4213, 4272, 4274, 5428 and 5431). Two other loci (RPPX04273 and 1161) possibly also belong to this system. In addition, chaperonins play an important role in protein folding, encoded by *dnaJ*, *groES*, *groEL*, *htpG*, and a gene coding for a 33-kDa chaperonin (RPPX07096). Upon toluene exposure of the wildtype strain, all those genes were down-regulated within the first two minutes, and subsequently up-regulated (Supplementary Results at the end of this chapter, Figures S4.7 and S4.8). In the ΔtrgI strain, most if not all genes were up-regulated to an even higher extent. These observations indicate that the damage-repair response as a result of solvent exposure is more severe in a *trgI*-knockout strain.

Deletion of *trgI* has a general effect on glucose and fructose metabolism

A remarkable physiological effect of the *trgI* deletion is loss of the ability to utilize glucose or fructose as the sole source of carbon and energy (141). Whereas fructose is imported via a PTS-type transporter and further metabolized in the cytoplasm as fructose-1-phosphate, the initial glucose metabolism is more complex in *Pseudomonads*. Glucose enters the periplasm via porins OprB-1 or OprB-2 and is subsequently transported directly into the cytoplasm via an ABC transporter encoded by *gtsABCD*, or oxidized in the periplasm via gluconate (catalyzed by Gcd, glucose dehydrogenase) to 2-ketogluconate (catalyzed by Gad) (23). Gluconate and 2-ketogluconate are transported to the cytoplasm via dedicated transporters encoded by *gntP*, respectively *kguT* and phosphorylated by GnuK, respectively, KguK.

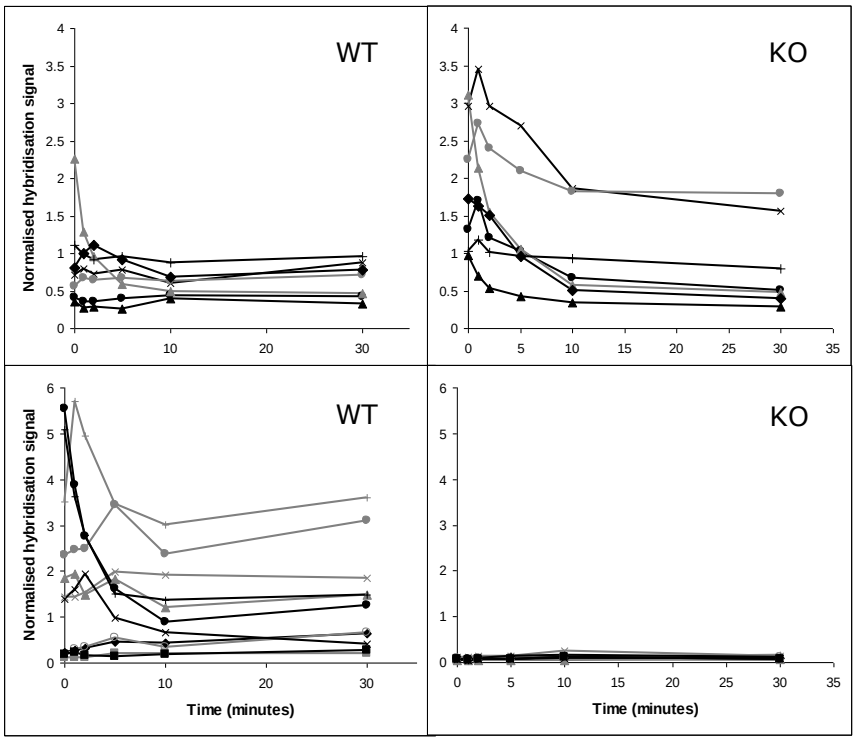


Figure 4.5 Normalised hybridisation signals of genes involved in glucose import and metabolism. (Upper panels) ♦ *gtsA*, ▲*gtsB,* ● *gtsC*, × *gtsD,* + *gcd*, ▲ *oprB-2,* ● *oprB-1*; (Lower panels) + *ptxS*, ○ *kguK*, ■ *kguT*, × *gad cytochrome* c *subunit*, ● *gad alpha chain*, ▲ *gad gamma chain*, + *gnuK*, ● *gntP*, × *gnuR*, ♦ *kguD,* ■ σ54-dependent transcriptional regulator. (WT) *P. putida* S12; (KO) *P. putida* S12ΔTrgI

The present study clearly indicated that the inability to utilize glucose and fructose was not caused by impaired transcription of sugar transport genes. Expression of the fructose transporter genes was about the same in both strains whereas transcript levels of *oprB-1*, *oprB-2* and *gtsABCD* were actually higher in the Δ*trgI* strain (Figure 4.5). *Gcd* was expressed at a slightly lower level whereas the *gad* gene was not detectably transcribed as were *gntP*, *gnuK*, *kguT* and *kguK*. As expression of these genes is known to be induced by gluconate and ketogluconate (36, 129), their lack of expression suggested that gluconate and ketogluconate were absent. Since *gcd* transcription the absence of gluconate can only be explained by impaired transport of glucose into the periplasm. In addition, the relatively high intrinsic expression level of *oprB-1*, *oprB-2* and *gtsABCD* in the mutant strain strengthened the finding of Castillo *et al.* (23) that transcription of these genes is induced by glucose in the outer medium. The impaired sugar transport may be caused by structural modification of the outer cell structure, which may affect the functioning of the membrane-embedded transport proteins as described for the impaired functioning of drugs efflux transporters of a mutant *P. putida* DOT-T1E strain with altered phospholipid head group composition (10).

Discussion

TrgI is a recently discovered toluene-responsive gene of *P. putida* S12 with unknown function. To obtain insight into the possible role of TrgI, the transcriptomic responses of wild type S12 and of *trgI*-deletion mutant S12ΔTrgI (141) were monitored during the first 30 min following addition of toluene. This presented a clear overview of genes and pathways that were differentially expressed upon the sudden addition of a dose of toluene that is lethal to non-solvent tolerant bacteria (60), and revealed initial insight into a possible regulatory role for TrgI.

Remarkably, the absolute number of differentially expressed genes in S12ΔTrgI was significantly smaller than in wildtype S12 in the first 5 min after addition of toluene. Moreover, already before the addition of toluene many genes of the mutant show altered expression levels compared to the wild type. This suggests that TrgI is not only involved in toluene-elicited responses but also determines basal expression levels of large groups of genes. Apparently, inactivation of *trgI* prepares the cells for a sudden addition of toluene by 'setting' basal expression of many genes to levels comparable with wild type

cells after toluene addition. However, since after 30 minutes the number of differentially expressed genes in the mutant is significantly higher than in the wildtype, a feedback loop appears to be disturbed as well.

Accumulation of solvent molecules in membranes may cause dissipation of the proton motive force (PMF) and hence, up-regulation of oxidative phosphorylation (140, 141). In line with this, NADH dehydrogenase- and cytochrome *c* oxidase genes of COG group C (Energy metabolism) were differentially expressed. The relatively high expression levels of the cbb_3-type cytochrome oxidase compared to the lower oxygen-affinity aa_3-type in both wildtype and mutant strains suggested that in cultures exposed to toluene, high-affinity cytochrome *c* oxidase is specifically needed to provide for an adequate proton gradient.

Cells exposed to organic solvents may change their fatty acid composition to strengthen their membranes by increasing the degree of saturation, by cis-trans isomerisation of unsaturated fatty acids or by newly synthesising long chain fatty acids (53, 159). Eventually, accumulation of solvent molecules in the membranes results in cell lysis. Lysing cells release fatty acids that trigger expression of the genes encoding the fatty acid degradation pathway (COG group I: Lipid metabolism) (26). However, we did not find evidence that fatty acid-synthesis was increased. This suggests that the capacity of the available fatty acid synthesis machinery is sufficiently high.

Genes encoding the Ttg2FEDC putative early toluene transport system were rapidly upregulated in response to toluene in *P. putida* S12. Previously, only the SrpABC solvent efflux pump was found to be differentially expressed in response to toluene (140, 141). As SrpABC becomes fully transcribed only after extended toluene exposure (66), Ttg2FEDC may cushion the first blow dealt by the sudden exposure to toluene. In *P. putida* S12ΔTrgI the maximum expression level of the Srp genes was reached much sooner, whereas the Ttg2 genes and the antibiotic pump ArpABC exhibited a similar expression pattern.

Remarkably, the ADI (arginine deiminase) pathway appeared to be upregulated in response to toluene, and to an even higher level in the *trgI*-knockout mutant than in the wildtype strain. Only one arginine-ornithine antiporter gene was down-regulated in the wildtype whereas both were down-regulated in the knock-out mutant, suggesting that *P. putida* S12ΔTrgI accumulates the polyamine ornithine in response to toluene, providing additional protection against solvent stress (112). Moreover, ornithine may act as a precursor for proline, a compatible solute implicated in protection against water and solvent stress (42). Since the cultures were in mid-exponential phase, it is unlikely that either anoxic or carbon-limiting conditions occurred, which are the normal conditions for inducing the ADI pathway (88).

Genes involved in oxidative protein folding and several chaperonin-encoding genes were up-regulated in both wildtype *P. putida* S12 and the trgI-knockout mutant upon addition of toluene. Presumably, the up-regulation of those COG O genes related to the occurrence of protein misfolding and random formation of disulfide bonds. These events may well occur in solvent-exposed cells as result of oxidative stress caused by impaired respiration. The genes involved in protein folding were up-regulated to a higher level in the trgI-knockout strain, suggesting that this strain is better capable of repairing misfolded proteins.

Transcriptomics analysis of *P. putida* S12 and *P. putida* S12ΔTrgI suddenly exposed to toluene has shed new light on solvent tolerance. The initial response to toluene in wildtype cells appeared to be fully geared towards survival before all solvent tolerance mechanisms are fully induced. The clear delay in timing of the overall transcriptional response in the *trgI* deletion mutant was indicative for a milder response to sudden toluene exposure. This would be in agreement with an intrinsically improved toluene tolerance. The impact of the *trgI* deletion thus appeared to be relatively broad.

The diversity of responses associated with *trgI*, as well as its wide impact on gene expression, suggest an important regulatory role. Analyses using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and SMART (http://smart.embl-heidelberg.de/) (data not shown) did not provide any clues to a potential function. A model of TrgI tertiary structure (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (114, 157, 158) showed highest structural similarity (TM-score of 0.6923) with a molybdate-dependent transcriptional regulator, *modE*, of *E. coli* (40), although TrgI is much smaller than ModE and does not exhibit clear DNA binding residues. Hence, TrgI may act as a modulator of other transcription factors.

Materials and methods

Bacterial strains

The bacterial strains used in this study are *Pseudomonas putida* S12, which was originally isolated as a styrene utilising bacterium (44) and *P. putida* S12ΔTrgI. *P. putida* S12ΔTrgI is a *trgI* knock-out mutant that was constructed as described previously (141).

Standard culture conditions

Luria broth (LB medium) (117) was used as the standard culturing medium. As a solid medium, LB with 1.5% (w/v) agar was used. Batch cultivation was routinely carried out in 100-ml Erlenmeyer flasks containing 25 ml of liquid medium, placed on a horizontally shaking incubator at 30 ºC.

Analysis of differential gene expression after a sudden addition of toluene

Culture conditions

Differential gene expression after a sudden addition of 5 mM toluene was analysed in early exponential phase cultures (optical density at 600 nm of 0.5-0.6) of *P. putida* strains S12 (wildtype) and S12ΔTrgI (141). Samples were drawn immediately before (t=0) and at set intervals (1, 2, 5, 10 and 30 min) after toluene exposure. Messenger RNA (mRNA) was isolated from the samples and microarray analysis was performed as described previously (141).

Transcriptome analysis

Sampling, mRNA isolation, cDNA preparation and hybridisation for transcriptome analysis were performed as described previously (141). The custom made high-density microarrays used were based on the newly available sequence of *P. putida* S12 (Ruijssenaars and de Winde, manuscript in preparation). The genome sequence of strain S12 contains 7107 open reading frames (orfs), of which 1903 are not annotated.

Data analysis

Microarray data were imported into the GeneSpring GX 7.3.1 software package (Agilent Technologies, Santa Clara, CA, USA) using the GC RMA algorithm. After normalisation (signals below 0.01 were taken as 0.01; per chip: normalise to 50th percentile; per gene: normalise to median) of the data, probesets representing control genes were removed, as well as absolute non-changing loci (between 0.667- and 1.334-fold change). The resulting set of 6164 differentially expressed loci was used for further investigation.

The overall transcriptional activity change during toluene exposure was quantified by calculating the total number of differentially expressed genes for each time point after toluene addition, using the transcript levels at t = 0 as reference (Figure 4.1).

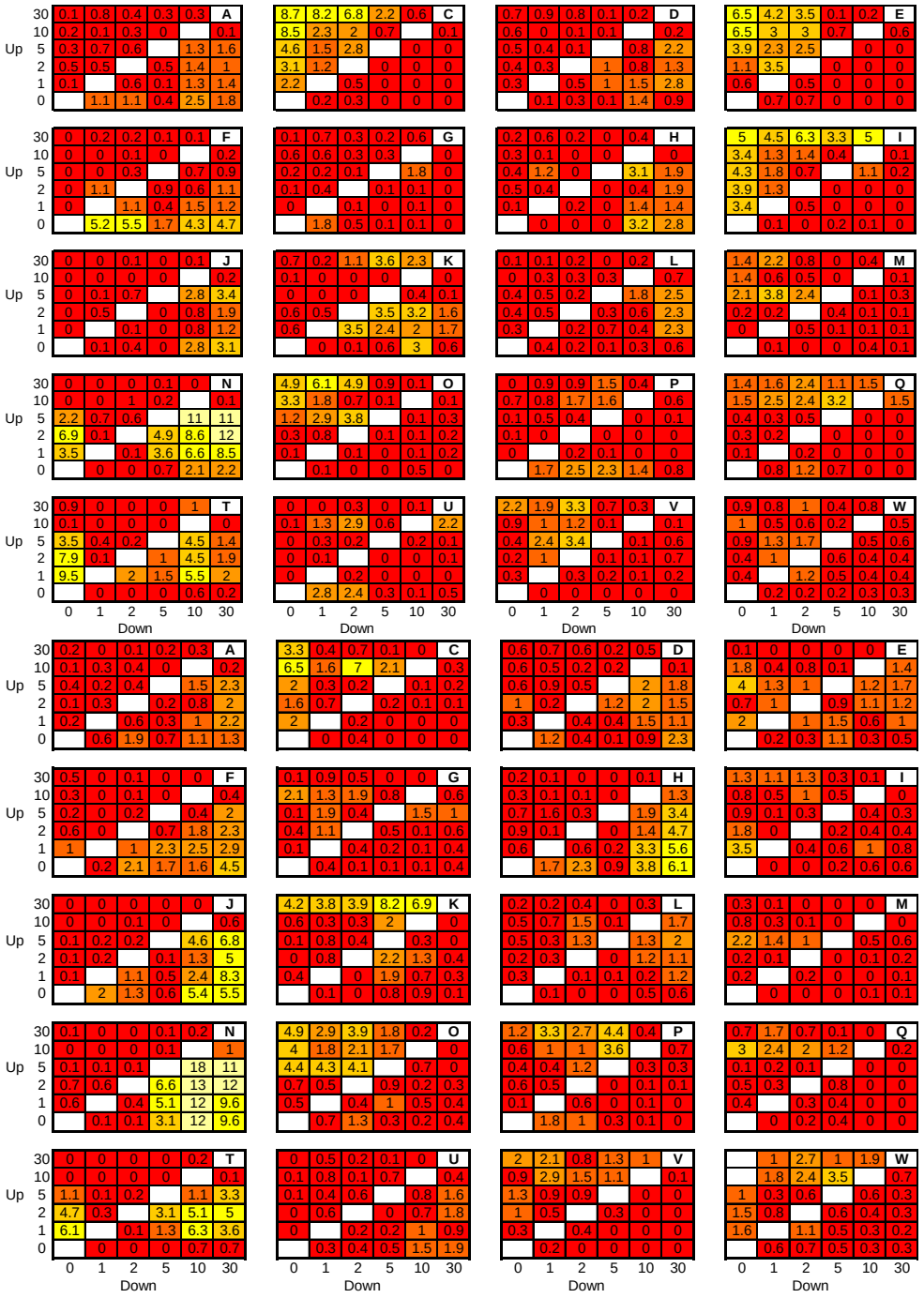
Overrepresentation and statistical analysis

Overrepresentation of specific groups of genes among the total response-groups was determined using the hyper-geometric test in the R statistical program environment. For example: if a group of genes selected for upregulation

following toluene exposure, the chance that an x-number of genes of COG group A are in that group of upregulated genes is being assessed. Or, in other words, it can be assessed what the chance is that this group of upregulated genes contains 50% COG group A while all genes contains 30% COG group A. The comparison between sets of two such groups (in this example 'up regulated' and 'COG group A') can be found in Table 4.1.

Supplementary Results

The supplementary results start on the next page.


A
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5
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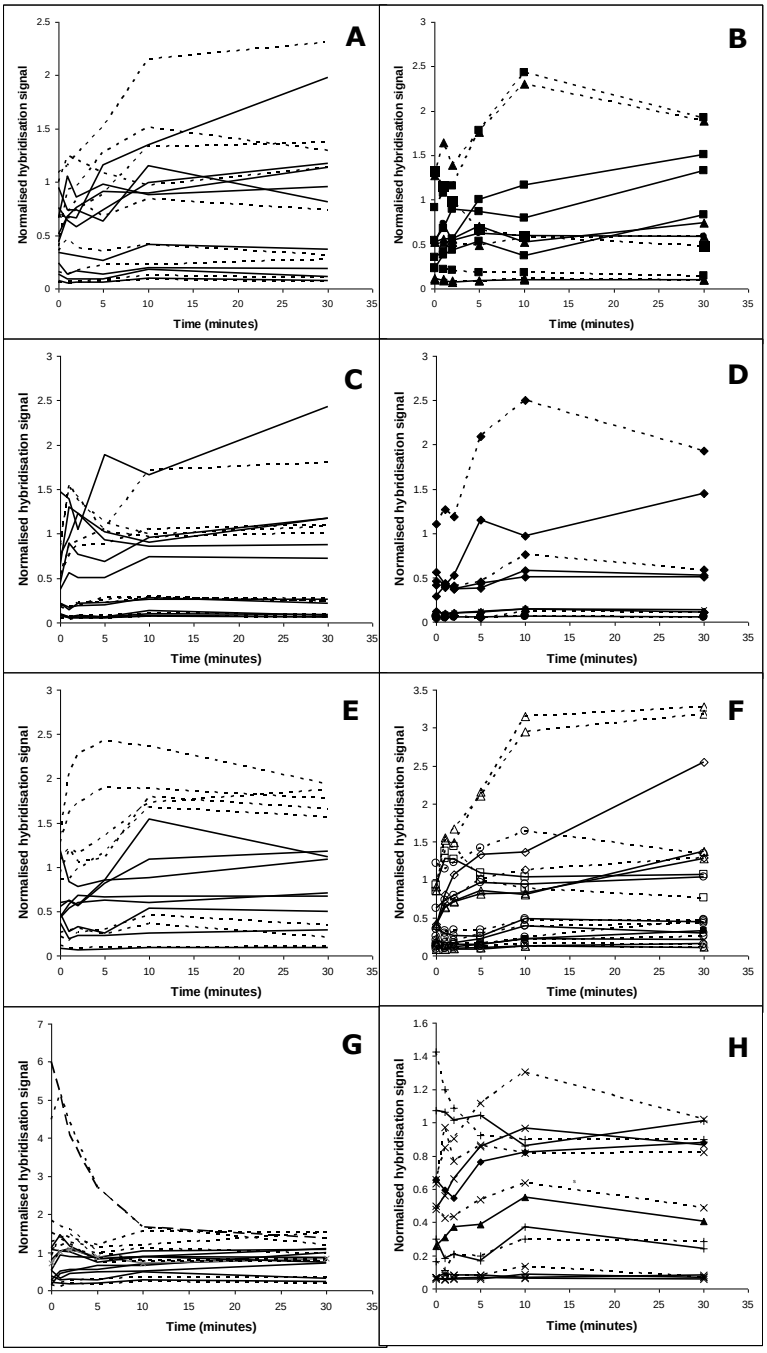
1
0
0 1 2 5 10 30
Down

Figure S4.1 (Page 84) Overrepresentation of COG groups per time-period in *P. putida* S12 (upper half of the figure) and *P. putida* S12ΔTrgI (lower half of the figure). Values are –log(*p*-value). Values above the diagonal represent significance of overrepresentation of up-regulated genes and below the diagonal down-regulated genes are represented. Genes without COG (43 genes) and genes belonging to COG's S (Function unknown, 2292 genes) and R (General function prediction only, 596 genes) are not shown. Abbreviations (with total amount of genes between brackets): A RNA processing and modification (16), C Energy production and conversion (260), D Cell division and chromosome partitioning (24), E Amino acid transport and metabolism (431), F Nucleotide transport and metabolism (90), G Carbohydrate transport and metabolism (164), H Coenzyme metabolism (131), I Lipid metabolism (166), J Translation, ribosomal structure and biogenesis (78), K Transcription (418), L DNA replication, recombination and repair (225), M Cell envelope biogenesis and outer membrane (214), N Cell motility (72), O Posttranslational modification, protein turnover and chaperones (117), P Inorganic ion transport and metabolism (246), Q Secondary metabolites biosynthesis, transport and catabolism (123), T Signal transduction mechanisms (239), U Intracellular trafficking and secretion (56), V Defence mechanisms (159), W Extracellular structure (4).

Figure S4.2 (Page 86) Normalised hybridisation signals of genes of the fatty acid degradation pathway. Solid lines: *P. putida* S12; Dotted lines *P. putida* S12ΔTrgI. (A) 3-ketoacyl-CoA thiolase; (B) ■ acetyl-CoA acetyltransferase and ▲ acyl-CoA dehydrogenase (1.3.99.-); (C) acyl-CoA dehydrogenase (EC 1.3.99.3); (D) ♦ acyl-CoA dehydrogenase, short-chain specific and ● alcohol dehydrogenase; (E) aldehyde dehydrogenase; (F) Δ enoyl-CoA hydratase/delta(3)-cis-delta(2)-trans-enoyl-CoA isomerase/3-hydroxyacyl-CoA dehydrogenase/3-hydroxybutyryl-CoA epimerase, ○ enoyl-CoA hydratase, ◊ glutaryl-CoA dehydrogenase and □ glutarate-CoA ligase; (G) long chain fatty acid-CoA ligase; (H) × membrane-bound aldehyde dehydrogenase iron-sulfur protein and + rubredoxin-NAD(+) reductase.


A
B
C
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E
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G
H
Normalised hybridisation signal
Time (minutes)

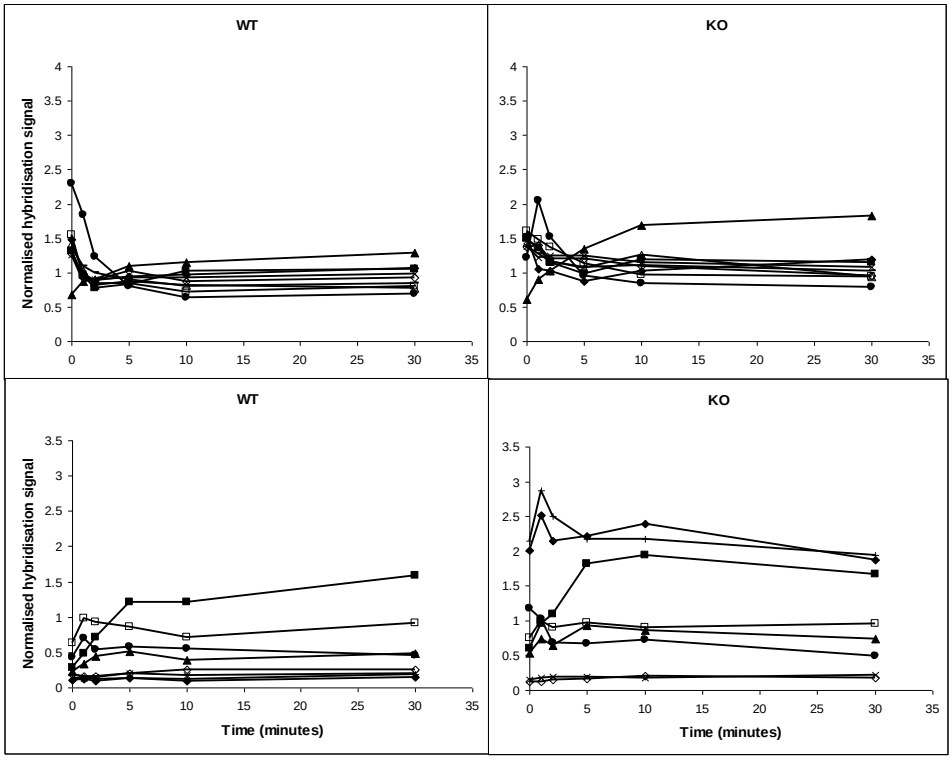


Figure S4.3 Normalised hybridisation signals of genes involved in the biosynthesis of fatty acids. Upper panels: ♦ *fabH* (RPPX01104), ■ *fabH* (RPPX01105), ▲ *fabH* (RPPX009460), ◊ *fabD* (RPPX02797), ● biotin carboxylase (RPPX06980), - biotin carboxylase (RPPX02405), Δ Biotin carboxyl carrier protein of acetyl-CoA carboxylase (RPPX02406), × Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha (RPPX03059), □ Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta (RPPX01172); Lower panels: ■ *fabG* (RPPX01047), ● *fabG* (RPPX04926), ▲ *fabG* (RPPX01366), ♦ *fabG* (RPPX00008), □ *fabG* (RPPX04304), × *fabG* (RPPX03087), + *fabB* (RPPX00004), ◊ *fabB* (RPPX01484); (WT) *P. putida* S12; (KO) *P. putida* S12ΔTrgI.

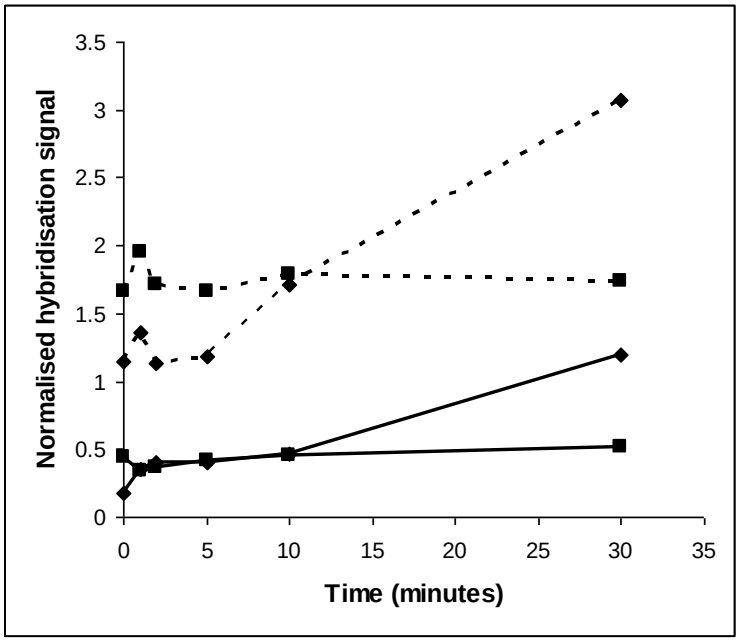
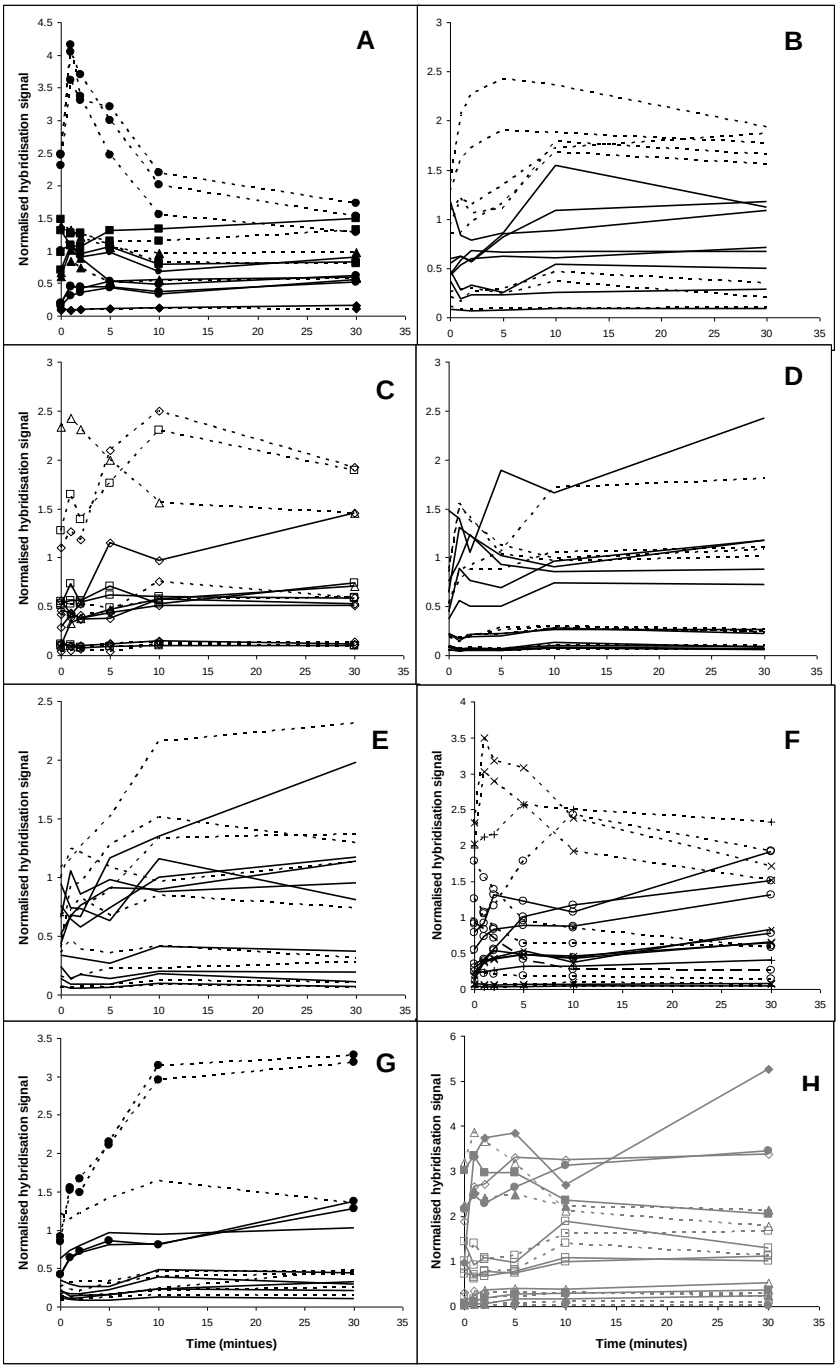


Figure S4.4 Normalised hybridisation signals of the genes of the glyoxylate shunt. Solid lines: *P. putida* S12, dotted lines: *P. putida* S12ΔTrgI. ■ malate synthase, ♦ isocitrate lyase.

Figure S4.5 (Page 89) Normalised hybridisation signals of genes of the valine, leucine and isoleucine degradation pathway. Solid lines: *P. putida* S12; Dotted lines *P. putida* S12ΔTrgI. (A) ■ 3-hydroxyisobutyrate dehydrogenase, ▲ leucine dehydrogenase, ♦ 3-hydroxyacyl CoA dehydrogenase, ● 2-oxoisovalerate dehydrogenase alpha or beta subunit; (B) aldehyde dehydrogenase; (C) Δ isovaleryl-CoA dehydrogenase, ◊ acyl-CoA dehydrogenase, short-chain specific, □ acyl-CoA dehydrogenase (EC 1.3.99.-); (D) acyl-CoA dehydrogenase (EC 1.3.99.3); (E) 3-ketoacyl-CoA thiolase; (F) × omega-amino acid--pyruvate aminotransferase, + dihydrolipoamide dehydrogenase, ○ acetyl-CoA acetyltransferase; (G) enoyl-CoA hydratase, ● enoyl-CoA hydratase / delta(3)-cis-delta(2)-trans-enoyl-CoA isomerase / 3-hydroxyacyl-CoA dehydrogenase / 3-hydroxybutyryl-CoA epimerase; (H) (Grey symbols) ● branched-chain amino acid aminotransferase, Δ methylcrotonyl-CoA carboxylase carboxyl transferase subunit, □ 3-hydroxyisobutyryl-CoA hydrolase, ◊ hydroxymethylglutaryl-CoA lyase, ♦ succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A, ▲ methylcrotonyl-CoA carboxylase biotin-containing subunit, ■ methylglutaconyl-CoA hydratase.


A
B
C
D
E
F
G
H
Normalised hybridisation signal
Time (mintues)
Time (minutes)

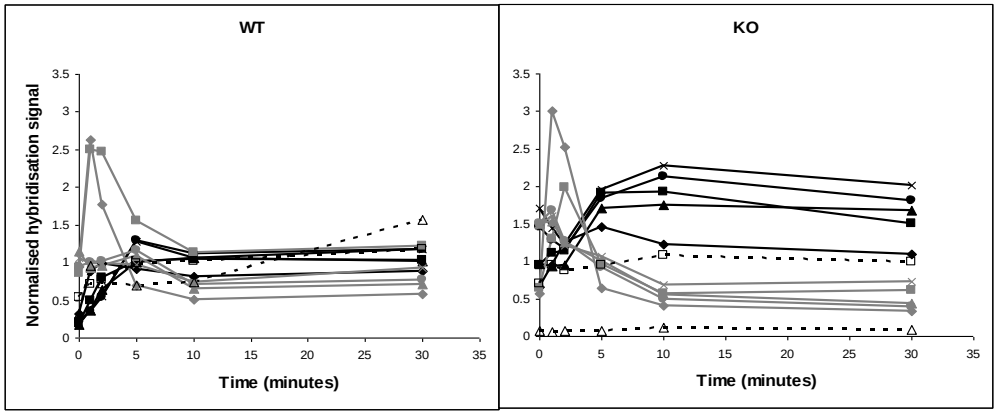


Figure S4.6 Normalised hybridisation signal of genes coding for the ADI pathway and arginine/ornithine transporter genes. Black symbols: ♦ arginine deiminase, ▲ornithine carbamoyltransferase, ■ ornithine carbamoyltransferase, ● carbamate kinase, × carbamate kinase, Δ ornithine cyclodeaminase (RPPX02309), □ ornithine cyclodeaminse family protein (RPPX01343); Grey symbols: ▲ *aotM*, × *aotP*, ● *aotQ*, ♦ arginine/ornithine antiporter, ■ arginine/ornithine antiporter. (WT) *P. putida* S12; (KO) *P. putida* S12ΔTrgI

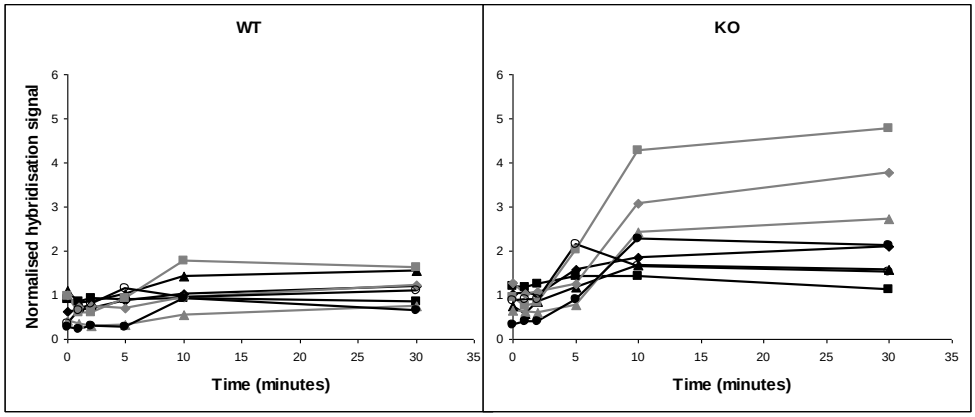


Figure S4.7 Normalised hybridisation signals of the oxidative protein folding genes. Black symbols: ♦ RPPX04213 *dsbA*, ▲RPPX02408 *dsbD*, ■ RPPX05431 *dsbC*, ○ RPPX01161 *tlpA*, ● RPPX04273 *tlpA*; grey symbols: ♦ RPPX05428 *dsbG*, ▲ RPPX04274 *dsbG*, ■ RPPX04272 *dsbD*. (WT) *P. putida* S12; (KO) *P. putida* S12ΔTrgI

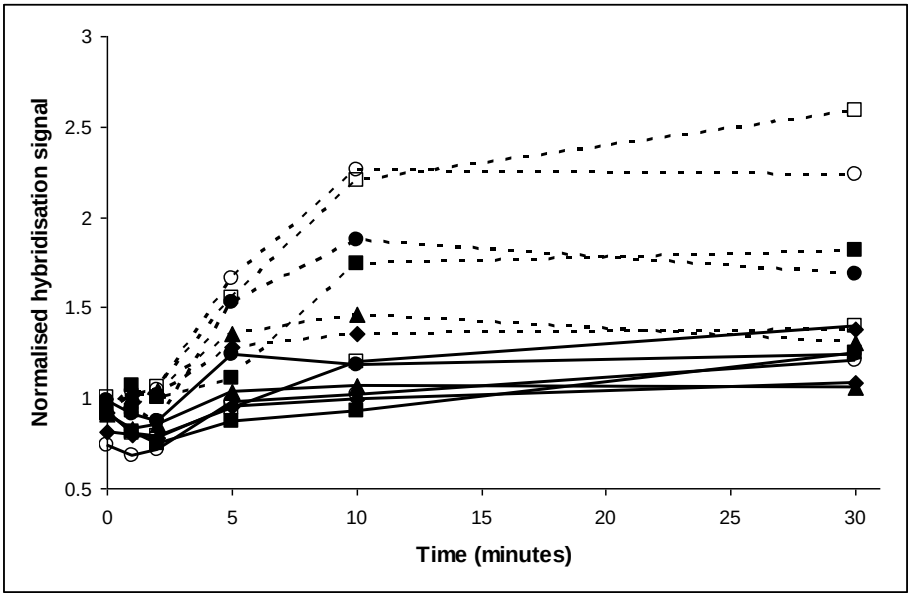


Figure S4.8 Normalised hybridisation signals of chaperonin genes. Solid lines: *P. putida* S12; Dotted lines *P. putida* S12ΔTrgI. ♦ *groEL*, ▲ *groES*, ● 33 kDa chaperonin (RPPX07096), ■ *dnaJ*, □ *htpG* (RPPX05623), ○ *htpG* (RPPX05624).

Isolation and genetic characterisation of an improved benzene-tolerant mutant of *Pseudomonas putida* S12

The extremely benzene tolerant mutant strain *Pseudomonas putida* S12.49 was obtained by laboratory evolutionary selection. Whereas wildtype *P. putida* S12 does not grow above 14 mM benzene, *P. putida* S12.49 tolerates up to 23 mM benzene in LB medium. The genetic basis for the strongly enhanced benzene tolerance was investigated by proteome and transcriptome analysis. Strikingly, the solvent extrusion pump SrpABC was constitutively expressed to the level of fully induced wild type strain. In addition, many transposase genes showed increased transcript levels, among which those associated with the indigenous mutator element IS*S12*. This response was further stimulated by the presence of benzene, suggesting an increased, benzene-controlled, insertion-element mediated mutation frequency in *P. putida* S12.49. Further analysis showed that the regulator gene *srpS* was interrupted by IS*S12*, causing the aberrant expression of SrpABC and suggesting that the improved benzene tolerance was acquired as a result of the increased mutation frequency. In addition, various gene and protein expression features relating to energy generation indicated system changes that accommodated, or compensated, the high-level expression of the solvent extrusion pump.

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Introduction

Benzene is an extremely toxic organic solvent that accumulates in bacterial cell membranes. The accumulation brings about multiple effects, ranging from dissipation of the proton motive force to loss-of-function of membrane-embedded enzymes (125). Despite its toxicity, several bacteria have been reported to survive the presence of extremely high amounts of benzene in liquid cultures, ranging from the near-saturating concentration of 21 mM (0.19 %) to even 90 % (31, 63, 93, 96, 137). The highest level of tolerance to benzene to our knowledge, was reported for *Rhodococcus opacus* B-4 (93). This strain can utilize benzene as the sole carbon and energy source; however, it was hypothesized that this utilization may not be essential for its high level of tolerance. Other extremely benzene tolerant species have been described that do not utilize benzene, like the deep-sea marine microorganisms reported by Kato et al. that are tolerant to 5-10 % of benzene (63).

Pseudomonas putida S12 is a gram-negative bacterium that is tolerant to 14 mM (0.13 %) of benzene and is not capable of degrading it. The benzene tolerance of *P. putida* S12 is not as high as for the strains mentioned above, but its general tolerance to organic solvents is very high. This was demonstrated by its ability to grow in the presence of a second phase of various solvents like toluene, styrene and octanol (146). The high solvent tolerance of *P. putida* S12, together with its metabolic versatility and easy genetic accessibility, makes it a useful strain for the bioproduction of (toxic) fine chemicals (98, 115, 139, 148, 151). Fundamental knowledge about the mechanisms of solvent tolerance is of importance to further improve the production of toxic fine chemicals and their removal using two-phase fermentation systems (139).

In this study, we describe the isolation of an extremely benzene tolerant mutant of *P. putida* S12 as a result of laboratory evolutionary selection. With the relatively simple method of repeated transfers of a solvent-exposed culture to fresh medium with increasing concentrations of solvent, we made full use of the plasticity of the bacterial genome to survive a changing and essentially lethal environment. The genetic basis of the improved benzene tolerance of the mutant strain *P. putida* S12.49 was investigated by transcriptome and proteome analyses.

Results

Isolation of a benzene hypertolerant strain of *P. putida* S12

An extremely solvent tolerant mutant of *P. putida* S12 was obtained by subjecting the wildtype strain to a laboratory scale evolutionary selection procedure. The procedure was designed to yield hyper-solvent tolerant mutants. The more toxic solvent benzene was chosen rather than the common model solvent toluene, since wildtype *P. putida* S12 tolerates saturating concentrations of toluene. In LB medium, wildtype *P. putida* S12 can grow in the presence of 14 mM benzene, whereas in minimal medium with glucose (MMg) only 10 mM benzene is tolerated. The maximum solubility of benzene is 25 mM.

P. putida S12 was cultured in a series of four different concentrations of benzene. Cells growing at the highest benzene concentration Y (visual inspection after 1-6 days) were used to inoculate five bottles containing fresh medium supplemented with (Y-1), Y, (Y+1), (Y+2), and (Y+3) mM of benzene. This process was repeated until newly inoculated cells would not grow further in higher benzene concentrations, indicating no further improvement in benzene-tolerance. Figure 5.1 shows the results of the laboratory evolution experiment in both LB medium and MMg.

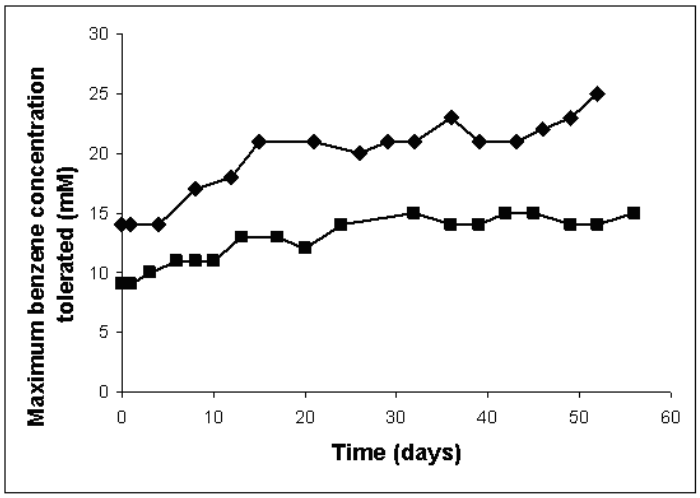

Figure 5.1 Laboratory evolution of *P. putida* S12 towards extreme benzene tolerance in batch cultures in LB medium (♦) and minimal medium with glucose (MMg) (■). At each data point the cells were transferred to fresh medium when growth was observed in the presence of the indicated concentration of benzene.

After 52 days of culturing and 15 subsequent transfers to increasing concentrations of benzene, a mutant strain was isolated and pure-cultured from an LB culture containing 25 mM benzene. This mutant, named *P. putida* S12.49, was stored in LB medium with 20 % glycerol at -80 ºC. In order to assess the phenotypic stability of strain S12.49, cells from the glycerol stock were cultured in LB medium without solvents for 28 generations. Following this treatment, growth was assessed in a range of benzene concentrations. No growth was observed above 23 mM benzene. Hence, we concluded that the stable intrinsic benzene tolerance of strain S12.49 was 23 mM rather than 25 mM.

The laboratory evolution experiment was repeated in minimal medium with glucose (MMg). However, after 56 days and 17 subsequent transfers to fresh medium with benzene, we could only isolate *P. Putida* colonies that would stably tolerate 10 mM of benzene in MMg, which is equal to the tolerance level of wildtype *P. putida* S12. Therefore, only strain S12.49 selected in LB medium was selected for further study.

Phenotypic characterisation of benzene hypertolerant *P. putida* S12.49

Several experiments were performed to further characterise the benzene hypertolerant strain *P. putida* S12.49. Table 5.1 shows the maximum growth rates of *P. putida* S12.49 compared to *P. putida* S12 in LB medium and MMg, both without and with different concentrations of benzene. Without solvent, the growth rate of strain S12.49 was lower than that of the wildtype strain. When cultured in the presence of benzene, the maximum growth rate of S12.49 was inversely proportional to the benzene concentration like for the wildtype, but the inhibiting effect was much less dramatic as shown by the relative growth rate (Table 5.1). Surprisingly, the effect of benzene on the relative maximum growth rate in MMg was comparable for wildtype *P. putida* S12 and *P. putida* S12.49 (Table 5.1). The maximum tolerance level was found not to exceed 10 mM, which is indeed the tolerance level of wildtype strain S12. The yield in MMg batch cultures without benzene was 0.59 g CDW/g glucose for the wildtype strain and 0.40 g CDW/g glucose for strain S12.49.

The apparent medium-dependence of benzene tolerance in strain S12.49 clearly suggested that certain medium components were required that are present in LB medium but absent in MMg. Therefore, strain S12.49 was cultured in MMg supplemented with 0.5 g/l yeast extract or a vitamin solution (PMI 1640 Vitamins Solution, Sigma-Aldrich). The tolerance to benzene slightly increased in MMg with yeast extract, from 10 mM to 12 mM, whereas vitamins supplementation had no effect on benzene tolerance. Neither yeast extract nor vitamins did have any effect on the benzene tolerance of wildtype

strain S12 in MMg. Moreover, by inoculating cultures on MMg with higher starting optical densities than usually used, we could conclusively show that the higher cell densities commonly reached in LB medium compared to MMg did not cause increased benzene tolerance (data not shown).

Table 5.1 Maximum growth rate μ (h^{-1}) and relative growth rate (% of μ without benzene of *P. putida* S12 and *P. putida* S12.49 in LB medium and MMg in the absence and presence of benzene. Values are the average of duplicate experiments.

	LB medium		
Strain	**0 mM**	**10 mM**	**12 mM**
S12	1.18 (100%)	0.32 (27%)	0.26 (22%)
S12.49	0.49 (100%)	0.35 (71%)	0.28 (5 %)
	MMg		
S12	1.01 (100%)	0.22 (22%)	0.19 (19%)
S12.49	0.61 (100%)	0.16 (2 %)	0.11 (18%)

Since solvent tolerance and antibiotics tolerance are often interlinked (39, 133), the MIC's for several antibiotics were determined for strain S12.49 (Table 5.2). Surprisingly, the mutant strain showed a decreased resistance to ß-lactams (ampicillin and piperacillin), compared to wildtype S12. The resistance to aminoglycoside antibiotics (streptomycin, kanamycin and spectinomycin, and gentamicin respectively) was also found to be decreased or equal. An increased resistance was only found for chloramphenicol and polymyxin B.

Table 5.2 MIC's (mg/L; see Experimental procedures) after one day of exposure to various antibiotics of *P. putida* S12 and *P. putida* S12.49. Values are the average of two experiments.

Antibiotic	***P. putida* S12**	***P. putida* S12.49**
Ampicillin	500	250
Piperacillin	375	125
Streptomycin	47	3.9
Gentamicin	3.9	3.9
Kanamycin	31.25	7.8
Spectinomycin	1500	250
Tetracyclin	16.5	16.5
Polymyxin B	5.9	16.5
Novobiocin	2000	1000
Chloramphenicol	500	>2000

Transcriptomics and proteomics of *P. putida* S12.49 – global analysis

To better understand the background of the improved benzene tolerance of *P. putida* S12.49, transcriptome and proteome analyses were carried out on both the wildtype and the mutant strain, in the absence and presence of benzene. Chemostat culturing was employed to ensure a reproducible and reliable comparison between the strains and conditions tested.

Since S12.49 did not exhibit its extremely tolerant phenotype in MMg, the strains were cultured in LB medium. Because standard LB medium caused foaming and other problems in chemostats, 0.25×LB medium was used. A phosphate buffer was added to allow stable maintenance of pH 7 and 10 mM glucose was added to sustain sufficiently high cell densities. Expectedly, the use of diluted LB medium resulted in lower benzene tolerance for both strains: in batch cultures, strain S12.49 tolerated 17 mM instead of 23 mM and S12 tolerated 10 mM instead of 12 mM. Still, in a chemostat at a dilution rate of 0.2 h^{-1}, even 17 mM benzene proved to be toxic for the mutant strain. Wash-out occurred and the benzene concentration was therefore lowered to 8 mM. Samples of duplicate chemostats were drawn at steady state and proteomes and transcriptomes were analysed.

We performed comparisons between the transcriptomes of *P. putida* S12.49 and *P. putida* S12 in the absence of solvent, and between the transcriptomes of S12.49 cultured in the absence and presence of 8 mM benzene. Compared to wild type *P. putida* S12, 545 ORFs were down-regulated (7.7 % of the total number of 7107 ORFs) and 346 (4.9 %) were up-regulated 2-fold or more in strain S12.49 under non-solvent exposed conditions. When strain S12.49 was cultured in the presence of 8 mM benzene, 646 orfs (9.1 %) were down-regulated and 866 (12.2 %) were up-regulated 2-fold or more compared to cultures without benzene. The effect of benzene on strain S12.49 may therefore be considered to be more dramatic than the effect of toluene on wildtype S12, which resulted in a mere 4.7 % of the genes being differentially expressed (141).

In the analyses described hereafter, only the 5204 annotated ORFs in the *P. putida* S12 genome will be taken into account and referred to as genes. All differentially expressed genes were sorted according to functional category, based on the available COG-data and on manual annotation when no COG data were available. In Figure 5.2, the percentage of differentially expressed orfs per functional category is presented. In Supplemental Table S1 in the original publication all differentially expressed genes are shown with their fold changes. The genes described in this paper are given in Table 5.3.

In addition to the transcriptome, the proteome of *P. putida* S12.49 was analysed using 2-D DIGE. Protein spots that showed ≥ 2-fold difference in expression were considered relevant. The comparison of S12.49 and S12 yielded 37 protein spots that were excised from the gel, comprising 28 different proteins. The genes corresponding to 13 of these proteins were also found to be differentially expressed in the transcriptome analysis. For the analysis of strain S12.49 grown with and without 8 mM benzene, 28 proteins spots were excised that comprised 19 different proteins. The corresponding genes of 11 of these proteins were also differentially expressed. In Table 5.4 a summary of the results is presented and in supplemental table S2 the complete results can be found.

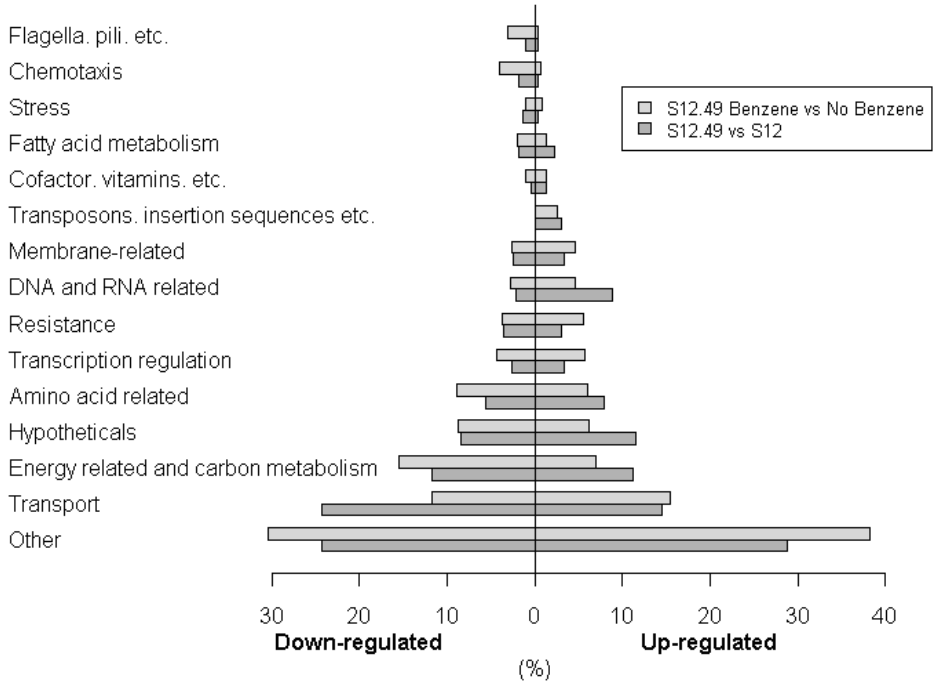

Figure 5.2 Percentage of differentially expressed genes per functional category, calculated as percentage of total number of differentially expressed ORFs minus the ORFs without annotation.

Table 5.3 Summary of the results of the transcriptome analyses. The genes are grouped according to functional category. Only categories that proved to be of interest in relation to solvent tolerance in previous studies (65, 141, 142, 147) were included in this summary. The complete results can be found in Supplemental Table S1 in the original publication.

Locus tag (RPPX0....)	Gene name	Average fold change[a]
P. putida S12.49 compared to P. putida S12		
Energy and carbon metabolism		
0531, 0532, 0534-5042, 2053	ATP synthase genes	0.35
1721-1723, 1725	Cellulose synthase	2.83
5482-5486, 5488, 5489, 7103	Cytochrome c oxidase genes, aa_3 type	0.18
6016, 6293, 6294, 6296	Cytochrome c oxidase genes, cbb_3 type	8.06
2055	Cytochrome c peroxidase	0.48
5058	Cytochrome c550	0.43
4540, 4541	Gluconate 2-dehydrogenase	4.42
1630	Glycogen phosphorylase	0.46
3198, 711	Ketogluconate dehydrogenase	0.21
5605, 7054	NADH dehydrogenase	2.99
Transposases and insertion sequences		
-[b]	ISS12, orf 1 and orf 2	2.51
0439, 1494, 1495, 2050, 4739, 6175, 6311	Transposases	2.59
Outer cell structure		
5954	Anhydromuramoyl-peptide exo-beta-N-acetylglucosaminidase	2.03
1685	AsmA family protein	2.54
0064	Class II Aldolase and Adducin proteins	0.45
2193	Glycosyltransferase (cell wall biogenesis)	0.45
5833	Outer membrane porin F	0.32
2364, 767, 1361, 6917	Outer membrane porin, oprD family	0.26
0656, 3184, 4714-4716	Outer membrane porin, oprD family	2.97
3394	Outer membrane protein	10.70
4432	Outer membrane protein nosA precursor	46.04
4713	Outer membrane siderophore receptor	2.52
3573	Peptidoglycan-specific endopeptidase, M23 family	0.41
Flagella and pili		
2855-2857	Outer membrane usher protein sefC	0.23
6901	Pili membrane scaffold protein pilV	2.48
Stress-related genes		
2854	Chaperone protein ecpD	0.19
3739	Cold shock protein	0.39
2340-2342	InaA protein	0.19
Transport		
4100, 4101	arpBC	0.31
1676-1679, 3832-3835, 4247-	Branched-chain amino acid transport ATP-	0.29

4249, 6838, 7031	binding protein livFGHM	
2365, 2363, 5948, 2362	Dipeptide binding protein	0.12
2366-2369	Dipeptide transport genes dppDFBC	0.06
5318-5321	General L-amino acid transport genes aapPMQ	0.14
1523-1525	Glycerol transport	0.21
6677-6679	Maltoporin	0.01
2061, 2062	Maltose/maltodextrin transport ATP-binding protein malK	0.05
0289	srpABC	10.49
0287	srpRS	0.31
5947, 2372, 2371, 6486, 2935, 2928, 2911	Transporter, drug/metabolite exporter family	1.62
P. putida S12.49 with 8 mM benzene compared to P. putida S12.49 without benzene		
Energy and carbon metabolism		
6454	1,4-alpha-glucan branching enzyme	0.21
6284	2-ketogluconate transporter	5.95
0955-0957	Aconitate hydratase	0.18
5324, 7068	Alpha-amylase family protein	0.40
0541, 0542	ATP synthase	2.25
2053	ATP synthase protein I	0.34
1721-1723, 1725	Cellulose synthase	4.82
1807, 3699, 4214, 4215, 4483, 5378, 6649	Cytochromes	0.50
6293, 6296, 6016, 6294	Cytochrome c oxidase, cbb_3 type	0.09
5489	Cytochrome c oxidase, aa_3 type	0.15
3236, 3238, 7076	Cytochrome bd oxidase, CIO	0.05
2055	Cytochrome c peroxidase	0.45
3198, 0711	Dehydrogluconate dehydrogenase	0.08
1382	Fumarate hydratase	0.45
5019	Gluconate 2-dehydrogenase	0.48
4540, 4541, 4539	Gluconate 2-dehydrogenase	2.98
4933, 5620-5652	Glucose transport	0.24
1630	Glycogen phosphorylase	0.31
3558	Glycogen synthase	0.15
3673	Malate dehydrogenase	0.34
5605	NADH dehydrogenase	2.11
5739, 5740	Poly(3-hydroxyalkanoate) polymerase	0.17
4916	Polyphosphate kinase	0.14
6650	Ubiquinol-cytochrome c reductase iron-sulfur subunit	0.18
Flagella and pili		
2503	Curli production assembly/transport component csgF precursor	2.26
1099, 1100, 1102, 1108, 1109, 1112-1114, 1116-1119, 1938, 3494, 3495, 4823, 6884	Flagellar genes	0.30

Continued on next page

Table 5.3 *Continued*

2855, 2857	Outer membrane usher protein sefC	0.43
2825	PapC-like porin protein involved in fimbrial biogenesis	2.05
1398	Usher protein cooC	3.10
Transposases and insertion sequences		
0333	Putative insertion sequence ATP-binding protein Y4BM/Y4KI/Y4TA	2.79
0033, 0099, 0107, 0271, 0334, 0523, 0524, 1213, 2050, 4376, 4513, 4739, 4801, 5384, 6860, 6860, 6861	Transposases	2.53
Outer cell structure		
2193	Glycosyltransferase (cell wall biogenesis)	0.42
2477	Inner membrane protein creD	2.01
3268	Integral membrane protein	2.78
3621	Lytic transglycosylase homolog yjbJ	2.42
1909	Membrane protein glpM	2.20
6257	Membrane-bound lytic murein transglycosylase B	2.16
6551, 5810	Murein hydrolase export	2.10
5422, 5423, 5420, 6958, 5424, 7050	Non-ribosomal peptide synthetase modules	0.10
7006	Outer membrane lipoprotein Blc	2.48
5833	Outer membrane porin F	0.08
6442	Outer membrane porin protein 32 precursor	2.13
5097, 2364	Outer membrane porin, oprD family	0.26
0683, 1467, 3184, 0154, 0656	Outer membrane porin, oprD family	11.56
6280	Outer membrane protein assembly factor yaeT	15.80
0121	Outer membrane protein ompK precursor	2.50
2147	Outer membrane siderophore receptor	0.10
5687, 3781, 5688, 2212, 2256, 4174, 2255, 4713, 6346, 6347	Outer membrane siderophore receptor	8.96
1185	Outer membrane usher protein focD	2.04
5764	Peptidoglycan binding protein (LysM domain)	0.07
1110	Peptidoglycan hydrolase flgJ	0.48
6689, 6690	Peptidoglycan-specific endopeptidase, M23 family / Alanine--glyoxylate aminotransferase	2.21
0560	Polysaccharide acetyltransferase	2.34
5926	Phospholipase	0.36
4785	Phospholipase	3.22
2479	UDP-2,3-diacylglucosamine hydrolase	2.07
Resistance		
4135, 4920, 7043, 4104, 0148, 1972, 4845, 4136, 7043	Multidrug resistance proteins A and B	2.24
2257	trgI	0.04
Stress-related genes		

1397	Chaperone protein cooB	2.21
2854	Chaperone protein ecpD	0.33
3739	Cold shock protein	0.15
2052	General stress protein 17O	0.30
2341	InaA protein	2.37
6920	Lipid A biosynthesis lauroyl acyltransferase	2.06
562	Thioredoxin	2.46
1018, 4724, 1557	Universal stress protein family	0.35
6385	Xenobiotic reductase xenA	2.11
Transport		
4101, 4103	arpBR	2.11
6838	Branched-chain amino acid transport ATP-binding protein livG	0.44
5319-5321	General L-amino acid transport ATP-binding protein aapPMQ	2.47
6677	Maltoporin	2.47
1483, 3719	Multidrug resistance transporter, bcr family	2.53
6984, 7020	Multidrug/protein/lipid ABC transporter family, ATP-binding and permease protein	2.49
6488, 2371, 2911, 2372, 5244, 2935	Transporter, drug/metabolite exporter family	2.56
5351, 5352, 5822, 6752	Na+/H+ antiporter nhaP	3.37

a) The average fold change is given when a gene is present in more than one copy in the genome or when a complex or group of genes is described.
b) The insertion sequence ISS12 is present in the genome in multiple copies, depending on the growth conditions (147), so no locus tag can be given.

Table 5.4 Summary of the results of the proteome analyses. Proteins without a known function are not shown. The fold-changes of proteins that were found in more than one spot are averaged. The complete results can be found in Supplemental Table S2 in the original publication.

Locus tag (RPPX0....)	Protein name	Average fold change[a]
***P. putida* S12.49 compared to *P. putida* S12**		
0538[b]	ATP synthase delta chain	0.26
2365, 2362[b]	Dipeptide-binding protein	0.11
5318[b]	General L-amino acid-binding protein	0.33
3128[b]	Glutaminase-asparaginase	2.03
1405[b]	Glycine betaine-binding protein	0.46
3463[b]	Hypothetical exported protein	0.40
6034[b]	Immunogenic protein	0.30
1675[b]	Leucine-, isoleucine-, valine-, threonine-, and alanine-binding protein	0.32
1566[b]	Methylisocitrate lyase	9.34
5445[b,c]	NADH-dependent flavin oxidoreductase	0.39
5445[c]	NADH-dependent flavin oxidoreductase	2.60

Continued on next page

Table 5.4 *Continued*

2364[b]	Outer membrane porin, OprD family	0.07
4250[d]	Leucine-, isoleucine-, valine-, threonine-, and alanine-binding protein	0.38
5170[d]	Putrescine-binding protein	0.47
5396[e]	Aromatic-amino-acid aminotransferase	2.60
5477[e]	Aspartate carbamoyltransferase	2.01
5632[e]	Dihydrolipoamide dehydrogenase	0.42
4933[e]	Glucose-binding protein	0.45
4939[e]	Glyceraldehyde 3-phosphate dehydrogenase	2.01
4833[e]	Hypothetical exported protein	0.43
4568[e]	LSU ribosomal protein L15P	0.31
5674[e]	Organic hydroperoxide resistance protein	0.50
0738[e]	Outer membrane protein OmpH	0.38
2665[e]	Peptide synthase	0.42
5654[e]	Porin	0.49
2432[e]	Signal peptidase I	4.39
5631[e]	Succinyl-CoA synthetase beta chain	0.50
***P. putida* S12.49 with 8 mM benzene compared to *P. putida* S12.49 without benzene**		
0984[b]	3-ketoacyl-CoA thiolase	0.39
1102[b]	Flagellin	0.25
4933[b]	Glucose-binding protein	0.22
3128[b]	Glutaminase-asparaginase	2.52
1405[b]	Glycine betaine-binding protein	0.44
5381[b]	Heteromeric transcriptional activator MvaT P16 subunit	2.06
3463[b]	Hypothetical exported protein	0.28
5674[b]	Organic hydroperoxide resistance protein	4.54
2364[b]	Outer membrane porin, OprD family	0.42
0093[b]	Penicillin acylase	0.34
0630[b]	Peroxiredoxin	2.85
2665[d]	Peptide synthase	0.30
2385[e]	Acetyltransferase, GNAT family	0.42
5632[e]	Dihydrolipoamide dehydrogenase	2.46
0469[e]	Hypothetical protein	3.57
4568[e]	LSU ribosomal protein L15P	3.44
0738[e]	Outer membrane protein OmpH	5.11
5630[e]	Succinyl-CoA synthetase alpha chain	3.86
5631[e]	Succinyl-CoA synthetase beta chain	2.40

a) Part of the proteins was found in more than one spot, in those occasions the average fold change is given.
b) Protein from which the corresponding gene was differentially expressed with the same trend according to transcriptome analysis.
c) This protein was found in two different spots with contradicting fold changes. Expression of the corresponding gene was down-regulated.
d) The genes coding for these proteins were not found in the transcriptome analyses, but another gene with the same function and the same trend in differential expression was.
e) The genes coding for these proteins were not found in the transcriptome analyses.

Comparison of the transcriptomes and proteomes of *P. putida* S12.49 and *P. putida* S12

A selection of the genes and proteins that were differentially expressed in *P. putida* S12.49 compared to *P. putida* S12 in the absence of benzene are presented in Tables 5.3 and 5.4 (please refer to supplemental tables S1 and S2 in the original publication for the complete dataset). A striking observation was the high level of expression of *srpABC* in the mutant strain S12.49. This operon encodes a proton-driven solvent extrusion pump that has previously been shown to be crucial for toluene tolerance in *P. putida* S12 (68). Compared to wildtype S12, the *srpABC* genes were up-regulated by a factor 11.2, 10.6 and 8.9, respectively, which corresponded to the levels usually observed in fully induced toluene-challenged wildtype S12. The high level of expression of *srpABC* is accompanied by low expression of *srpRS* (0.5 and 0.1-fold down-regulated), which is in good accordance with their previously suggested role as repressors for *srpABC* (147). Two genes of the Arp antibiotic transporter, *arpBC*, were lowly expressed in S12.49. Arp removes, amongst others, ampicillin, novobiocin and streptomycin from the cell and downregulation is therefore in good accordance to the observed decrease in antibiotic resistance in this mutant. In addition, three transporter genes of the drug/metabolite exporter family were down-regulated, in good agreement with the observed reduced antibiotic resistance of strain S12.49.

One-hundred and forty seven transporter genes were differentially expressed, the majority of which was down-regulated. Many of the down-regulated transporter genes (~40 %) represented amino acid and dipeptide transporters. Accordingly, proteome analysis of strain S12.49 revealed down-regulation of substrate binding proteins partly associated with amino acid and dipeptide transporters. Also glycerol and maltose transport genes were found to be down-regulated. These observations may partly explain the impeded growth of strain S12.49 compared to the wildtype on LB medium.

Various genes coding for outer membrane porins and proteins were differentially expressed. The membrane protein OmpH was down-regulated, as was OprF. The highest up-regulated gene in *P. putida* S12.49, encoding a NosA homologue, was also among the differentially expressed outer membrane-related genes. NosA is a copper-binding membrane channel that provides the copper cofactor of nitrite reductase in *Pseudomonas stutzeri* (77). Enhanced expression of this NosA homologue might indicate the involvement of copper in improved benzene tolerance. However, adding a ten-fold increased concentration of copper to wildtype S12 cells growing in MMg medium did not result in an elevated level of solvent tolerance (data not shown).

Several genes encoding ATP synthase subunits were down-regulated in S12.49 compared to S12, as also confirmed by proteome analysis. A group of genes coding for cytochrome *c* assembly proteins and cytochrome *c* polypeptides I, II and III, all part of cytochrome aa_3 or *c* oxidase, was down-regulated, as was cytochrome c550. By contrast, four genes encoding subunits of membrane bound cytochrome *c* oxidase of the cbb_3-type were up-regulated (coding for an oxidase polypeptide I, two diheme subunits and one monoheme subunit). Proteome analysis furthermore showed that the beta chain subunit of the citric acid cycle enzyme succinyl-CoA synthetase was downregulated.

Amongst the differentially expressed transposase genes, the two orfs of the insertion element IS*S12* were found to be up-regulated (also see below). This may imply increased transcriptional activity of the IS*S12* transposases and, therefore, an increased chance of insertion events. Alternatively, it may also be caused by an increased transcriptional activity of the loci at which IS*S12* was inserted. This could not be verified, since many of the insertion locations could not be verified in the *P. putida* S12 genome sequencing data. This was caused by the multiple occurrence of these sequences (147) that furthermore carry internal repeats, which complicated genome assembly and annotation. In addition, the up-regulated transposases named RPPX06311, 1494, 1495, 6175 and 0439 all have at least a partial resemblance to IS*S12*.

Response of *P. putida* S12.49 to benzene

The proteomic and transcriptomic responses of *P. putida* S12.49 to benzene are summarized in Tables 5.3 and 5.4 (complete data are available in supplemental tables S1 and S2 in the original publication). Upon addition of benzene, the intrinsically elevated expression level of *srpABC* in strain S12.49 did not increase further. Also the expression of *srpRS* did not change. These observations suggest that the expression of the solvent extrusion pump in the benzene hypertolerant mutant strain S12.49 was constitutively high and has therefore become independent of the presence of organic solvents. The recently described new gene of solvent tolerance *trgI* (141) was down-regulated 25-fold in 8 mM benzene, whereas this gene was down-regulated only 4-fold in wildtype S12 in the presence of toluene (141). This dramatic difference in expression between the two strains clearly confirms that the level of *trgI* expression correlates with the extent of solvent tolerance.

Two groups of differentially expressed genes in response to benzene clearly attracted attention: transposases and transporters. S12 harbours 78 transposase genes, of which 16 (21 %) were up-regulated and none were down-regulated in strain S12.49 exposed to benzene. In wildtype S12 cells, no transposases were differentially expressed in the presence of toluene (141).

Although the interpretation of the up-regulation of transposase-encoding genes was complicated for the reasons pointed out above, the massive response does suggest a higher genetic flexibility in S12.49 that is furthermore stimulated by the presence of benzene. This would endow strain S12.49 with more possibilities to cope with solvents at the genetic level.

In wildtype *P. putida* S12 cultured in the presence of toluene, more transporters were down-regulated than up-regulated (141). Strain S12.49 showed an opposite response when exposed to benzene: 109 genes were up-regulated and 68 were down-regulated. About 40 % of the down-regulated transporter genes comprises of amino acid and dipeptide transporters. Aromatic amino acids resemble benzene chemically and the down-regulation of transporters for these amino acids may prevent benzene entry. Ten of the up-regulated transport genes were drug/metabolite export genes and four were multidrug transport genes. Four other up-regulated genes were copies of the Na+/H+ antiporter *nhaP*. Since the mutant is not extremely tolerant to benzene in MMg, it was hypothesized that the high concentration of Na^+-ions in LB medium plays a role in the build-up and maintenance of the H^+-gradient via NhaP. However, the addition of 5 g/l NaCl to MMg did not increase the benzene tolerance of S12.49 in this medium. Hence, it appeared unlikely that the H^+-gradient is maintained through the import of Na^+ via NhaP.

In the category of energy-related genes and proteins, several genes coding for cytochrome *c* oxidase subunits of the cbb_3 and aa_3 type and cytochrome *c* encoding genes were unexpectedly down-regulated in the presence of benzene. The down-regulation amounted to such an extent that the expression levels were even lower than in the wildtype strain in the absence of benzene (see Supplemental Table S1 in the original publication). One cytochrome *c* subunit and also a gene encoding part of NADH dehydrogenase were found to be up-regulated in 8 mM benzene. Two citric-acid-cycle proteins were found to be up-regulated: succinyl-CoA synthetase alpha chain and beta chain as well as a part of the pyruvate dehydrogenase complex (dihydrolipoamide dehydrogenase). While these responses suggested a higher TCA cycle activity, the genes coding for fumarate hydratase, malate dehydrogenase and aconitate hydratase were found to be down-regulated.

The unexpected response of a number of energy-related genes and proteins to benzene exposure suggests that the mutant strain disposes of an unusual but effective way of maintaining the proton gradient that is required to provide the SrpABC solvent extrusion pump with energy. Moreover, a fierce down-regulation of the genes of the arginine deiminase pathway was observed (RPPX04947-RPPX04949, RPPX02731 and RPPX02732, see Table S1 in the original publication), which provides an indication that the cells are well

energized (105). In order to shed some light onto this matter, the effect of the energy-uncoupling protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on benzene tolerance was assessed. This compound specifically dissipates the proton gradient from which the solvent extrusion pump derives its energy. Addition of 250 µM CCCP to benzene-adapted S12 cells decreased the benzene tolerance to from 14 to 8 mM. Addition of 250 µM of CCCP to S12.49 cells resulted in a benzene tolerance decrease of 23 to 14 mM. Thus, the relative decrease in benzene tolerance level was similar for both wildtype and mutant strain. However, the mutant strain still maintained the wildtype level of benzene tolerance in the presence of the uncoupling protonophore. This provided strong indication that this strain does have additional ways of generating and maintaining the proton gradient over the wildtype strain.

Seven genes related to storage of carbon were down-regulated, whereas genes involved in carbon source uptake and utilization were up-regulated (2-ketogluconate transporter, gluconate- and glucose dehydrogenase). By contrast, an ATP-driven glucose transporter was down-regulated, suggesting that energy-demanding carbon-source uptake was diminished in favour of transporters that do not require energy for substrate uptake. The up-regulation of carbon uptake systems was confirmed by proteomics showing up-regulation of a glucose binding protein.

Since organic solvents act on membranes (125), membrane- and other outer cell structure-associated genes are often differentially expressed in response to these compounds. In strain S12.49, three murein-related genes were up-regulated (membrane-bound lytic murein transglycosylase B, murein hydrolase export regulator and murein hydrolase exporter), as well as two medium-chain-fatty-acid CoA ligases, whereas a glycosyltransferase involved in cell wall biogenesis was down-regulated. Several outer membrane porins were differentially expressed, amongst which OprD was also found in the proteome analysis. Remarkably, several of the OprD family outer membrane proteins that were intrinsically expressed to higher, respectively, lower levels in strain S12.49. These trends in expression level were reinforced in response to benzene exposure, which observation suggests that specific OprD-family proteins contribute to solvent tolerance whereas others may have adverse effects. Amongst the differentially expressed resistance genes, a group of nine multidrug resistance genes was up-regulated. Additionally, various genes involved in resistance to, *e.g.*, copper and fusaric acid were up-regulated.

Insertion sequence IS*S12*

In *P. putida* S12.49 the two open reading frames *orf1* and *orf2* of the indigenous insertion sequence IS*S12,* both encoding transposases, were up-

regulated 2-fold compared to wildtype strain S12. In a previous study, it was proposed that IS*S12* acts as a mutator element enabling *P. putida* S12 to cope with sudden severe and even lethal stress (147). The authors isolated a mutant strain that had survived a sudden toluene shock and carried eight copies of IS*S12* in its genome instead of the seven copies normally associated with wildtype *P. putida* S12. This copy was inserted in *srpS*, a regulatory gene of *srpABC*, leading to constitutive expression of the solvent extrusion pump.

In view of the up-regulation of the IS*S12* orfs and the constitutive expression of the solvent removal pump in strain S12.49, it was hypothesized that also in this mutant one of the regulatory genes *srpRS* may be interrupted by IS*S12*. Southern blot analysis confirmed that *P. putida* S12.49 harboured an extra copy of IS*S12* (Figure 5.3), which was at the same spot as reported by Wery *et al.* (2001). As a control, wildtype *P. putida* S12 was subjected to a toluene shock and the surviving population also showed the extra IS*S12* signal at the expected spot (Figure 5.3).

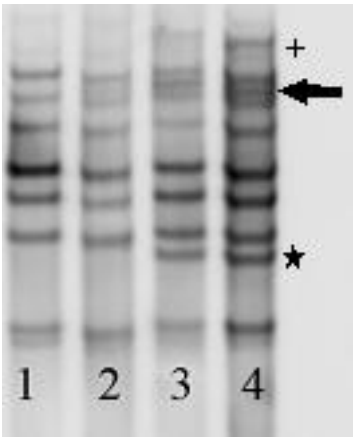

Figure 5.3 Southern blot with a probe for insertion sequence IS*S12*. *P. putida* S12 before (1) and after (2) addition and culturing with 1% toluene, *P. putida* S12.49 before (3) and after (4) addition and culturing with 1% toluene. The DNA fragment harbouring IS*S12* in *srpS* is marked by the arrow. (*) indicates a band that is present in strain S12.49 but not in S12. (+) indicates a signal that occurred in the population of strain S12.49 that survived the toluene shock treatment.

Sequence analysis confirmed that an extra copy of IS*S12* was inserted in *srpS* of strain S12.49. The exact location at which *srpS* was interrupted by IS*S12*, however, was different from that reported by Wery *et al.* (147) and the orientation of IS*S12* was opposite (Figure 5.4). Apparently, 8 nucleotides of *srpS* had been duplicated during the insertion event as they were present upstream as well as downstream of IS*S12*. The final three nucleotides of IS*S12* (GCA) were also present in the sequence of s*rpS*, immediately downstream of the eight nucleotides that were duplicated.

The interruption of *srpS* by IS*S12* suggests that strain S12.49 should be relatively tolerant to a sudden toluene shock. Therefore, a 1 % (v/v) toluene shock was administered to early logarithmic phase cultures of both *P. putida* S12.49 and the wildtype strain. The survival frequency determined 30 min. after the shock treatment was higher by a factor 10,000 for the mutant. Upon prolonged incubation of toluene-shocked cultures, wildtype S12 required two days to grow to a high cell density, whereas strain S12.49 required only a single day. These results reconfirmed that the interruption of *srpS* by IS*S12* confers increased tolerance to a sudden toluene shock treatment. However, the exact location or the orientation of the insertion element did not appear to be essential.

In addition to the IS*S12* signal relating to the insertion in *srpS*, an additional IS*S12* band was observed by Southern analysis of strain S12.49 (Figure 5.3). This band was absent from the wildtype strain, suggesting that another IS*S12* insertion event had occurred at a yet unidentified locus in the genome of the benzene-tolerant mutant. Moreover, an additional IS*S12* signal was observed in the population of strain S12.49 that had survived a toluene shock (Figure 5.3). Also for this insertion, the locus has yet to be identified.

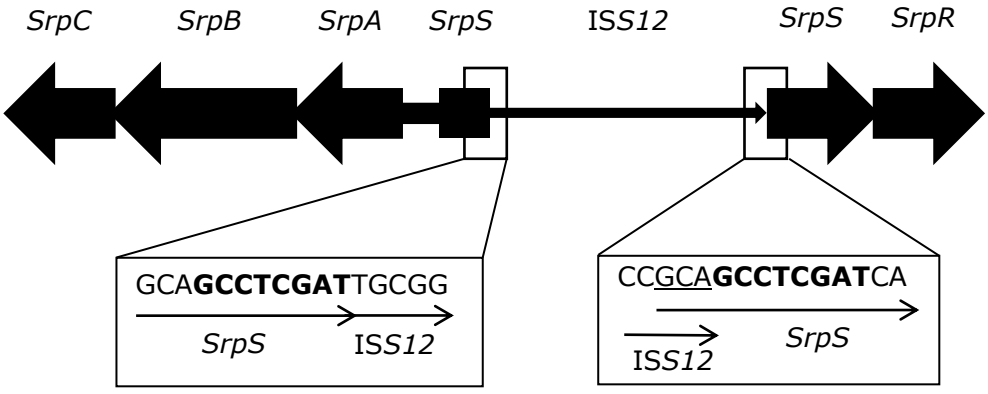

Figure 5.4 Schematic representation of insertion of ISS12 in *srpS*. Nucleotides in boldface are part of *srpS* and were duplicated during insertion. The underlined nucleotides are part of *srpS* and are also part of IS*S12*.

Discussion

The extremely benzene tolerant mutant strain *P. putida* S12.49 was isolated in a laboratory evolution experiment and is able to grow in the presence of 23 mM benzene, whereas the wildtype *P. putida* S12 tolerates only 14 mM. The genetic basis of the extreme benzene tolerance was investigated by a transcriptomics and proteomics approach. Global analysis of the results showed that the laboratory evolutionary selection procedure apparently had a severe impact at the systems level and provided further evidence that solvent tolerance is based on a complex system of responses and mechanisms.

A key element in the improved tolerance to benzene is the constitutive expression of the solvent extrusion pump SrpABC. This pump has previously been shown to be crucial for solvent tolerance in *P. putida* S12 (68), but the induction is rather slow (69, 141). In order to cope with severe or even lethal solvent stress, such as a sudden toluene shock, the pump must be expressed constitutively (147). In strain S12.49 the pump regulator gene *srpS* was interrupted by insertion element IS*S12* which ensured constitutive expression of the solvent extrusion pump.

This study reconfirmed the important role of insertion element IS*S12* in genetic adaptability to lethal stress. In strain S12.49, the occurrence of IS*S12* insertions appeared to be more frequent than in the wildtype strain. This finding was in good agreement with the up-regulation of the IS*S12*-genes that suggests a higher activity of the IS*S12*-associated transposases, and which was even further stimulated by the presence of benzene. Also other transposase-encoding genes were transcribed at higher levels, suggesting that strain S12.49 has an intrinsically increased mutation frequency and should therefore perhaps be considered a mutator strain. The recently reported insertion sequence IS*Ppu21* (127) apparently has a similar function as IS*S12.* We found the complete sequence of IS*Ppu21* three times in the *P. putida* S12 draft genome sequence (loci RPPX01944-1945, RPPX03877-3878 and RPPX05012) (Ruijssenaars and de Winde, manuscript in preparation). Although this confirms the findings of Sun and Dennis (127) we found a fourth isolated copy of the transposase encoding gene of IS*Ppu21* (RPPX06107). Since none of the above loci were differentially expressed in strain S12.49, there is no indication that this particular insertion element plays a role in the genetic adaptability of this strain.

Although crucial for improved benzene tolerance, the constitutive expression of SrpABC alone is not sufficient. Even fully induced wildtype cells

are not able to grow in the presence of 23 mM benzene like the mutant strain. This implicates that other systems are essential to support solvent tolerance in S12.49. It is of key importance to realise that the presence of benzene is the 'normal' condition for the mutant strain, *i.e.*, the condition under which the strain has evolved. This 'normal' situation requires continuous activity of the solvent efflux pump SrpABC. This, together with the dissipation of the proton-motive force caused by the accumulation of benzene molecules in the membranes, puts a heavy burden on the energy systems of the cells. Therefore, adjustments to these systems may be expected to be required to sustain the imposed energy demand.

Indeed, several genes involved in energy-dependent glucose uptake were down-regulated whereas genes involved in energy-independent uptake were up-regulated. Also carbon storage-related genes were down-regulated. These responses suggest that more (energy-wise cheap) glucose was taken up to provide energy. Also, the generation of reducing power through the citric-acid-cycle appeared to be increased. In the proteomics results, an up-regulation of succinyl-CoA synthetase (both alpha and beta chain subunits) was found, as well as an up-regulation of dihydrolipoamide dehydrogenase which is part of the pyruvate dehydrogenase complex that supplies the citric-acid-cycle with acetyl-CoA. On the other hand, transcriptome analysis showed a down-regulation of the genes coding for fumarate hydratase, malate dehydrogenase and aconitate hydratase. In addition, many genes encoding subunits of terminal oxidases of the respiratory chain were heavily down-regulated in response to benzene. These observations suggest that strain S12.49 disposes of efficient alternative ways to generate energy. This was partly confirmed by the relatively mild impact of the protonophore CCCP on benzene tolerance in strain S12.49. However, the exact nature of the mechanism by which strain S12.49 meets the extra energy demand imposed by the improved benzene tolerance remains elusive, as does the involvement of medium components from LB. These challenging questions, as well as the implications of the second additional copy of IS*S12* in *P. putida* S12.49, are the subject of further study.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study are *Pseudomonas putida* S12, which was originally isolated as a styrene utilising bacterium (44), *P. putida* S12.49 (this study) and *P. putida* S12.62 (this study).

Luria broth (LB) (117) was used as the standard culturing medium. As a solid medium, LB with 1.5 % (w/v) agar was used. Batch cultivation was routinely carried out in 100-ml Erlenmeyer flasks containing 25 ml of liquid medium, placed on a horizontally shaking incubator at 30 ºC. As a minimal medium, mineral salts medium with 2 g/l glucose as the carbon source (MMg), was used (44).

Batch culturing for laboratory evolution was carried out in Boston bottles with Mininert valves (Alltech, Deerfield, IL, USA) in 10 ml medium (either LB or MMg (141)).

Continuous cultivation of *P. putida* S12 and *P. putida* S12.49 for proteomics and transcriptomics analysis was performed in duplicate in chemostats (BioFloIIc, New Brunswick Scientific, NJ, USA) with a working volume of 1.0 l. The pH was kept constant at 7.0 and the stirring speed was set at 350 rpm. The temperature was kept at 30 ºC and pure oxygen was supplied at 0.6 l h^{-1}. The dilution rate was set at 0.2 h^{-1}. The culturing medium used was 4×-diluted LB with 37 mM K-phosphate buffer pH 7 (44) and 10 mM glucose. Benzene was added separately using a KD Scientific syringe pump (Applikon) to a final concentration of 8 mM in the chemostat. Samples were drawn at steady state, which was reached after five volume changes. For transcriptomics analysis one sample was drawn and analysed per chemostat, for proteomics analysis three samples were drawn and analysed per chemostat.

Transcriptome analysis

Sampling, mRNA isolation, cDNA preparation and hybridisation for transcriptome analysis were performed as described previously (141). The microarray used was a custom made high-density microarray based on the genome sequence of *P. putida* S12 (Ruijssenaars and de Winde., manuscript in preparation). The genome sequence of strain S12 contains 7107 open reading frames (orfs), of which 1903 are not annotated.

Microarray data were imported into the GeneSpring GX 7.3.1 software package (Agilent Technologies) using the GC RMA algorithm. After normalisation of the data (signals below 0.01 were taken as 0.01; per chip:

normalise to 50th percentile; per gene: normalise to median) probesets representing control genes were removed as well as non-changing genes (between 0.667- and 1.334-fold change).
The resulting set of 5408 differentially expressed genes was used for further analysis. Fold-changes between *P. putida* S12 and *P. putida* S12.49 in the absence of benzene were calculated, as well as between *P. putida* S12.49 in the absence and presence of 8 mM benzene.

Proteome analysis

Proteomics analysis was performed using the 2D-DIGE (two dimensional difference-in-gel-electrophoresis) method, according to Wijte et al. (153). In brief, protein samples were labelled with CyDyes Cy3 and Cy5 and the standard, existing of a mixture of all samples in the experiment was labelled with Cy2 (all GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Iso-electric focussing of the pI 4-7L and pI 6-11L Immobiline Dry-Strips was done on an IPGphor (GE Healthcare) for a total of 60750 Vh and 30750 Vh respectively. Second-dimension polyacrylamide lab-cast gels were run at 1 W per gel, for 1 h, followed by 13 W per gel until the bromophenol blue had migrated to the bottom of the gel. Analysis of the gels was done using the DeCyder 2D Software version 6.5 with the DeCyder Extended Data Analysis module version 1.0 (both GE Healthcare). Fold-changes were calculated as described for the transcriptomics analysis. Spots representing proteins with a fold-change ≥ 2 or ≤ 0.5 were excised from a preparative gel and digested in-gel with trypsin. Protein identification was performed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry and microliquid chromatography electrospray tandem mass spectrometry (μlC-ESI MS/MS).

Analytical methods

Determination of survival frequencies after toluene shock were performed as described before (141). Briefly, exponentially growing cells were incubated for 30 min with a second phase of toluene. Survival frequency was determined by counting the number of colony-forming units before and after the toluene shock.

Antibiotic resistance was assessed as previously described (141). The minimal inhibiting concentration (MIC) was defined as the antibiotic concentration at which no growth was observed.
Southern blot analysis of IS*S12* transposition events was performed as described by Wery *et al.* (147). In short, genomic DNA of *P. putida* S12 and *P. putida* S12.49 before and after a 1 % toluene shock was digested overnight with *Kpn*I. The digested DNA was separated on a 0.8 % agarose gel and

subsequently blotted onto a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) that was hybridised overnight with a DIG labelled probe complementary to the insertion element IS*S12*. The probe was synthesized according to the manufacturers' protocol (PCR DIG Probe Synthesis Kit, Roche Applied Science, Almere, The Netherlands) with primers 5'-CTGCGCTCAATGCACAAGGGC-3' and 5'-GCACGCTGTAGCCCTCCCGG-3'. The hybridised probe was detected using a DIG Nucleic Acid Detection Kit (Roche) in combination with CPD-star (Roche) and visualized using a G:BOX geldoc system (Syngene, Cambridge, UK).

6 Discussion

The remarkable solvent tolerance properties exhibited by *Pseudomonas putida* S12 have intrigued researchers since the 1990's (*e.g.* (50, 69, 146)). Twelve years ago it was acknowledged that these properties could be very useful for the bioproduction of toxic fine chemicals (148). In this thesis, for the first time the global response of *Pseudomonas putida* S12 to organic solvents is investigated using proteomics and transcriptomics analysis. The results of this research are instrumental to further delineate and understand the mechanism of solvent tolerance in this intriguing microorganism. Accordingly, these new insights promise to be useful in improving *P. putida* S12 for the production of fine chemicals.

In 2005, Feder and Walser published a paper where they questioned the use of transcriptomics in elucidating stress response mechanisms (33). The authors stated that 'mRNA abundance provides little information on protein activity and fitness' and therefore 'cannot substitute for detailed functional and ecological analyses of candidate genes'. Frankly speaking, the same holds true for protein abundance: the mere presence of a protein does not give any information about its functioning and its contribution to fitness. Moreover, the relationship between protein/enzyme abundance and metabolite concentrations is not straightforward, since amongst other issues protein turnover and enzyme kinetics play an important part as well.

Seen in this light, no single technique is suitable to elucidate stress response mechanisms since each individual technique can only zoom in on a part of the process. Of course, transcriptomics data tell little or nothing about protein activity, since the technique was specifically developed to measure mRNA abundance. However, it is certainly not true that transcriptomics analysis is useless for elucidating stress response mechanisms. Changes in specific mRNA abundances that are apparent upon addition of for example an organic solvent can well be correlated with a stress response. Subsequently, a thorough physiological interpretation of the effects observed is of course eminent. Likewise, changes in the abundance of specific proteins can also be correlated with a stress response. Proteomics and transcriptomics mainly monitor responses on the molecular level. These responses will, via cascades of various interactions and reactions, eventually lead to responses that are detectable on a physiological level.

Summarizing, transcriptomics and proteomics methodologies have clear limitations in elucidating stress response mechanisms. However, both techniques are indispensable in the search for global mechanisms applied by microbial cells in response to stress. In addition, proteomics and transcriptomics are the techniques of choice to select key genes that play an important role in stress response mechanisms and are worth while studying in

(much) more detail. This thesis presents several interesting examples of such an approach.

Proteomics analysis of *P. putida* S12 exposed to toluene revealed a dramatic decrease in abundance of the porin OprF (**Chapter 2**). It was hypothesised that since toluene is likely to enter the cell via OprF, a decrease in the abundance of this porin would be highly favourable. Recently OprF was functionally characterised, embedded in a lipopolysaccharide (LPS) membrane by computer simulation (126). This simulation yielded another view on the role that OprF possibly plays in solvent tolerance that can very well exist next to the porin hypothesis. In the simulation OprF caused small patches of the membrane to be positively charged, as opposed to the usual negative charge of the LPS of Gram-negative bacteria. This negative charge forms an effective barrier to hydrophobic compounds (126), which is apparently locally counteracted by OprF. Hence, decreasing the abundance of OprF would further enhance the effective hydrophilic barrier. In other studies described in this thesis the *oprF* gene was not differentially expressed, suggesting that it is indeed regulated at the protein level. This apparently is additionally true for OprH. This porin also showed an increase in protein abundance in the presence of toluene, whereas the *oprH* gene was not differentially expressed in chemostats with toluene (**Chapter 3**). Unfortunately, during the annotation of the *P. putida* S12 genome, *oprH* was not recognised as a gene, and hence it was not put on the S12-microarray.

P. putida S12.49 reproducibly tolerates 20 mM of benzene (**Chapter 5**), *i.e.* the growth behaviour in the presence of 20 mM of benzene is always identical. This strain also tolerates up to 24 mM benzene, however, growth behaviour is not reproducible in 21, 22 or 23 mM benzene. This observation indicates that an important part of the improved solvent tolerance of this strain relates to adaptation at the physiological level, in addition to (a) –genetic- mutation(s). In accordance, the adaptation mechanism appears to fail at concentrations well above 20 mM. The fact that it was not possible to isolate a mutant capable of growing in the presence of a second phase of benzene shows that there is a limit to solvent tolerance, even for *P. putida* S12. This limit most likely is caused by the maximal expression and activity of SrpABC, together with the high demand for energy (which cannot be fulfilled above a certain concentration), and an increasing weakness of the membranes in increasing concentrations of solvent. Laboratory evolution experiments however, clearly indicate the efficient plasticity of the genome of *P. putida* S12 towards solvent tolerance. In addition, the outstanding abilities of S12 to cope with and adapt to toxic organic solvents are in agreement with the relatively

low number of stress-related genes and/or proteins that were differentially expressed in each of the four studies described in this Thesis.

In **Chapter 4**, changes in genome-wide expression in *P. putida* S12 and *P. putida* S12ΔTrgI is monitored as a function of time, following the sudden addition of toluene to the growth medium. In this chapter, the role of TrgI as a potential regulator is further supported. Both in the presence and absence of toluene, the expression of a considerable number of genes was found to be influenced by deletion of *trgI*. Moreover, the number of genes affected by deletion of *trgI* changed upon addition of toluene, suggesting that the presence of solvent influences the regulatory action by *trgI*. In Figure 6.1, four different mechanisms through which genes may be affected by toluene and/or TrgI are schematically depicted. The first mode of regulation (I) is the most simple: the expression of a target gene is only directly affected by TrgI. In the second mode (II), also toluene plays a role, although independent of TrgI. In mode III, toluene influences both TrgI and the target gene. Toluene may act on TrgI at the level of transcription or translation, or directly on the TrgI protein. In mode IV, toluene affects TrgI but not the target gene. The exact mode of action of TrgI has not been elucidated, and different modes of action may even exist in parallel depending on the target gene. Moreover, it cannot be excluded that target genes are co-regulated by other regulatory genes.

Recently, the regulatory mechanism of *srpABC* by *srpRS* was investigated by Sun *et al.* (128). As already suggested in **Chapter 3**, they observed that toluene binds to SrpS, limiting its ability to bind to the operator site and enabling transcription of *srpABC* and *srpRS*. It also became clear that SrpR acts as a derepressor as well, allowing for moderate expression in the absence of solvents. The results presented in **Chapter 4**, clearly suggest a role for TrgI in this mechanism since the expression of *srpRSABC* increases very fast in the *trgI*-knockout strain to a maximum level that is well above that in the wildtype strain. It can be imagined that TrgI influences the binding of SrpS to the promotor region, in a way that encourages SrpS to bind to the DNA. Upon addition of toluene, the influence of TrgI would become smaller as the expression of *trgI* is down-regulated. Furthermore, toluene may bind to TrgI, promoting its release from SrpS. Subsequently, toluene would bind to SrpS as proposed by Sun et al., making the promotor region available for the transcriptional machinery. In the mutant strain S12ΔTrgI, toluene can bind to SrpS immediately, causing the level of transcription of *srpABC* and *srpRS* to rise very fast to a high level because no time is lost to alleviate the influence of TrgI on SrpS. This hypothesis of *srpRSABC* regulation by TrgI is an example of mode III regulation.

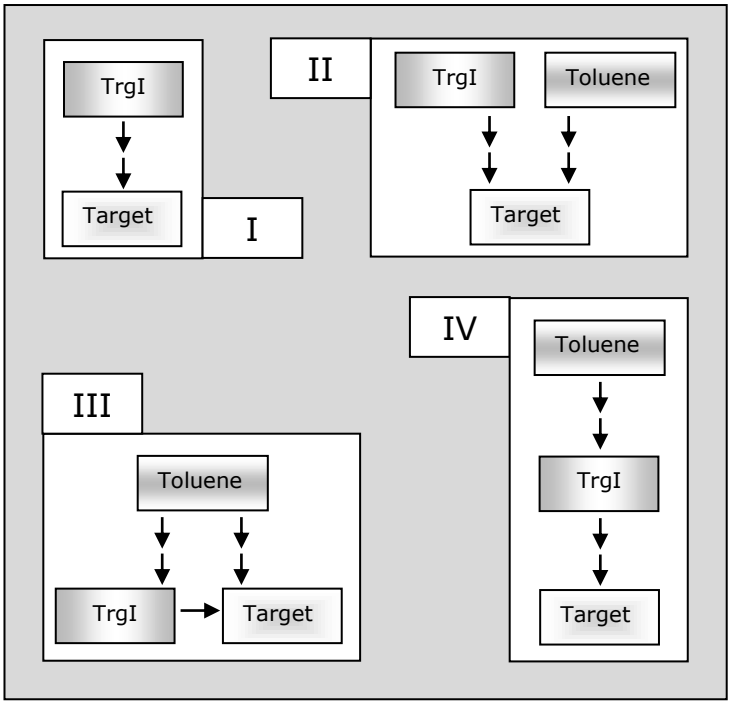

Figure 6.1 Schematic representation of the possible (transcriptional) regulation of target genes by TrgI and/or toluene, in modes I till IV that are described in the main text.

Modelling of the TrgI tertiary structure through the I-Tasser website (**Chapter 4**) yields a good basis for its presumed regulatory role. TrgI itself is not likely to bind to DNA and regulate gene expression directly. It may form a complex or otherwise cooperate with other regulatory proteins that do bind the DNA. Candidate co-regulators are expected to have the same expression profile as *trgI*. The expression profiles of one hundred and thirty five genes exhibits a correlation coefficient with expression of *trgI* of 0.8 or higher in wildtype S12 cells grown in batch cultures and suddenly exposed to toluene. In this list, eight well annotated transcriptional regulators are found (Table 6.1), which were all annotated as members of specific gene families (AraC, AsnC, IclR, LysR and MarR) and were not further characterised. The members of these families are known to be either positive or negative regulators, or both. Three transcriptional regulators are particularly interesting, namely RPPX04118 (AraC family), RPPX03392 (MarR family) and RPPX02247 (IclR family).

Members of the AraC family typically are involved in regulation of carbon metabolism, response to environmental stress and pathogenesis (29, 134).

Table 6.1 Transcriptional regulators that have an expression profile that is similar (correlation coefficient of 0.8 or higher) to that of *trgI* in *P. putida* S12 grown in batch cultures and suddenly exposed to toluene (also see Chapter 5).

Gene	Name	C.C.[a]	Regulation of:
RPPX04118	Transcriptional regulator, AraC family	0.89	Carbon metabolism, response to environmental stress and pathogenesis (29, 134)
RPPX04109	Transcriptional regulator, LysR family	0.87	Virulence, metabolism, quorum sensing, motility (82)
RPPX02288	Transcriptional regulator, LysR family	0.86	See RPPX04109
RPPX02310	Transcriptional regulator, AsnC family	0.86	General: amino acid metabolism and related processes; *E. coli*: global regulator (13)
RPPX03392	Transcriptional regulator, MarR family	0.84	Antibiotic resistance, antimicrobial agents, sensing of aromatic compounds and virulence (30)
RPPX02247	Transcriptional regulator, IclR family	0.82	Glyoxylate bypass, multidrug resistance, degradation of aromatics, inactivation of quorum-sensing signals, plant pathogenicity and sporulation (90)
RPPX06665	Transcriptional regulator, LysR family	0.81	See RPPX04109
RPPX03720	Transcriptional regulator, LysR family	0.80	See RPPX04109

a) C.C. = Correlation Coefficient
The swift down-regulation of *trgI* in *P. putida* S12 upon a sudden solvent shock, appears to be an initial measure against the devastating effects of the solvent. Several strains of *P. putida* and a few other *Pseudomonas* species appear to have a gene similar to *trgI* (**Chapter 3**). None of those strains (*P. putida* KT2440, BIRD-1, GB-1, W619 and S16, *P. entomophila* L48 and *P. fluorescens* Pf-5 and Pf0-1) can degrade toluene or benzene, nor have they been shown to withstand the same high solvent concentrations as S12. This means that, next to *trgI*, additional traits are necessary to make a *Pseudomonas* solvent tolerant, presenting additional evidence for the regulatory role of this gene.

Since toluene can be considered an environmental stress, it is possible that *trgI* cooperates with RPPX04118. Cooperation with RPPX03392 is also likely because MarR family regulators are, amongst others, involved in the sensing of

aromatic compounds (30). In view of the functions associated with IclR family regulators, RPPX02247 is the most promising putative regulator. Members of this family are involved in, e.g., regulation of the glyoxylate bypass, multidrug resistance and degradation of aromatics (90). The differentially expressed genes in wildtype S12 and S12ΔTrgI cells that were exposed to sudden toluene addition (**Chapter 4**) overlap with these functionalities. One of the regulators of the solvent pump genes *srpABC* in S12, *srpS,* as well as the regulators of the solvent pump genes *ttgDEF* and *ttgGHI* in *P. putida* DOT-T1E, *ttgT* and *ttgV*, belong to the IclR family (90). The differences in expression of *srpABC*, *arpABC* and *ttg2CD* between S12 and S12ΔTrgI encourage the idea of a cooperation between *trgI* and RPPX02247.

The solvent extrusion pump SrpABC was long considered one of the most important properties that confer solvent tolerance in S12. It uses electrons to transfer solvent molecules out of the cell. This, together with the dissipation of the proton motive force that occurs when solvent molecules accumulate in the membranes of the cell, leads to a decreased cell yield in the presence of solvents (62). The two chemostat studies of wildtype S12 cells cultured in the presence of toluene (**Chapters 2 and 3**) showed that most aspects of the energy metabolism are involved in keeping toluene-exposed cells well energised. The mutant strain with an elevated level of solvent tolerance, *P. putida* S12.49 that is described in **Chapter 5**, seems to have a completely altered energy metabolism. Taking together these observations, an interesting picture emerges about the plasticity of the energy metabolism of *P. putida* S12 and the immense importance of this quality for solvent tolerance. Without the property of being able to alter its energy metabolism, S12 could not exploit the solvent extrusion pump to its full potential. Therefore, the flexibility of its energy metabolism appears to be crucial for the solvent tolerance properties of *P. putida* S12.

Literature

1. **Alekshun, M. N., and S. B. Levy.** 1999. The mar regulon: multiple resistance to antibiotics and other toxic chemicals. Trends in microbiology **7:**410-413.
2. **Alsaker, K. V., T. R. Spitzer, and E. T. Papoutsakis.** 2004. Transcriptional analysis of spo0A overexpression in Clostridium acetobutylicum and its effect on the cell's response to butanol stress. J Bacteriol **186:**1959-1971.
3. **Aono, R., M. Ito, A. Inoue, and K. Horikoshi.** 1992. Isolation of novel toluene-tolerant strain of *Pseudomonas aeruginosa*. Biosci Biotechnol Biochem **56:**145-146.
4. **Aono, R., and H. Kobayashi.** 1997. Cell surface properties of organic solvent-tolerant mutants of *Escherichia coli* K-12. Applied and environmental microbiology **63:**3637-3642.
5. **Ballerstedt, H., R. J. M. Volkers, A. E. Mars, J. E. Hallsworth, V. A. Martins dos Santos, J. Puchalka, J. Van Duuren, G. Eggink, K. N. Timmis, J. A. M. De Bont, and J. Wery.** 2007. Genomotyping of *Pseudomons putida* strains using *P. putida* KT2440-based high-density DNA microarrays: implications for transcriptomics studies. Applied microbiology and biotechnology **75:**1133-1142.
6. **Bell, A., M. Bains, and R. E. Hancock.** 1991. *Pseudomonas aeruginosa* outer membrane protein OprH: expression from the cloned gene and function in EDTA and gentamicin resistance. J Bacteriol **173:**6657-6664.
7. **Benndorf, D., M. Thiersch, N. Loffhagen, C. Kunath, and H. Harms.** 2006. Pseudomonas putida KT2440 responds specifically to chlorophenoxy herbicides and their initial metabolites. Proteomics **6:**3319-3329.
8. **Berg, J. M., J. L. Tymoczko, and L. Stryer.** 2002. Biochemistry, 5 ed. W.H. Freeman and Company, New York.
9. **Bernal, P., J. Munoz-Rojas, A. Hurtado, J. L. Ramos, and A. Segura.** 2007. A Pseudomonas putida cardiolipin synthesis mutant exhibits increased sensitivity to drugs related to transport functionality. Environ Microbiol **9:**1135-1145.
10. **Bernal, P., A. Segura, and J. L. Ramos.** 2007. Compensatory role of the cis-trans-isomerase and cardiolipin synthase in the membrane fluidity of Pseudomonas putida DOT-T1E. Environ Microbiol **9:**1658-1664.
11. **Boer, V. M., J. H. de Winde, J. T. Pronk, and M. D. Piper.** 2003. The genome-wide transcriptional responses of *Saccharomyces cerevisiae* grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. J Biol Chem **278:**3265-3274.
12. **Bogdanov, M., P. N. Heacock, and W. Dowhan.** 2002. A polytopic membrane protein displays a reversible topology dependent on membrane lipid composition. The EMBO journal **21:**2107-2116.
13. **Brinkman, A. B., T. J. Ettema, W. M. de Vos, and J. van der Oost.** 2003. The Lrp family of transcriptional regulators. Molecular microbiology **48:**287-294.
14. **Brown, S. D., M. R. Thompson, N. C. Verberkmoes, K. Chourey, M. Shah, J. Zhou, R. L. Hettich, and D. K. Thompson.** 2006. Molecular dynamics of the *Shewanella oneidensis* response to chromate stress. Mol Cell Proteomics **5:**1045-1071.
15. **Budde, I., L. Steil, C. Scharf, U. Volker, and E. Bremer.** 2006. Adaptation of *Bacillus subtilis* to growth at low temperature: a combined transcriptomic and proteomic appraisal. Microbiology (Reading, England) **152:**831-853.
16. **Campbell, J. W., and J. E. Cronan, Jr.** 2001. Escherichia coli FadR positively regulates transcription of the fabB fatty acid biosynthetic gene. J Bacteriol **183:**5982-5990.
17. **Capitani, G., O. Eidam, R. Glockshuber, and M. G. Grutter.** 2006. Structural and functional insights into the assembly of type 1 pili from Escherichia coli. Microbes and infection / Institut Pasteur **8:**2284-2290.
18. **Cartwright, C. P., J.-R. Juroszek, M. J. Beavan, M. F. S. Ruby, S. M. F. d. Morais, and A. H. Rose.** 1986. Ethanol dissipates the proton motive force across the plasma membrane of *Saccharomyces cereviseae*. J Gen Microbiol **132:**369-377.
19. **Choi, H. J., S. A. Kim, D. W. Kim, J. Y. Moon, Y. K. Jeong, and W. H. Joo.** 2008. Characterization of Pseudomonas sp. BCNU 171 tolerant to organic solvents. Journal of basic microbiology **48:**473-479.

20. **Collet, J. F., and J. C. Bardwell.** 2002. Oxidative protein folding in bacteria. Molecular microbiology **44:**1-8.
21. **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. Applied and environmental microbiology **58:**2723-2729.
22. **de Bont, J. A. M.** 1998. Solvent-tolerant bacteria in biocatalysis. Trends Biotechnol **16:**493-499.
23. **del Castillo, T., J. L. Ramos, J. J. Rodriguez-Herva, T. Fuhrer, U. Sauer, and E. Duque.** 2007. Convergent peripheral pathways catalyze initial glucose catabolism in Pseudomonas putida: genomic and flux analysis. J Bacteriol **189:**5142-5152.
24. **Denef, V. J., M. A. Patrauchan, C. Florizone, J. Park, T. V. Tsoi, W. Verstraete, J. M. Tiedje, and L. D. Eltis.** 2005. Growth substrate- and phase-specific expression of biphenyl, benzoate, and C1 metabolic pathways in Burkholderia xenovorans LB400. J Bacteriol **187:**7996-8005.
25. **Desmond, C., G. F. Fitzgerald, C. Stanton, and R. P. Ross.** 2004. Improved stress tolerance of GroESL-overproducing *Lactococcus lactis* and probiotic *Lactobacillus paracasei* NFBC 338. Applied and environmental microbiology **70:**5929-5936.
26. **DiRusso, C. C., T. L. Heimert, and A. K. Metzger.** 1992. Characterization of FadR, a global transcriptional regulator of fatty acid metabolism in Escherichia coli. Interaction with the fadB promoter is prevented by long chain fatty acyl coenzyme A. J Biol Chem **267:**8685-8691.
27. **Dominguez-Cuevas, P., J. E. Gonzalez-Pastor, S. Marques, J. L. Ramos, and V. de Lorenzo.** 2006. Transcriptional tradeoff between metabolic and stress-response programs in *Pseudomonas putida* KT2440 cells exposed to toluene. J Biol Chem **281:**11981-11991.
28. **Duque, E., A. Segura, G. Mosqueda, and J. L. Ramos.** 2001. Global and cognate regulators control the expression of the organic solvent efflux pumps TtgABC and TtgDEF of Pseudomonas putida. Molecular microbiology **39:**1100-1106.
29. **Egan, S. M.** 2002. Growing repertoire of AraC/XylS activators. J Bacteriol **184:**5529-5532.
30. **Ellison, D. W., and V. L. Miller.** 2006. Regulation of virulence by members of the MarR/SlyA family. Curr Opin Microbiol **9:**153-159.
31. **Fang, Y., Z. Lu, F. Lv, X. Bie, S. Liu, Z. Ding, and W. Xu.** 2006. A newly isolated organic solvent tolerant Staphylococcus saprophyticus M36 produced organic solvent-stable lipase. Current microbiology **53:**510-515.
32. **Fay, J. P., and R. N. Farias.** 1977. Inhibitory action of a non-metabolizable fatty acid on the growth of *Escherichia coli*: role of metabolism and outer membrane integrity. J Bacteriol **132:**790-795.
33. **Feder, M. E., and J. C. Walser.** 2005. The biological limitations of transcriptomics in elucidating stress and stress responses. J Evol Biol **18:**901-910.
34. **Fraga Muller, J., A. M. Stevens, J. Craig, and N. G. Love.** 2007. Transcriptome analysis reveals that multidrug efflux genes are upregulated to protect *Pseudomonas aeruginosa* from pentachlorophenol stress. Applied and environmental microbiology **73:**4550-4558.
35. **Fredrickson, J. K., D. L. Balkwill, G. R. Drake, M. F. Romine, D. B. Ringelberg, and D. C. White.** 1995. Aromatic-degrading Sphingomonas isolates from the deep subsurface. Applied and environmental microbiology **61:**1917-1922.
36. **Frunzke, J., V. Engels, S. Hasenbein, C. Gatgens, and M. Bott.** 2008. Co-ordinated regulation of gluconate catabolism and glucose uptake in Corynebacterium glutamicum by two functionally equivalent transcriptional regulators, GntR1 and GntR2. Molecular microbiology **67:**305-322.
37. **Fujita, K., A. Matsuyama, Y. Kobayashi, and H. Iwahashi.** 2004. Comprehensive gene expression analysis of the response to straight-chain alcohols in *Saccharomyces cerevisiae* using cDNA microarray. J Appl Microbiol **97:**57-67.
38. **Griffin, T. J., S. P. Gygi, T. Ideker, B. Rist, J. Eng, L. Hood, and R. Aebersold.** 2002. Complementary profiling of gene expression at the transcriptome and proteome levels in *Saccharomyces cerevisiae*. Mol Cell Proteomics **1:**323-333.
39. **Guazzaroni, M. E., W. Teran, X. Zhang, M. T. Gallegos, and J. L. Ramos.** 2004. TtgV bound to a complex operator site represses transcription of the promoter for the multidrug and solvent extrusion TtgGHI pump. J Bacteriol **186:**2921-2927.

40. **Hall, D. R., D. G. Gourley, G. A. Leonard, E. M. Duke, L. A. Anderson, D. H. Boxer, and W. N. Hunter.** 1999. The high-resolution crystal structure of the molybdate-dependent transcriptional regulator (ModE) from Escherichia coli: a novel combination of domain folds. The EMBO journal **18:**1435-1446.
41. **Hallsworth, J. E., S. Heim, and K. N. Timmis.** 2003. Chaotropic solutes cause water stress in *Pseudomonas putida*. Environ Microbiol **5:**1270-1280.
42. **Hallsworth, J. E., B. A. Prior, Y. Nomura, M. Iwahara, and K. N. Timmis.** 2003. Compatible solutes protect against chaotrope (ethanol)-induced, nonosmotic water stress. Applied and environmental microbiology **69:**7032-7034.
43. **Hancock, R. E., and F. S. Brinkman.** 2002. Function of *Pseudomonas* porins in uptake and efflux. Annu Rev Microbiol **56:**17-38.
44. **Hartmans, S., J. Smits, M. van de Werf, F. Volkering, and J. A. M. de Bont.** 1989. Metabolism of styrene oxide and 2-phenyl ethanol in the styrene degrading *Xanthobacter* strain 124X. Applied and environmental microbiology **55:**2850-2855.
45. **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. Applied and environmental microbiology **56:**1347-1351.
46. **Havlis, J., H. Thomas, M. Sebela, and A. Shevchenko.** 2003. Fast-response proteomics by accelerated in-gel digestion of proteins. Anal Chem **75:**1300-1306.
47. **Hayashi, S., R. Aono, T. Hanai, H. Mori, T. Kobayashi, and H. Honda.** 2003. Analysis of organic solvent tolerance in *Escherichia coli* using gene expression profiles from DNA microarrays. J Biosci Bioeng **95:**379-383.
48. **Hedstrom, L.** 1999. IMP dehydrogenase: mechanism of action and inhibition. Curr Med Chem **6:**545-560.
49. **Hegde, P. S., I. R. White, and C. Debouck.** 2003. Interplay of transcriptomics and proteomics. Curr Opin Biotechnol **14:**647-651.
50. **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. Applied and environmental microbiology **60:**4440-4444.
51. **Heipieper, H. J., P. de Waard, P. van der Meer, J. A. Killian, S. Isken, J. A. M. de Bont, G. Eggink, and F. A. de Wolf.** 2001. Regiospecific effect of 1-octanol on cis-trans isomerization of unsaturated fatty acids in the solvent-tolerant strain Pseudomonas putida S12. Applied microbiology and biotechnology **57:**541-547.
52. **Heipieper, H. J., G. Meulenbeld, Q. van Oirschot, and J. de Bont.** 1996. Effect of Environmental Factors on the trans/cis Ratio of Unsaturated Fatty Acids in Pseudomonas putida S12. Applied and environmental microbiology **62:**2773-2777.
53. **Heipieper, H. J., G. Neumann, S. Cornelissen, and F. Meinhardt.** 2007. Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. Applied microbiology and biotechnology **74:**961-973.
54. **Heipieper, H. J., G. Neumann, N. Kabelitz, M. Kastner, and H. H. Richnow.** 2004. Carbon isotope fractionation during cis- trans isomerization of unsaturated fatty acids in Pseudomonas putida. Applied microbiology and biotechnology.
55. **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.
56. **Hoskisson, P. A., and G. Hobbs.** 2005. Continuous culture--making a comeback? Microbiology (Reading, England) **151:**3153-3159.
57. **Hua, Q., C. Yang, T. Oshima, H. Mori, and K. Shimizu.** 2004. Analysis of gene expression in Escherichia coli in response to changes of growth-limiting nutrient in chemostat cultures. Applied and environmental microbiology **70:**2354-2366.
58. **Inoue, A., and K. Horikoshi.** 1989. A *Pseudomonas* thrives in high concentration of toluene. Nature **338:**264-266.
59. **Isken, S., and J. A. M. de Bont.** 1996. Active efflux of toluene in a solvent-resistant bacterium. J Bacteriol **178:**6056-6058.
60. **Isken, S., and J. A. M. de Bont.** 1998. Bacteria tolerant to organic solvents. Extremophiles **2:**229-238.

61. **Isken, S., and J. A. M. de Bont.** 2000. The solvent efflux system of *Pseudomonas putida* S12 is not involved in antibiotic resistance. Applied microbiology and biotechnology **54:**711-714.
62. **Isken, S., A. Derks, P. F. F. Wolffs, and J. A. M. de Bont.** 1999. Effect of organic solvents on the yield of solvent-tolerant *Pseudomonas putida* S12. Applied and environmental microbiology **65:**2631-2635.
63. **Kato, C., A. Inoue, and K. Horikoshi.** 1996. Isolating and characterizing deep-sea marine microorganisms. Trends Biotechnol **14:**6-12.
64. **Kazakov, A. E., D. A. Rodionov, E. Alm, A. P. Arkin, I. Dubchak, and M. S. Gelfand.** 2009. Comparative genomics of regulation of fatty acid and branched-chain amino acid utilization in proteobacteria. J Bacteriol **191:**52-64.
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 microbiology letters **198:**117-122.
66. **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 (Reading, England) **147:**43-51.
67. **Kieboom, J., and J. A. M. de Bont.** 2000. Mechanisms of organic solvent tolerance in bacteria, p. 393-402. *In* G. H.-A. Storz, R. (ed.), Bacterial stress responses. ASM press, Washington.
68. **Kieboom, J., J. J. Dennis, J. A. M. de Bont, and G. Zylstra.** 1998. Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J Biol Chem **273:**85-91.
69. **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.
70. **Kim, K., S. Lee, K. Lee, and D. Lim.** 1998. Isolation and characterization f toluene-sensitive mutants from the toluene-resistant bacterium *Pseudomonas putida* GM73. J Bacteriol **180:**3692-3696.
71. **Kobayashi, H., H. Takami, H. Hirayama, K. Kobata, R. Usami, and K. Horikoshi.** 1999. Outer membrane changes in a toluene-sensitive mutant of toluene-tolerant *Pseudomonas putida* IH-2000. J Bacteriol **181:**4493-4498.
72. **Kongpol, A., J. Kato, and A. S. Vangnai.** 2008. Isolation and characterization of Deinococcus geothermalis T27, a slightly thermophilic and organic solvent-tolerant bacterium able to survive in the presence of high concentrations of ethyl acetate. FEMS microbiology letters **286:**227-235.
73. **Krabbe, M., N. Weyand, and D. Low.** 2000. Environmental control of pilus gene expression, p. 305-321. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress respones. ASM Press, Washington, D.C.
74. **Kuhner, S., L. Wohlbrand, I. Fritz, W. Wruck, C. Hultschig, P. Hufnagel, M. Kube, R. Reinhardt, and R. Rabus.** 2005. Substrate-dependent regulation of anaerobic degradation pathways for toluene and ethylbenzene in a denitrifying bacterium, strain EbN1. J Bacteriol **187:**1493-1503.
75. **Laane, C., S. Boeren, K. Vos, and C. Veeger.** 1987. Rules for optimization of biocatalysis in organic solvents. Biotechnol bioeng **30:**81-87.
76. **Leao, C., and N. Van Uden.** 1984. Effects of ethanol and other alkanols on passive proton influx in the yeast Saccharomyces cerevisiae. Biochim Biophys Acta **774:**43-48.
77. **Lee, H. S., R. E. Hancock, and J. L. Ingraham.** 1989. Properties of a Pseudomonas stutzeri outer membrane channel-forming protein (NosA) required for production of copper-containing N2O reductase. J Bacteriol **171:**2096-2100.
78. **Letunic, I., R. R. Copley, B. Pils, S. Pinkert, J. Schultz, and P. Bork.** 2006. SMART 5: domains in the context of genomes and networks. Nucleic Acids Res **34:**D257-260.
79. **Li, L., T. Komatsu, A. Inoue, and K. Horikoshi.** 1995. A toluene-tolerant mutant of *Pseudomonas aeruginosa* lacking the outer membrane protein F. Biosci Biotechnol Biochem **59:**2358-2359.
80. **Li, X. Z., L. Zhang, and K. Poole.** 1998. Role of the multidrug efflux systems of *Pseudomonas aeruginosa* in organic solvent tolerance. J Bacteriol **180:**2987-2991.
81. **Loh, K.-C., and B. Cao.** 2008. Paradigm in biodegradation using *Pseudomonas putida* - A review of proteomics studies. Enzyme Microb Technol **43:**1-12.

82. **Maddocks, S. E., and P. C. Oyston.** 2008. Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. Microbiology (Reading, England) **154:**3609-3623.
83. **Majtan, T., G. Bukovska, and J. Timko.** 2004. DNA microarrays--techniques and applications in microbial systems. Folia Microbiol (Praha) **49:**635-664.
84. **Marouga, R., S. David, and E. Hawkins.** 2005. The development of the DIGE system: 2D fluorescence difference gel analysis technology. Anal Bioanal Chem **382:**669-678.
85. **Matsui, K., T. Hirayama, K. Kuroda, K. Shirahige, T. Ashikari, and M. Ueda.** 2006. Screening for candidate genes involved in tolerance to organic solvents in yeast. Applied microbiology and biotechnology **71:**75-79.
86. **Matsumoto, M., J. A. M. de Bont, and S. Isken.** 2002. Isolation and characterization of the solvent-tolerant *Bacillus cereus* strain R1. J Biosci Bioeng **94:**45-54.
87. **Mengin-Lecreulx, D., J. van Heijenoort, and J. T. Park.** 1996. Identification of the mpl gene encoding UDP-N-acetylmuramate: L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase in Escherichia coli and its role in recycling of cell wall peptidoglycan. J Bacteriol **178:**5347-5352.
88. **Mercenier, A., J.-P. Simon, C. Vander Wauven, D. Haas, and V. Stalon.** 1980. Regulation of enzyme synthesis in the arginine deiminase pathway of *Pseudomonas aeruginosa*. J Bacteriol **144:**159-163.
89. **Miller, I., J. Crawford, and E. Gianazza.** 2006. Protein stains for proteomic applications: which, when, why? Proteomics **6:**5385-5408.
90. **Molina-Henares, A. J., T. Krell, M. Eugenia Guazzaroni, A. Segura, and J. L. Ramos.** 2006. Members of the IclR family of bacterial transcriptional regulators function as activators and/or repressors. FEMS Microbiol Rev **30:**157-186.
91. **Moriya, K., and K. Horikoshi.** 1993. A benzene-tolerant bacterium utilizing sulfur compounds isolated from deep sea. J Ferment Bioeng **76:**397-399.
92. **Mostertz, J., C. Scharf, M. Hecker, and G. Homuth.** 2004. Transcriptome and proteome analysis of *Bacillus subtilis* gene expression in response to superoxide and peroxide stress. Microbiology (Reading, England) **150:**497-512.
93. **Na, K. S., A. Kuroda, N. Takiguchi, T. Ikeda, H. Ohtake, and J. Kato.** 2005. Isolation and characterization of benzene-tolerant *Rhodococcus opacus* strains. J Biosci Bioeng **99:**378-382.
94. **Nelson, K. E., C. Weinel, I. T. Paulsen, R. J. Dodson, H. Hilbert, V. A. P. Martins dos Santos, D. E. Fouts, S. R. Gill, M. Pop, M. Holmes, L. Brinkac, M. Beanan, R. T. DeBoy, D. S., J. Kolonay, R. Maupu, W. Nelson, O. White, J. Peterson, H. Khouri, I. Hance, P. Chris Lee, E. Holtzapple, D. Scanlan, K. Tran, A. Moazzez, T. Utterback, M. Rizzo, K. Lee, D. Kosack, D. Moestl, H. Wedler, J. Lauber, D. Stjepandic, J. Hoheisel, M. Straetz, S. Heim, C. Kiewitz, J. Eisen, K. N. Timmis, A. Dusterhoft, B. Tummler, and C. M. Fraser.** 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ Microbiol **4:**799-808.
95. **Neumann, G., Y. Veeranagouda, T. B. Karegoudar, O. Sahin, I. Mausezahl, N. Kabelitz, U. Kappelmeyer, and H. J. Heipieper.** 2005. Cells of Pseudomonas putida and Enterobacter sp. adapt to toxic organic compounds by increasing their size. Extremophiles **9:**163-168.
96. **Nielsen, L. E., D. R. Kadavy, S. Rajagopal, R. Drijber, and K. W. Nickerson.** 2005. Survey of extreme solvent tolerance in gram-positive cocci: membrane fatty acid changes in *Staphylococcus haemolyticus* grown in toluene. Applied and environmental microbiology **71:**5171-5176.
97. **Nijkamp, K., N. van Luijk, J. A. M. de Bont, and J. Wery.** 2005. The solvent-tolerant *Pseudomonas putida* S12 as host for the production of cinnamic acid from glucose. Applied microbiology and biotechnology **69:**170-177.
98. **Nijkamp, K., R. G. Westerhof, H. Ballerstedt, J. A. M. de Bont, and J. Wery.** 2007. Optimization of the solvent-tolerant Pseudomonas putida S12 as host for the production of p-coumarate from glucose. Applied microbiology and biotechnology **74:**617-624.
99. **Nudleman, E., and D. Kaiser.** 2004. Pulling together with type IV pili. Journal of molecular microbiology and biotechnology **7:**52-62.
100. **Ogino, H., K. Yasui, T. Shiotani, T. Ishihara, and H. Ishikawa.** 1995. Organic Solvent-Tolerant Bacterium Which Secretes an Organic Solvent-Stable Proteolytic Enzyme. Applied and environmental microbiology **61:**4258-4262.

101. **Osborne, S. J., J. Leaver, M. K. Turner, and P. Dunnill.** 1990. Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. Enzyme Microb Technol **12:**281-291.
102. **Paje, M. L., B. A. Neilan, and I. Couperwhite.** 1997. A Rhodococcus species that thrives on medium saturated with liquid benzene. Microbiology (Reading, England) **143 (Pt 9):**2975-2981.
103. **Perkins, D. N., D. J. Pappin, D. M. Creasy, and J. S. Cottrell.** 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis **20:**3551-3567.
104. **Pessione, E., M. G. Giuffrida, L. Prunotto, C. Barello, R. Mazzoli, D. Fortunato, A. Conti, and C. Giunta.** 2003. Membrane proteome of Acinetobacter radioresistens S13 during aromatic exposure. Proteomics **3:**1070-1076.
105. **Poolman, B., A. J. Driessen, and W. N. Konings.** 1987. Regulation of arginine-ornithine exchange and the arginine deiminase pathway in Streptococcus lactis. J Bacteriol **169:**5597-5604.
106. **Quandt, J., and M. F. Hynes.** 1993. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. Gene **127:**15-21.
107. **Radniecki, T. S., M. E. Dolan, and L. Semprini.** 2008. Physiological and transcriptional responses of Nitrosomonas europaea to toluene and benzene inhibition. Environ Sci Technol **42:**4093-4098.
108. **Ramos, J. L., E. Duque, M. T. Gallegos, P. Godoy, M. I. Ramos-Gonzalez, A. Rojas, W. Teran, and A. Segura.** 2002. Mechanisms of solvent tolerance in gram-negative bacteria. Ann Rev Microbiol **56:**743-768.
109. **Ramos, J. L., E. Duque, M. J. Huertas, and A. Haidour.** 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.
110. **Ramos, J. L., E. Duque, J.-J. Rodriguez-Herva, P. Godoy, A. Haidour, F. Reyes, and A. Fernandez-Barrero.** 1997. Mechanisms for solvent tolerance in bacteria. J Biol Chem **272:**3887-3890.
111. **Ramos, J. L., M. Martinez-Bueno, A. J. Molina-Henares, W. Teran, K. Watanabe, X. Zhang, M. T. Gallegos, R. Brennan, and R. Tobes.** 2005. The TetR family of transcriptional repressors. Microbiol Mol Biol Rev **69:**326-356.
112. **Rhee, H. J., E. J. Kim, and J. K. Lee.** 2007. Physiological polyamines: simple primordial stress molecules. Journal of cellular and molecular medicine **11:**685-703.
113. **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.
114. **Roy, A., A. Kucukural, and Y. Zhang.** 2010. I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc **5:**725-738.
115. **Ruijssenaars, H. J., E. M. Sperling, P. H. Wiegerinck, F. T. Brands, J. Wery, and J. A. M. de Bont.** 2007. Testosterone 15beta-hydroxylation by solvent tolerant *Pseudomonas putida* S12. Journal of biotechnology **131:**205-208.
116. **Sabirova, J. S., M. Ferrer, D. Regenhardt, K. N. Timmis, and P. N. Golyshin.** 2006. Proteomic insights into metabolic adaptations in Alcanivorax borkumensis induced by alkane utilization. J Bacteriol **188:**3763-3773.
117. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Press.
118. **Sanchez, B., M. C. Champomier-Verges, P. Anglade, F. Baraige, C. G. de Los Reyes-Gavilan, A. Margolles, and M. Zagorec.** 2005. Proteomic analysis of global changes in protein expression during bile salt exposure of *Bifidobacterium longum* NCIMB 8809. J Bacteriol **187:**5799-5808.
119. **Santos, P. M., D. Benndorf, and I. Sa-Correia.** 2004. Insights into *Pseudomonas putida* KT2440 response to phenol-induced stress by quantitative proteomics. Proteomics **4:**2640-2652.
120. **Santos, P. M., V. Roma, D. Benndorf, M. von Bergen, H. Harms, and I. Sa-Correia.** 2007. Mechanistic insights into the global response to phenol in the phenol-biodegrading strain Pseudomonas sp. M1 revealed by quantitative proteomics. Omics **11:**233-251.

121. **Schultz, J., F. Milpetz, P. Bork, and C. P. Ponting.** 1998. SMART, a simple modular architecture research tool: identification of signaling domains. Proceedings of the National Academy of Sciences of the United States of America **95:**5857-5864.
122. **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.
123. **Segura, A., P. Godoy, P. van Dillewijn, A. Hurtado, N. Arroyo, S. Santacruz, and J. L. Ramos.** 2005. Proteomic analysis reveals the participation of energy- and stress-related proteins in the response of *Pseudomonas putida* DOT-T1E to toluene. J Bacteriol **187:**5937-5945.
124. **Sikkema, J., J. A. M. de Bont, and B. Poolman.** 1994. Interactions of cyclic hydrocarbons with biological membranes. J Biol Chem **269:**8022-8028.
125. **Sikkema, J., J. A. M. de Bont, and B. Poolman.** 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev **59:**201-222.
126. **Straatsma, T. P., and T. A. Soares.** 2009. Characterization of the outer membrane protein OprF of Pseudomonas aeruginosa in a lipopolysaccharide membrane by computer simulation. Proteins **74:**475-488.
127. **Sun, X., and J. J. Dennis.** 2009. A novel insertion sequence derepresses efflux pump expression and preadapts *Pseudomonas putida* S12 for extreme solvent stress. J Bacteriol **DOI:10.1128/JB.00832-09**.
128. **Sun, X., Z. Zahir, K. H. Lynch, and J. J. Dennis.** 2011. An antirepressor, SrpR, is involved in transcriptional regulation of the SrpABC solvent tolerance efflux pump of *Pseudomonas putida* S12. J Bacteriol **193:**2717-2725.
129. **Swanson, B. L., P. Hager, P. Phibbs, Jr., U. Ochsner, M. L. Vasil, and A. N. Hamood.** 2000. Characterization of the 2-ketogluconate utilization operon in Pseudomonas aeruginosa PAO1. Molecular microbiology **37:**561-573.
130. **Tam, L. T., C. Eymann, D. Albrecht, R. Sietmann, F. Schauer, M. Hecker, and H. Antelmann.** 2006. Differential gene expression in response to phenol and catechol reveals different metabolic activities for the degradation of aromatic compounds in Bacillus subtilis. Environ Microbiol **8:**1408-1427.
131. **Tatusov, R. L., N. D. Fedorova, J. D. Jackson, A. R. Jacobs, B. Kiryutin, E. V. Koonin, D. M. Krylov, R. Mazumder, S. L. Mekhedov, A. N. Nikolskaya, B. S. Rao, S. Smirnov, A. V. Sverdlov, S. Vasudevan, Y. I. Wolf, J. J. Yin, and D. A. Natale.** 2003. The COG database: an updated version includes eukaryotes. BMC Bioinformatics **4:**41.
132. **Tatusov, R. L., E. V. Koonin, and D. J. Lipman.** 1997. A genomic perspective on protein families. Science **278:**631-637.
133. **Teran, W., A. Felipe, A. Segura, A. Rojas, J.-L. Ramos, and M. Trinidad-Gallegos.** 2003. Antibiotic-dependent induction of *Pseudomonas putida* DOT-T1E TtgABC efflux pump is mediated by the drug binding repressor TtgR.
134. **Tobes, R., and J. L. Ramos.** 2002. AraC-XylS database: a family of positive transcriptional regulators in bacteria. Nucleic Acids Research **30:**318-321.
135. **Tomas, C. A., J. Beamish, and E. T. Papoutsakis.** 2004. Transcriptional analysis of butanol stress and tolerance in *Clostridium acetobutylicum*. J Bacteriol **186:**2006-2018.
136. **Trautwein, K., S. Kuhner, L. Wohlbrand, T. Halder, K. Kuchta, A. Steinbuchel, and R. Rabus.** 2008. Solvent stress response of the denitrifying bacterium "Aromatoleum aromaticum" strain EbN1. Applied and environmental microbiology **74:**2267-2274.
137. **Urai, M., T. Aizawa, H. Anzai, J. Ogihara, N. Iwabuchi, B. Neilan, I. Couperwhite, M. Nakajima, and M. Sunairi.** 2006. Structural analysis of an extracellular polysaccharide produced by a benzene tolerant bacterium, *Rhodococcus* sp. 33. Carbohydrate research **341:**616-623.
138. **Verhoef, S., H. J. Ruijssenaars, J. A. M. de Bont, and J. Wery.** 2007. Bioproduction of p-hydroxybenzoate from renewable feedstock by solvent-tolerant Pseudomonas putida S12. Journal of biotechnology **132:**49-56.
139. **Verhoef, S., N. Wierckx, R. G. Westerhof, J. H. de Winde, and H. J. Ruijssenaars.** 2009. Bioproduction of *p*-hydroxystyrene from glucose by the solvent-tolerant bacterium *Pseudomonas putida* S12 in a two-phase water-decanol fermentation. Applied and environmental microbiology **75:**931-936.

140. **Volkers, R. J., H. Ballerstedt, J. H. de Winde, and H. Ruijssenaars.** 2010. Isolation and genetic characterization of an improved benzene tolerant mutant of *Pseudomonas putida* S12 Environ Microbiol Reports.
141. **Volkers, R. J., H. Ballerstedt, H. Ruijssenaars, J. A. M. de Bont, J. H. de Winde, and J. Wery.** 2009. TrgI, toluene repressed gene I, a novel gene involved in toluene-tolerance in Pseudomonas putida S12. Extremophiles **13:**283-297.
142. **Volkers, R. J. M., A. L. de Jong, A. G. Hulst, B. L. M. van Baar, J. A. M. de Bont, and J. Wery.** 2006. Chemostat-based proteomic analysis of toluene-affected Pseudomonas putida S12. Environ Microbiol **8:**1674-1679.
143. **von Wallbrunn, A., H. J. Heipieper, and F. Meinhardt.** 2002. Cis/trans isomerisation of unsaturated fatty acids in a cardiolipin synthase knock-out mutant of *Pseudomonas putida* P8. Applied microbiology and biotechnology **60:**179-185.
144. **von Wallbrunn, A., H. H. Richnow, G. Neumann, F. Meinhardt, and H. J. Heipieper.** 2003. Mechanisms of *cis-trans* isomerization of unsaturated fatty acids in *Pseudomonas putida*. J Bacteriol **185:**1730-1733.
145. **Weber, F. J., and J. A. M. de Bont.** 1996. Adaptation mechanisms of microorgansms to the toxic effects of organic solvents on membranes. Biochim Biophys Ac **1286:**228-245.
146. **Weber, F. J., L. P. Ooijkaas, R. M. Schemen, S. Hartmans, and J. A. M. de Bont.** 1993. Adaptation of Pseudomonas putida S12 to high concentrations of styrene and other organic solvents. Applied and environmental microbiology **59:**3502-3504.
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., 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. Applied microbiology and biotechnology **54:**180-185.
149. **Westermeier, R., and R. Marouga.** 2005. Protein detection methods in proteomics research. Biosci Rep **25:**19-32.
150. **Wierckx, J. P. N., H. Ballerstedt, J. A. M. de Bont, J. H. de Winde, H. Ruijssenaars, and J. Wery.** 2008. Transcriptome analysis of a phenol-producing *Pseudomonas putida* S12 construct: genetic and physiological basis for improved production. J Bacteriol **190:**2822-2830.
151. **Wierckx, J. P. N., J. A. M. de Bont, and J. Wery.** 2005. Engineering of solvent-tolerant *Pseudomonas putida* S12 for bioproduction of phenol from glucose. Applied and environmental microbiology **71:**8221-8227.
152. **Wierckx, N. J., H. Ballerstedt, J. A. M. de Bont, J. H. de Winde, H. J. Ruijssenaars, and J. Wery.** 2008. Transcriptome analysis of a phenol-producing Pseudomonas putida S12 construct: genetic and physiological basis for improved production. J Bacteriol **190:**2822-2830.
153. **Wijte, D., A. L. de Jong, M. A. Mol, B. L. van Baar, and A. J. Heck.** 2006. ProteomIQ blue, a potent post-stain for the visualization and subsequent mass spectrometry based identification of fluorescent stained proteins on 2D-gels. Journal of proteome research **5:**2033-2038.
154. **Wohlbrand, L., B. Kallerhoff, D. Lange, P. Hufnagel, J. Thiermann, R. Reinhardt, and R. Rabus.** 2007. Functional proteomic view of metabolic regulation in "Aromatoleum aromaticum" strain EbN1. Proteomics **7:**2222-2239.
155. **Wu, J., N. Zhang, A. Hayes, K. Panoutsopoulou, and S. G. Oliver.** 2004. Global analysis of nutrient control of gene expression in Saccharomyces cerevisiae during growth and starvation. Proceedings of the National Academy of Sciences of the United States of America **101:**3148-3153.
156. **Zahir, Z., K. D. Seed, and J. J. Dennis.** 2006. Isolation and characterization of novel organic solvent-tolerant bacteria. Extremophiles **10:**129-138.
157. **Zhang, Y.** 2008. I-TASSER server for protein 3D structure prediction. BMC Bioinformatics **9:**40.
158. **Zhang, Y.** 2009. I-TASSER: fully automated protein structure prediction in CASP8. Proteins **77 Suppl 9:**100-113.
159. **Zhang, Y. M., and C. O. Rock.** 2008. Membrane lipid homeostasis in bacteria. Nature reviews **6:**222-233

Summary

Systems analysis of solvent tolerance mechanisms in *Pseudomonas putida* S12

Importance of energy metabolism and the functional identification of the TrgI regulator

Hydrophobic organic solvents, like benzene and toluene, are mainly toxic for bacteria because of their accumulation in the membranes of the cells. The accumulation of solvent molecules in a membrane lowers its rigidity and increases its fluidity and permeability, resulting in an increased rate of cell lysis. Moreover, the functioning of proteins and enzymes embedded in the membranes is negatively affected. In addition, accumulation of solvents causes dissipation of the proton motive force (PMF) as well as a decrease in the energy status of the cell.

In 1990 the isolation of the remarkably solvent tolerant bacterium *Pseudomonas putida* S12 was reported. Research into the solvent tolerance properties of *P. putida* S12 initially focused on the fatty acid composition of the membranes and how it is influenced by the cell. Later, it was discovered that *P. putida* S12 is able to actively extrude solvent molecules from the cell by an energy-dependent efflux system that was named Srp, for solvent resistance pump. Furthermore, an insertion sequence named IS*S12* was found to play a role in solvent tolerance and a relationship between flagella and solvent tolerance was established.

All of the above mentioned mechanisms were elucidated by a traditional reductionist approach. This approach does not provide insight into specific nor global cellular responses related to solvent stress and the interactive dynamics of solvent tolerance mechanisms upon solvent exposure. Systems-level analysis techniques such as transcriptomics and proteomics are very promising tools for elucidating these aspects of solvent tolerance mechanisms.

A 2D-DIGE proteomics study of S12 cultured in the presence of 3 mM (sub-lethal) and 5 mM (lethal to non-solvent tolerant bacteria) toluene in chemostats showed that proteins involved in the energy metabolism of the cell play an important role in solvent tolerance (**Chapter 2**). Five enzymes that are part of the citric acid cycle appeared to be more abundant in toluene-exposed cells than in non-exposed cells. Moreover, other energy-household related proteins showed differential abundances, according to the theory of a higher

need for energy in solvent-exposed cells. Among those was AtpF, which is part of the ATP synthase complex, which had a lower abundance in the presence of toluene. Other interesting findings comprised two outer membrane proteins, OprF and OprH, of which the latter one showed the most dramatic increase in abundance. OprH was hypothesised to have a function in positive support of membrane stabilisation. A hypothetical protein, PP3611, appeared to be less abundant in the presence of toluene.

The expression of the corresponding gene *PP3611* was accordingly shown to be decreased in the presence of toluene as well, in a subsequent transcriptomics experiment (**Chapter 3**). The gene was renamed *trgI* (*toluene repressed gene I*) and knock-out and over-expression mutants were constructed. Analysis of these *trgI* mutants confirmed the correlation of this gene with solvent tolerance. qPCR analysis led to the hypothesis that *trgI* plays an important role in the first defence against solvents since its expression decreased immediately after addition of toluene. The knock-out mutant S12ΔTrgI added to this hypothesis with its increased survival frequency after a 1% toluene shock. The mutant also seemed to exhibit improved lysis resistance as well as a rounded cell morphology and an altered resistance to antibiotics after addition of toluene, suggesting an additional membrane-related function for TrgI. Other results of the transcriptomics analysis of S12 cultured in the presence of toluene comprised differential expression of genes involved in cellular energy-household, as well as genes relating to membrane-associated functions and the outer cell structure. As expected, *srpABCRS*, encoding the solvent efflux pump and its regulatory genes, was up-regulated. In addition, several flagella- and pili-associated genes were differentially expressed.

The function of *trgI* was further investigated by sudden exposure of S12 and S12ΔTrgI to 5 mM toluene in batch cultures, and following global expression between 1 and 30 minutes after addition of toluene (**Chapter 4**). The global transcriptome response revealed large differences between wildtype and *trgI* deletion mutant. The timing of the overall transcriptional response was delayed in *P. putida* S12ΔTrgI. Genes belonging to specific functional groups, *i.e.* energy production and conversion, amino acid transport and metabolism, lipid metabolism and posttranslational modification, protein turnover and chaperones, some of which have an established relationship with solvent tolerance, were not overrepresented in the *trgI* deletion mutant after sudden toluene exposure. Specific groups of genes overrepresented amongst the *trgI*-dependent toluene responsive genes were the same as the groups mentioned above, except for posttranslational modification, protein turnover and chaperones. Moreover, the genes belonging to these groups were consistently overexpressed in the *trgI* deletion mutant. Hence, in the presence of solvent

trgI affected a large group of genes with a large diversity in functions. Analysis of the presumed tertiary structure of the protein TrgI suggested an involvement in regulation of gene expression, which in the light of the broad effects of deletion of the corresponding gene, appears very likely.

An improved benzene-tolerant mutant of *P. putida* S12 was obtained by using laboratory evolution (**Chapter 5**). S12 was cultured in increasing concentrations of benzene in LB medium for a period of two months, resulting in strain *P. putida* S12.49 that tolerates up to 24 mM benzene (also see **Chapter 6**). This strain was cultured in chemostats with and without benzene and global gene expression and protein abundances were compared to those of the wildtype S12 strain. The solvent efflux pump SrpABC was constitutively expressed in S12.49, which appeared to be caused by the insertion of the transposable element IS*S12* in the regulatory gene *srpS*. Since SrpABC is energy-dependent, constitutive expression together with the dissipation of the proton motive force caused by accumulation of benzene molecules in the cell membranes, should hypothetically lead to enhanced expression of genes and proteins related to energy metabolism. Unexpectedly, an overall downregulation of terminal cytochrome *c* oxidases was observed in S12.49. The additional downregulation of genes involved in the arginine deiminase pathway and the fact that S12.49 still has the wildtype level of benzene tolerance after addition of the energy-uncoupling protonophore CCCP further supported the proposition that S12.49 harbours alternative mechanisms to generate and maintain a proton gradient, or is dramatically more efficient in doing so than the wild-type strain.

The research presented in this thesis clearly showed that indeed systems-level analysis techniques such as transcriptomics and proteomics are very useful in identifying the global cellular responses of *P. putida* S12 to toxic organic solvents (**Chapter 6**). The thus identified genes can be further investigated using a more classic and targeted approach. This was done for *trgI* that was hypothesised to be involved in the first line of defence against solvents. Analysis of a knock-out mutant of this gene showed its large influence on gene expression in the presence of solvents. The possible function of the corresponding protein was therefore proposed to be that of a regulator of gene expression. This regulatory function is likely to be influenced by organic solvents.

The solvent efflux pump SrpABC has long been considered to constitute the most important mechanism of solvent tolerance in S12. In this thesis it is clearly shown that the intrinsic flexibility of the energy generating mechanisms is at least as important. Without this flexibility, S12 would not be able to make full use of the efflux pump. The combination and interplay of the features that

make *P. putida* S12 solvent tolerant would not have been revealed without the use of transcriptomics and proteomics analyses.

Samenvatting

Systeemanalyse van de oplosmiddeltolerante bacterie *Pseudomonas putida* S12

Importantie van het energiemetabolisme en de functionele identificatie van de TrgI regulator

Hydrofobe organische oplosmiddelen, zoals benzeen en tolueen, zijn vooral giftig voor bacteriën omdat ze ophopen in de membranen van de cel. Deze ophoping veroorzaakt een vermindering van de stevigheid en een verhoging van de vloeibaarheid en doorlaatbaarheid van de membranen, met als resultaat een verhoogde mate van cellysis. Ook het functioneren van de eiwitten en enzymen die zich in de membranen bevinden wordt negatief beïnvloed. Daarnaast vinden dissipatie van de protonengradiënt en een verlaging van de energiestatus van de cel plaats.

In 1990 werd de isolatie van de opvallend oplosmiddelresistente bacterie *Pseudomonas putida* S12 beschreven. Het onderzoek naar de resistentie-eigenschappen van *P. putida* S12 richtte zich in eerste instantie op de vetzuursamenstelling van de membranen en op hoe deze wordt beïnvloed door de cel. Later werd ontdekt dat *P. putida* S12 in staat is om actief oplosmiddelmoleculen uit de cel te pompen met behulp van een energie-afhankelijke pomp genaamd Srp (*solvent resistance pump)*. Verder werd gevonden dat een insertiesequentie genaamd IS*S12* een rol speelt bij oplosmiddeltolerantie en dat er een relatie bestaat tussen flagellen en oplosmiddeltolerantie.

Alle hierboven genoemde eigenschappen werden ontdekt door gebruik te maken van traditionele reductionistische onderzoeksmethoden. Deze methoden gaven en geven echter geen inzicht in de algemene cellulaire responsen die gerelateerd zijn aan oplosmiddelstress en in de interacties tussen de verschillende resistentiemechanismen na blootstelling aan oplosmiddelen. Technieken waarmee systeembrede analyses gedaan kunnen worden, zoals transcriptomics en proteomics, lijken zeer geschikt te zijn om juist deze eigenschappen van oplosmiddeltolerantie te onderzoeken.

Een 2D-DIGE proteomics studie van S12 gekweekt in chemostaten in aanwezigheid van 3 mM (sub-lethaal) en 5 mM (lethaal voor niet-tolerante bacteriën) tolueen liet zien dat eiwitten die betrokken zijn bij het energiemetabolisme van de cel een belangrijke rol spelen bij oplosmiddeltolerantie (**Hoofdstuk 2**). Vijf enzymen die deel uitmaken van de

citroenzuurcyclus bleken in grotere mate aanwezig te zijn in aan tolueen blootgestelde cellen dan in niet aan tolueen blootgestelde cellen. Daarnaast werden variërende hoeveelheden van energiehuishoudingseiwitten gevonden, wat overeenkomt met de theorie dat aan oplosmiddelen blootgestelde cellen meer energie nodig hebben. Een van deze eiwitten was AtpF, een deel van het ATP-synthase complex, dat in aanwezigheid van tolueen in duidelijk kleinere hoeveelheden voorkwam. Ander interessante bevindingen waren de variërende hoeveelheden van twee buitenmembraan eiwitten, OprF en OprH, waarbij de laatste de meest verhoogde hoeveelheid liet zien. De aanname is dat OprH een rol speelt in het ondersteunen van de membraanstabiliteit. Een hypothetisch eiwit, PP3611, bleek in een kleinere hoeveelheid aanwezig te zijn in cellen die in contact stonden met tolueen dan in cellen waarbij dat niet zo was.

De expressie van het corresponderende gen *PP3611* was ook lager in de aanwezigheid van tolueen, zoals bleek uit transcriptomics experimenten (**Hoofdstuk 3**). Het gen kreeg de nieuwe naam *trgI* (*toluene repressed gene I*) en deletie- en overexpressiemutanten werden geconstrueerd. Analyse van deze mutanten bevestigde de relatie van het gen met oplosmiddeltolerantie. Resultaten van een qPCR analyse leidden tot de hypothese dat *trgI* een belangrijke rol speelt tijdens de eerste momenten van verdediging tegen oplosmiddelen omdat het expressieniveau van het gen onmiddellijk na toevoeging van tolueen sterk omlaag ging. Deze hypothese werd bevestigd door de verhoogde overlevingsfrequentie van de deletiemutant S12ΔTrgI na een 1% tolueen schok. De mutant bleek ook een waarschijnlijk verhoogde lysisresistentie te hebben, naast een rondere celvorm na toevoeging van tolueen en een veranderde antibioticaresistentie. De hypothese over de functie van *trgI* werd daarom uitgebreid met het hebben van een functie die te maken heeft met de membranen van S12. Andere resultaten van de transcriptomics analyse van S12 gekweekt in de aanwezigheid van tolueen bestonden uit de differentiële expressie van genen die betrokken zijn bij de energiehuishouding van de cellen en differentiële expressie van genen die te maken hebben met aan de membranen en buitenste celstructuren gerelateerde functies. Zoals verwacht kwamen *srpABCRS*, de genen die coderen voor de oplosmiddelpomp en de bijbehorende regulerende genen, verhoogd tot expressie. Daarnaast bleken ook verschillende aan flagellen en pili gerelateerde genen differentieel tot expressie te komen.

De functie van *trgI* werd verder onderzocht in een experiment waarin S12 en S12ΔTrgI plotseling werden blootgesteld aan 5 mM tolueen in batch cultures. De genexpressie van beide stammen werd gevolgd tussen 1 en 30 minuten na toevoeging van tolueen (**Hoofdstuk 4**). De algemene analyse van de transcriptionele respons op tolueenblootstelling liet grote verschillen zien

tussen wildtype *P. putida* S12 en de *trgI* deletiemutant. De timing van de algemene transcriptionele respons bleek vertraagd te zijn in *P. putida* S12ΔTrgI. Ook was de overrepresentatie van tot verschillende functionele groepen behorende genen (energieproductie en omzetting; aminozuurtransport en metabolisme; verzuurmetabolisme; posttranslationele modificatie, eiwitomzetting en chaperones), waarvan sommigen een bewezen rol spelen bij oplosmiddeltolerantie, afwezig in de *trgI* deletiemutant tijdens blootstelling aan tolueen. De expressieprofielen van alle genen werd voor beide stammen vergeleken. De eerste drie bovengenoemde specifieke groepen van genen bleken overgerepresenteerd te zijn binnen de groep van door *trgI* beïnvloedde en op tolueen reagerende genen. Verder bleken de genen binnen deze groepen vooral intrinsiek tot overexpressie te komen in de deletiemutant vergeleken met wildtype *P. putida* S12. In conclusie heeft *trgI* in aanwezigheid van oplosmiddelen een effect op de expressie van een groot aantal genen met zeer diverse functies. Analyse van de tertiaire structuur van het eiwit TrgI laat zien dat het misschien betrokken is bij de regulatie van genexpressie wat gezien de grootte van het effect van de deletie van het gen *trgI*, een zeer waarschijnlijke hypothese is.

Met behulp van laboratoriumevolutie werd een mutant van *P. putida* S12 verkregen die een verhoogde tolerantie voor benzeen heeft (**Hoofdstuk 5**). S12 werd gekweekt in oplopende concentraties benzeen in LB medium gedurende bijna twee maanden, resulterend in stam *P. putida* S12.49 die benzeenconcentraties tot 24 mM tolereert (zie ook **Hoofdstuk 6**). Deze stam werd vervolgens gekweekt in chemostaten met en zonder benzeen en de algemene genexpressie en eiwithoeveelheden werden vergeleken met die van de wildtype S12 stam. De oplosmiddelpomp SrpABC bleek constitutief tot expressie te komen in S12.49, wat werd veroorzaakt door de insertie van het transposon IS*S12* in de regulator *srpS*. Omdat SrpABC van energie afhankelijk is zou deze constitutieve expressie, samen met de door ophoping van benzeenmoleculen in de celmembranen veroorzaakte dissipatie van de protonengradiënt, hypothetisch gezien leiden tot verhoging van de expressie van genen en de hoeveelheid van eiwitten die gerelateerd zijn aan het energiemetabolisme. Onverwacht werd echter een algemene afname van expressie van terminale cytochroom *c* oxidase genen waargenomen in S12.49. Daarnaast werd een afname van de expressie van genen die betrokken zijn bij de arginine deiminase route waargenomen en bleek dat S12.49 het niveau van benzeen tolerantie van het wildtype behoudt na toevoeging van de energie-ontkoppelaar CCCP. Deze waarnemingen ondersteunden allemaal de aanname dat S12.49 alternatieve mechanismen bezit om een protonengradiënt aan te

leggen en te onderhouden, of dat S12.49 zeer veel efficiënter opereert met betrekking tot deze eigenschappen dan wildtype S12.

Het onderzoek beschreven in dit proefschrift laat duidelijk zien dat de systeem-brede analyse technieken transcriptomics en proteomics inderdaad goed bruikbaar zijn voor het volgen van de algemene cellulaire responsen van *P. putida* S12 op giftige organische oplosmiddelen (**Hoofdstuk 6**). De aldus geïdentificeerde genen kunnen dan verder onderzocht worden door gebruik te maken van een meer klassieke en gerichte aanpak. Dit werd gedaan voor *trgI* waarvan vermoed werd dat het betrokken is bij de eerste momenten van verdediging tegen oplosmiddelen. Analyse van een deletiemutant van dit gen liet zien dat het een immens grote invloed heeft op genexpressie in aanwezigheid van oplosmiddelen. Het werd daarom voorgesteld dat de mogelijke functie van het corresponderende eiwit dat van een regulator van genexpressie is. Deze regulerende functie wordt waarschijnlijk beïnvloed door oplosmiddelen.

De oplosmiddelpomp SrpABC wordt sinds lange tijd beschouwd als het meest belangrijke mechanisme van oplosmiddeltolerantie in S12. Uit dit proefschrift blijkt dat de intrinsieke flexibiliteit van de mechanismen die energie genereren nog belangrijker is. Zonder deze flexibiliteit zal S12 niet in staat zijn om optimaal gebruik te maken van de oplosmiddelpomp. De combinatie en het samenspel van de eigenschappen die *P. putida* S12 oplosmiddeltolerant maken zou niet bekend zijn geworden zonder gebruik te maken van transcriptomics en proteomics analyses.

Samenvatting voor leken

Systeemanalyse van de oplosmiddeltolerante bacterie *Pseudomonas putida* S12

Importantie van het energiemetabolisme en de functionele identificatie van de TrgI regulator

Allerlei chemicaliën worden op traditionele wijze uit olie geproduceerd door de chemische industrie. De processen die daarvoor gebruikt worden, leveren voor het milieu schadelijk afval op en bovendien raken de oliereserves in de bodem langzaam maar zeker uitgeput. Het is dus zaak op zoek te gaan naar alternatieve methoden om chemicaliën te vervaardigen. Een van die alternatieven bestaat uit het laten maken van deze stoffen door bacteriën en ander micro-organismen. Micro-organismen hebben geen olie nodig en maken geen schadelijk afval. Ze eten bijvoorbeeld suikers uit plantafval en zijn in staat die suikers om te zetten in allerlei voor ons nuttige stoffen. Een bekend voorbeeld is de productie van alcohol in bier en wijn door gist. Voor de productie van allerlei andere chemicaliën zijn of worden op dit moment processen ontwikkeld waarbij (een gedeelte van) het productieproces wordt overgenomen door bacteriën. Twee bacteriële eigenschappen zijn daarbij van belang. Ten eerste moeten de bacteriën zelf goed bestand zijn tegen het beoogde product, ze mogen er niet door vergiftigd worden. Ten tweede is het van belang dat de bacteriën het proces waarbij de producten uit het groeimedium (de 'soep' waarin de bacteriën, hun voedsel en het product zich bevinden) worden opgezuiverd overleven, zodat ze hergebruikt kunnen worden. Bij de zuivering en winning van het product worden vaak organische oplosmiddelen gebruikt. Kennis over de manieren waarop bacteriën met dit soort oplosmiddelen omgaan, hoe ze zich ertegen wapenen, is belangrijk om efficiënte productieprocessen te kunnen ontwikkelen. Bestaande productie-organismen kunnen met deze kennis genetisch aangepast worden zodat ze beter tegen giftige producten of extractiemethoden kunnen. Omgekeerd kunnen organismen die die eigenschappen al bezitten aangepast worden tot efficiënte producenten.

Dit proefschrift gaat over de mechanismen die de bacterie *Pseudomonas putida* S12 gebruikt om te kunnen overleven in de aanwezigheid van giftige organische oplosmiddelen. Organische oplosmiddelen zijn vloeistoffen die voor het grootste deel bestaan uit koolstofatomen en waterstofatomen. Sommige van deze oplosmiddelen, bijvoorbeeld benzeen en

tolueen, zijn waterafstotend (hydrofoob) en lossen dus slecht op in water. Ze lossen echter wel goed op in vettige stoffen. De buitenkant van bacteriën, het celmembraan, bestaat uit een vettige stof. De moleculen van hydrofobe organische oplosmiddelen nestelen zich daarom graag in de celmembranen van bacteriën (zie Figuur 1.1 in Hoofdstuk 1). Dit maakt ze om een aantal redenen giftig. In de celmembranen bevinden zich allerlei eiwitten en enzymen die de bacterie nodig heeft om zijn omgeving te verkennen, om te communiceren met andere bacteriën en om bijvoorbeeld voedingsstoffen binnen te halen of afvalstoffen uit te scheiden. Voor de werking van deze enzymen en eiwitten is een goede inbedding in het celmembraan belangrijk. Oplosmiddelmoleculen verstoren die goede inbedding. Als de hoeveelheid oplosmiddelmoleculen groot wordt, vallen er gaten in de membranen. Hierdoor kunnen allerlei stoffen (goede en slechte) vrijelijk in en uit de cel stromen. Als de gaten groot genoeg worden, zullen de membranen hun stevigheid verliezen en valt de bacterie uit elkaar. Dat wordt lyseren genoemd, de bacterie gaat dan dood.

Pseudomonas putida S12 is relatief ongevoelig voor de giftige werking van organische oplosmiddelen, hij heeft een hoge mate van oplosmiddeltolerantie. Toen men het onderzoek naar de mechanismen van oplosmiddeltolerantie begon, in de jaren 1990, was de aandacht vooral gericht op de celmembranen. *P. putida* S12 bleek de membranen op verschillende manieren te kunnen veranderen en verstevigen. In een steviger membraan kunnen minder oplosmiddelmoleculen zitten en het zal ook minder snel uit elkaar vallen. Kort nadat dit onderzoek gestart was, kwam men achter het bestaan van een moleculaire pomp die *P. putida* S12 naar behoefte aan kan leggen in de membranen en waarmee hij oplosmiddelmoleculen naar buiten kan pompen. Deze pomp heet SrpABC. Als de bacterie merkt dat er oplosmiddelen aanwezig zijn, zal hij, afhankelijk van de hoeveelheid daarvan, een aantal exemplaren van SrpABC in de membranen aanleggen. De aanleg en vervolgens het gebruik van de pompen kost energie. Het veranderen van de membranen kost ook energie. Voor andere processen, zoals celdeling, is daardoor minder energie beschikbaar. Aan oplosmiddelen blootgestelde *P. putida* S12 cellen groeien dan ook minder snel dan niet-blootgestelde cellen.

Bovenstaande mechanismen van oplosmiddeltolerantie werden ontdekt door gericht op zoek te gaan naar de genen en eiwitten die het verstevigen van het membraan bewerkstelligen en door het bestuderen van mutanten met een afwijkende oplosmiddeltolerantie. Een mutant met een verlaagde oplosmiddeltolerantie bleek bijvoorbeeld een defect te hebben in een van de genen die verantwoordelijk zijn voor de opbouw van SrpABC, waardoor men op het spoor van deze pomp kwam.

De gerichte aanpak van het oplosmiddeltolerantie-onderzoek heeft veel nieuwe inzichten opgeleverd, maar niet hoe de verschillende mechanismen zich tot elkaar verhouden en of er nog andere mechanismen een rol spelen. Om hier meer over te weten te komen zou een overzicht van alles wat er zich in de cellen afspeelt zeer bruikbaar zijn. Er zijn verschillende technieken beschikbaar om zo'n overzicht te maken, waarvan er twee zijn gebruikt bij het onderzoek dat in dit proefschrift beschreven wordt: transcriptomics en proteomics.

De genen die in ieder organisme op het DNA liggen kunnen uit of aan staan. Als ze uit staan, zal het eiwit waarvoor het gen codeert niet gemaakt worden. Als ze aan staan zal dat wel gebeuren. Als een gen aan staat zegt men dat het gen tot expressie komt. De mate van expressie kan variëren, afhankelijk van de omstandigheden waarin het organisme zich bevindt. Met transcriptomics kan de mate van expressie van (bijna) alle genen in een organisme gemeten worden. De hoeveelheid eiwit of enzym die vervolgens gemaakt wordt, kan gemeten worden met proteomics. Het is niet zo dat de ene techniek de andere overbodig maakt omdat de relatie tussen de expressie van een gen en de hoeveelheid geproduceerd eiwit niet rechtevenredig is. De hoeveelheid actieve eiwitten en enzymen hangt van meer af dan van de genexpressie alleen, het wordt bijvoorbeeld ook beïnvloed door andere eiwitten en enzymen.

Proteomics is gebruikt bij het onderzoek dat beschreven staat in Hoofdstuk 2. Hierbij is *P. putida* S12 een aantal dagen lang gekweekt in aan- en afwezigheid van het oplosmiddel tolueen en zijn de hoeveelheden van zoveel mogelijk eiwitten onder beide omstandigheden met elkaar vergeleken. Wat onder andere opviel was dat een aantal eiwitten welke te maken hebben met de productie van energie in verschillende hoeveelheden aanwezig waren. In *P. putida* S12 cellen die in aanraking waren gekomen met tolueen waren meer van die eiwitten aanwezig. Dat betekent hoogstwaarschijnlijk dat die cellen meer energie produceren dan cellen die niet in aanraking komen met tolueen. Hiermee is bevestigd dat de verdediging tegen oplosmiddelen energie kost. Een andere vondst betrof de hoeveelheid van twee eiwitten die zich bevinden in de celmembranen van *P. putida* S12. Van het ene eiwit (OprF) wordt gedacht dat het tolueenmoleculen de cel binnenlaat, het andere eiwit (OprH) zorgt voor stabilisatie van de membranen. De hoeveelheid OprF is lager in aan tolueen blootgestelde cellen, zodat er waarschijnlijk minder tolueen in de cel terecht komt. De hoeveelheid OprH is juist hoger in die cellen, zodat de membranen steviger zijn. Als laatste viel op dat in de aan tolueen blootgestelde cellen een relatief kleine hoeveelheid aanwezig was van een eiwit waarvan de functie niet bekend is (een 'hypothetisch' eiwit).

In Hoofdstuk 3 is het experiment met een aantal dagen lang in aan- en afwezigheid van tolueen gekweekte cellen herhaald, nu om de genexpressie te vergelijken. Hiervoor is transcriptomics gebruikt. De genen die coderen voor de oplosmiddelpomp SrpABC bleken aan te staan in aanwezigheid van tolueen, precies zoals verwacht. Ook werd een verhoogde expressie gevonden van allerlei genen die te maken hebben met energieproductie. Daarnaast bleken genen die te maken hebben met de opslag van suikers (het voedsel van de bacterie) verlaagd tot expressie te komen. Dat betekent dat de cellen in aanwezigheid van tolueen alle suiker direct nodig hebben om energie te maken, ze kunnen het zich niet permitteren om het op te slaan. De meest opvallende vinding was de verlaagde expressie van het gen dat codeert voor het hypothetische eiwit dat in Hoofdstuk 2 was gevonden. Het verschil in expressie met niet aan tolueen blootgestelde cellen was erg groot, wat een goede reden was om eens in meer detail naar dit gen te kijken. Uit een volgend experiment waarbij plotseling tolueen aan de cellen werd toegevoegd bleek dat ze de expressie van het gen onmiddellijk daarna zeer snel omlaag brachten. Blijkbaar was het voor *P. putida* S12 gunstig om het gen op een laag pitje te zetten. Om te bewijzen dat het gen echt van invloed is op oplosmiddeltolerantie werd een mutant gemaakt waarin het gen, dat inmiddels de naam *trgI* had gekregen, uit was geschakeld. De mutant, *P. putida* S12ΔTrgI, bleek minder snel dood te gaan aan tolueenvergiftiging dan de oorspronkelijke *P. putida* S12. Daarmee is bewezen dat *trgI* inderdaad een rol speelt bij oplosmiddeltolerantie. De snelle verlaging van de genexpressie na toevoegen van tolueen leidde tot de hypothese dat *trgI* een rol speelt bij de eerste verdediging tegen oplosmiddelen.

Het onderzoek dat beschreven staat in de Hoofdstukken 2 en 3 is gedaan met *P. putida* S12 cellen die gedurende een paar dagen gekweekt waren in aanwezigheid van tolueen. Deze cellen waren kortom gewend geraakt aan tolueen, waardoor de mechanismen van oplosmiddeltolerantie volop in werking waren. In Hoofdstuk 4 is vervolgens gekeken naar wat er gebeurd in cellen die plotseling te maken krijgen met tolueen. De genexpressie in deze cellen is gedurende het eerste halve uur na toevoeging van tolueen gevolgd met behulp van transcriptomics. Dat dit interessante gegevens op zou kunnen leveren was al gebleken uit het feit dat de expressie van *trgI* zeer snel omlaag ging na toevoeging van tolueen (zie hierboven). De mutant waarin *trgI* is uitgeschakeld, *P. putida* S12ΔTrgI, werd op dezelfde manier gevolgd om meer te weten te komen over de functie van *trgI*. Het aantal genen dat in een andere (hogere of lagere) mate tot expressie kwam in *P. putida* S12ΔTrgI ten opzichte van *P. putida* S12 was erg groot. Dat gold voor het moment vlak voor het toevoegen van tolueen en voor het halve uur erna. Hieruit kon geconcludeerd worden dat *trgI* een grote invloed heeft op de expressie van veel andere genen.

Ook bleek wederom dat genen die een rol spelen bij de productie van energie in *P. putida* S12 reageren op tolueen. Het uitschakelen van *trgI* zorgde ervoor dat een aantal van deze genen al voor de toevoeging van tolueen een andere expressie hadden. In feite zijn deze cellen alvast voorbereid op de aanwezigheid van tolueen. Andere opvallende zaken hadden te maken met bescherming tegen de effecten die oplosmiddelen hebben op bijvoorbeeld eiwitvouwing. Eiwitten bestaan uit lange ketens van aminozuren en ze worden pas actief als ze op een bepaalde manier zijn opgevouwen. Oplosmiddelen kunnen de eiwitvouwing verstoren. Een aantal genen welke te maken hebben met het weer goed vouwen van eiwitten na verstoring kwam hoger tot expressie in *P. putida* S12 na blootstelling aan tolueen. In *P. putida* S12ΔTrgI was de expressie zelfs nog hoger. Het bleek ook dat de genen die verantwoordelijk zijn voor SrpABC, de oplosmiddelpomp, veel sneller hoog tot expressie komen in de mutant zonder *trgI* dan in de oorspronkelijke *P. putida* S12. Twee moleculaire pompen die lijken op SrpABC, maar die voor zover bekend geen tolueen transporteren, reageerden op dezelfde manier in *P. putida* S12 en *P. putida* S12ΔTrgI, waaruit de voorzichtige conclusie getrokken kan worden dat *trgI* van invloed is op de mate van expressie van deze pompen.

Hoofdstuk 5 begint met de beschrijving van het maken van een mutant van *P. putida* S12 die nog beter tegen oplosmiddelen kan. Mutanten kunnen gemaakt worden door genen uit te schakelen of juist harder aan te zetten, zoals hierboven beschreven. Het is echter ook mogelijk om bacteriën te dwingen zich te evolueren richting een gewenste eigenschap. Om mutanten te verkrijgen die extra goed tegen oplosmiddelen kunnen is niets anders nodig dan ze steeds een beetje meer oplosmiddel geven. In de loop van twee maanden (zie Figuur 5.1 in Hoofdstuk 5) werd aan dezelfde bacterie populatie behalve vers voedsel, ook iedere keer een iets hogere concentratie benzeen gegeven. De aldus ontstane mutant, genaamd *P. putida* S12.49, was in staat te leven in groeimedium met 1,67 maal zoveel benzeen als waartegen *P. putida* S12 zelf kan. Andere verrassende eigenschappen van de mutant waren de lagere groeisnelheid in afwezigheid van benzeen en de verlaagde resistentie tegen diverse antibiotica ten opzichte van *P. putida* S12. Transcriptomics en proteomics werden gebruikt om de genexpressie en de eiwithoeveelheden in *P. putida* S12.49 te vergelijken met die van *P. putida* S12. Een van de meest opvallende observaties was de zeer hoge expressie van de genen die coderen voor de oplosmiddelpomp SrpABC, in *P. putida* S12.49 wanneer die gekweekt werd zonder benzeen in het medium. In de oorspronkelijke *P. putida* S12 komen die genen pas tot expressie als er oplosmiddel aanwezig is en niet al daarvoor. Na toevoegen van benzeen ging de expressie van deze genen in *P. putida* S12.49 niet verder omhoog. Na verder onderzoek bleek dat een van de genen die verantwoordelijk is voor de

regulatie van de genexpressie van de pomp uitgeschakeld was door een zogenaamd insertie element. Zo'n element kan door het DNA 'springen' en als het middenin een gen terechtkomt, zal het gen z'n werk niet meer kunnen doen. Naast deze vondst bleken ook genen betrokken bij de celmembranen en bij de opname en de opslag van koolstofverbindingen (voedsel) anders tot expressie te komen in de mutant dan in *P. putida* S12. Diverse genen die met de productie van energie te maken hebben kwamen ook op verrassend andere wijze tot expressie in de mutant. Sommigen bleken veel minder actief te zijn in *P. putida* S12.49 dan in *P. putida* S12 zelf, terwijl de verwachting was dat dat juist tegenovergesteld zou zijn omdat de strijd tegen oplosmiddelen veel energie kost (zie eerder in dit hoofdstuk). De belangrijkste conclusie van het onderzoek in Hoofdstuk 5 was dan ook dat *P. putida* S12 zeer flexibel met zijn energieproductie systemen om kan gaan en dat hij misschien wel systemen bezit waar we nog niet van afweten of dat hij bestaande systemen op een alternatieve manier gebruikt.

In het laatste hoofdstuk van dit proefschrift, Hoofdstuk 6, worden een aantal zaken uit de voorgaande hoofdstukken nader bediscussieerd. De belangrijkste discussiepunten betreffen de functie van het gen *trgI* en de vraag waarom *P. putida* S12 zo goed tegen oplosmiddelen kan. Uit de transcriptomics analyse van de mutant waarin *trgI* uitstaat, *P. putida* S12ΔTrgI, bleek dat het gen waarschijnlijk invloed heeft op de expressie van een groot aantal genen. Een gen met die invloed heet een regulator. Een computersimulatie van de manier waarop het eiwit TrgI gevouwen zou kunnen zijn en een vergelijking van die vouwing met de vouwing van andere eiwitten leverde meer bewijs op voor deze stelling. Het bleek echter ook dat TrgI een cruciaal onderdeel mist, waardoor het niet op zichzelf actief kan zijn als regulator. Samenwerking met een ander eiwit ligt dus voor de hand. Het is te verwachten dat het gen dat codeert voor dit andere eiwit zich hetzelfde gedraagt als *trgI* als er tolueen aan de bacteriecultuur wordt toegevoegd. Nadere bestudering van de transcriptomics resultaten leverde drie kandidaten op voor samenwerking met TrgI. Er zijn een aantal andere bacteriesoorten die een gen bevatten dat op *trgI* lijkt. Geen van deze soorten is zo goed bestand tegen oplosmiddelen als *P. putida* S12. Er is ook een soort bekend met een oplosmiddelpomp die vergelijkbaar is met SrpABC. Deze soort is ook niet zo goed bestand tegen oplosmiddelen als *P. putida* S12. De flexibiliteit van het productiesysteem voor energie in *P. putida* S12 zorgt ervoor dat hij de oplosmiddelpomp en andere energie kostende tolerantiemechanismen ten volle kan benutten. De hoge tolerantie voor oplosmiddelen van *P. putida* S12 wordt kortom veroorzaakt door een combinatie van eigenschappen die tot nu toe niet in andere bacteriën is gevonden.

Wageningen, september 2012

Beste allemaal,

Eindelijk komt er dan toch een einde aan een 'kleurrijke' periode. Een periode die om precies te zijn negen jaar, acht maanden en zes dagen duurde, want op 1 maart 2003 begon ik als aio bij TNO in Apeldoorn en op 6 november 2012 zal ik hopelijk promoveren. Eigenlijk begon het al in 2002, in december had ik een sollicitatiegesprek met Peter Letitre, Jan de Bont en naar ik meen ook Jan Wery (of was dat tijdens het tweede gesprek, ergens in januari? Het is alweer zo lang geleden...). Deze drie heren ben ik als eerste dank verschuldigd, zij hebben mij tenslotte aangenomen als aio, Peter, Jan en Jan bedankt daarvoor.

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Rita

Curriculum vitae

Rita Volkers was born on March 11, 1977 in Hoorn, The Netherlands. In 1989 she started her secondary education at R.S.G. Wiringherlant in Wieringerwerf, where she received her athenaeum diploma in 1995. In that same year she started her study of Chemistry at the University of Amsterdam (UvA). At the end of the second year she decided to specialise in biochemistry and microbiology. Her first internship was performed at the UvA, where she did experiments with the photoactive yellow protein of the bacterium *Rhodobacter sphaeroides*. After that, she went to Plant Research International (PRI) in Wageningen to do her second internship. During this internship she worked with microarrays for the first time, in a project concerning the production of astaxanthin in the unicellular algae *Chlorella zofingiensis*. In 2000, she received her MSc degree in Biomolecular Chemistry, after which she worked as a technician in various projects at PRI for half a year. In 2002 she got another job as a technician at PRI, this time in a project about the spreading of the weed wild lettuce in Europe. Her PhD research began in 2003 at the Dutch Institute for Applied Scientific Research (TNO) in Apeldoorn. In 2007, a few months after her department moved to the Kluyver Laboratory of the Technical University of Delft her PhD-contract finished. She continued to work at TNO with *P. putida* S12 as a researcher for two more years. Since April 2010 she is working as a postdoc researcher in the Laboratory of Nematology at Wageningen University and Research Centre.

Publications

Bhaganna, P. R.J.M. Volkers, A.N.W. Bell, K. Kluge, D.J. Timson, J.W. McGrath, H.J. Ruijssenaars, and J.E. Hallsworth. 2010. Hydrophobic substances induce water stress in microbial cells. Microbial biotechnology **3**(6):701-716

Verhoef, S., H. Ballerstedt, R.J.M. Volkers, J.H. de Winde and H.J. Ruijssenaars. 2010. Comparative transcriptomics and proteomics of p-hydroxybenzoate producing *Pseudomonas putida* S12: novel responses and implications for strain improvement. Applied microbiology and biotechnology **87**(2):679-690

Volkers, R.J.M., H. Ballerstedt, J.H. de Winde, and H.J. Ruijssenaars. 2010. Isolation and genetic characterization of an improved benzene-tolerant mutant of *Pseudomonas putida* S12. Environmental microbiology reports **3**:456-460

Volkers R.J.M., H. Ballerstedt, H.J. Ruijssenaars, J.A.M. de Bont, J.H. de Winde, and J. Wery. 2009. *TrgI*, toluene repressed gene I, a novel gene involved in toluene-tolerance in *Pseudomonas putida* S12. Extremophiles **13**(2):283-297

Ballerstedt H., R.J.M. Volkers, A.E. Mars, J.E. Hallsworth, V.A.M. Martins dos Santos, J. Puchalka, J. van Duuren, G. Eggink, K.N. Timmis, J.A.M. de Bont, and J. Wery. 2007. Genomotyping of *Pseudomonas putida* S12 using a *P. putida* KT2440-based high-density DNA microarrays: implications for transcriptomics studies. Applied microbiology and biotechnology **75**(5):1133-1142

Volkers R.J.M., A.L. de Jong, A.G. Hulst, B.L.M. van Baar, J.A.M. de Bont, and J. Wery. 2006. Chemostat-based proteomic analysis of toluene-affected *Pseudomonas putida* S12. Environmental microbiology **8**(9):1674-1679