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ORIGINAL PAPER

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

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Abstract *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 *t*oluene-*r*epressed *g*ene, *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.

Keywords *Pseudomonas putida* S12 · Solvent-tolerance · Toluene · *trgI* · Transcriptomics

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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 (Isken and de Bont 1998; Kieboom and de Bont 2000; Ramos et al. 2002). Organic solvents accumulate in lipid membranes of living cells, causing an increase of the membrane fluidity and a decrease of bilayer stability (Sikkema et al. 1995; Weber and de Bont 1996). 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 (Sikkema et al. 1995). Three types of membrane-associated solvent tolerance mechanisms have been elucidated (Heipieper and de Bont 1994; Weber and de Bont 1996; Ramos et al. 1997; Isken and de Bont 1998; Kieboom and de Bont 2000; Ramos et al. 2002): (1) cytoplasmic membrane changes, (2) outer membrane changes, and (3) 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 (Volkers et al. 2006). This and another recent proteomics study on *P. putida* DOT-T1E (Segura et al. 2005) 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 (Hayashi et al. 2003; Fujita et al. 2004; Matsui et al. 2006). In yeast, the genes involved in isooctane tolerance were found to be constitutively expressed rather than being induced by the solvent (Matsui et al. 2006). Fujita et al. (2004) 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 (Hayashi et al. 2003). 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. (2006) 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 (Griffin et al. 2002; Mostertz et al. 2004; Brown et al. 2006; Budde et al. 2006).

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 (Volkers et al. 2006). Our finding that the hypothetical protein PP3611 was down-regulated (Volkers et al. 2006) 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.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are shown in Table 1. *P. putida* S12 was originally isolated as a styrene utilising bacterium (Hartmans et al. 1989). 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 (Nijkamp et al. 2007) and pJNNmcs(t) (Nijkamp et al. 2005). The vectors and the PCR-amplified *trgI* were restricted with *Not*I and *Eco*RI. Ligation of *trgI* into the vectors yielded plasmids pJTTtrgI and pJNNtrgI(t). *P. putida* S12 was transformed with pJTTtrgI, resulting in *P. putida* S12ptrgI. The knockout mutant *P. putida* S12ΔtrgI was constructed as follows: oligonucleotides 5′-CGCGGATCCGCTGCACGCACCCATCC-3′ (forward), 5′-CGCTCTAGAGAAAGCGACTGAAGAGTCC-3′ (reverse), 5′-CGCTCTAGACGTGTCACGTAGATGTCG-3′ (forward) and 5′-CGCGCGGCCGCCGACACGCTCGATGTTGG-3′ (reverse) were designed on the published sequence of *P. putida* KT2440 (GenBank accession no. NC_002947) including restriction sites (*Bam*HI, *Xba*I, *Xba*I and *Not*I, respectively) 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 (Quandt and Hynes 1993). 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. Oligonucleotides used for construction of S12ΔpyrR were 5′-CCGGGATCCGCTGACCACGTCACAGG-3′ (forward), 5′-GGCTCTAGACCTTCGACCTCGAACGG-3′ (reverse), 5′-CCGTCTAGAAACTGTTCGATTACGGCCG-3′ (forward) and 5′-GGCGCGGCCGCGATGGCGTCAGGCTTGG-3′ (reverse). Oligonucleotides used for construction of *P. putida* S12Δat were 5′-GCGTCTAGATCGGCGATGAAGGCCGCGAC-3′ (forward), 5′-G

Table 1 Bacterial strains and plasmids used in this study

	Characteristics	Source or reference
Strains		
P. putida S12	Wild type	Hartmans et al. (1990)
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	Nijkamp et al. (2007)
pJNNmcs(t)	Expression vector with inducible promoter nagAa	Wierckx et al. (2008)
pJQ200SK	Suicide vector, Gm^R	Quandt and Hynes (1993)
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

CGGCGGCCGCGCCGCCAGCCTACTGTGTGG-3′ (reverse), 5′-GCGGGATCCGATGTAGTCCGACCAGTTATAG-3′ (forward) and 5′-GCGTCTAGAAAGGTGAAGGAGATCCTCGCC-3′ (reverse).

Culture conditions

Mineral salts medium (MM) (Hartmans et al. 1989) 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 30°C; *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) (Sambrook et al. 1982) 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, IL, 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 min 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 h 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, NJ, USA) with a working volume of 1.0 l, 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 five 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. Ten microliter of an exponentially growing culture was added to 90 µl of LB medium supplemented with antibiotic. Growth was assessed by visual inspection after 1, 2 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 (Wierckx et al. 2008). In brief, 1-ml culture samples were quenched in 1 ml ice-cold methanol, centrifuged and 1 ml RNAlater (Ambion, Foster City, CA, 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, CA, 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 (Ballerstedt et al. 2007; Wierckx et al. 2008). 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, 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 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, UK). Glucose, gluconate, 2-keto-gluconate and ammonium concentrations were determined as described previously (Nijkamp et al. 2005). 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 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 a NanoDrop-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). All primers (Table 2) were purchased from Eurofins MWG Operon (Ebersberg, Germany). Targets for q-RT-PCR and qPCR were the genes and corresponding mRNAs

Table 2 Primers used for qPCR analysis

Target gene	Primer	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	

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 2) 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, CA, 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 (55–95°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 tenfold 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 10–10^7 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.

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, e.g. *Saccharomyces cerevisiae* (Boer et al. 2003; Wu et al. 2004) and *E. coli* (Hua et al. 2004). 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 (Kieboom et al. 1998a). The transcriptomes were analysed using custom Affymetrix high-density microarrays (Ballerstedt et al. 2007).

The effect of the culture conditions on global gene expression was determined by principal component analysis (PCA) (Fig. 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 (Volkers et al. 2006), the decrease in the biomass yield in the presence of toluene (data not shown) was comparable to earlier findings (Isken

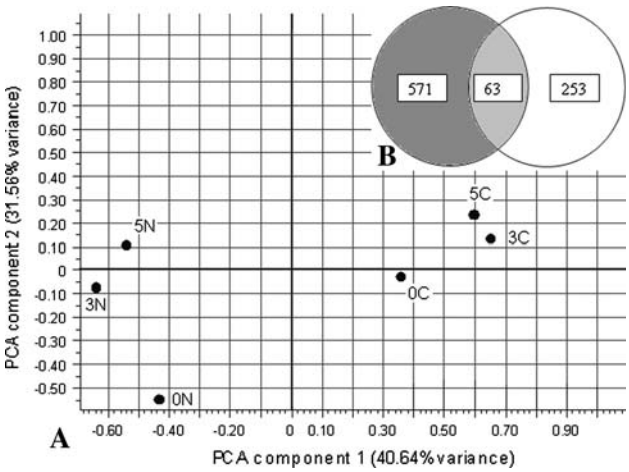

Fig. 1 **a** Principle component analysis (*PCA*) of global gene expression profiles on the following conditions: mineral salts medium with carbon limitation (*C*); mineral salts medium with nitrogen limitation (*N*); 0 mM toluene, 3 mM toluene, 5 mM toluene (0, 3, 5). Values are the average of two microarrays). **b** Venn diagram of 2-way ANOVA results. *Dark grey* 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

et al. 1999). 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, 4 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 (Fig. 1b). The genes differentially expressed as a result of a nutrient limitation-dependent response on toluene formed a group of 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 (Fig. 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 and 4 the genes in the most relevant functional categories, based on a previously conducted proteomics experiment, are shown (Volkers et al. 2006). 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 (S1). Genes are sorted by predicted function and their probable relation with solvent tolerance.

The defence against solvents is highly energy demanding (Isken et al. 1999; Segura et al. 2005; Volkers et al. 2006). 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 (Volkers et al. 2006). 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 (Kobayashi et al. 1999). 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 (Volkers et al. 2006). 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.

Table 3 Genes that are up-regulated in *P. putida* S12 in the presence of 3 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
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
Membrane					
PP1036		Periplasmic binding domain transglycosylase SLT domain fusion protein	1.46	1.58	N
PP1871	HtpX[h]	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

Table 3 continued

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]
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

[a] Locus tag based on genome of *P. putida* KT2440 (http://www.pseudomonas.com)

[b] NCBI database no

[c] Ratio of expression in 3 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 (Dominguez-Cuevas et al. 2006)

[g] Refers to (Volkers et al. 2006)

[h] General stress response gene

nih.gov/BLAST/) indicated that TrgI is also present in *P. putida* F1 (99% identity), *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 31% identity. SignalP in SMART (Schultz et al. 1998; Letunic et al. 2006) (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), 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 (Alekshun and Levy 1999).

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 (Kieboom et al. 1998a) that *srpB* is maximally expressed up to several hours after toluene exposure and can therefore be considered a late response. Toluene (5 mM) was 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 2 minutes upon addition of toluene (Fig. 2). At that time point, the transcription of *srpB* started to increase, reaching a maximum after 20 min.

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 wild-type 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 (Fig. 3).

Table 4 Genes that are down-regulated in *P. putida* S12 in the presence of 3 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 peptide-binding protein	0.3	0.23	N
PP0885		Dipeptide ABC transporter, periplasmic peptide-binding protein	0.36	0.27	C[f]
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

Table 4 continued

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]
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[h]	Cold-shock protein CspD	0.34	0.35	N

[a] Locus tag based on genome of *P. putida* KT2440 (http://www.pseudomonas.com)

[b] NCBI database no

[c] Ratio of expression in 3 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 (Dominguez-Cuevas et al. 2006)

[g] Refers to (Volkers et al. 2006)

[h] General stress response gene

The above-mentioned strains were also subjected to a 30-min 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.

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 5). *P. putida* S12ΔtrgI showed an increased initial level of resistance against the β-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.

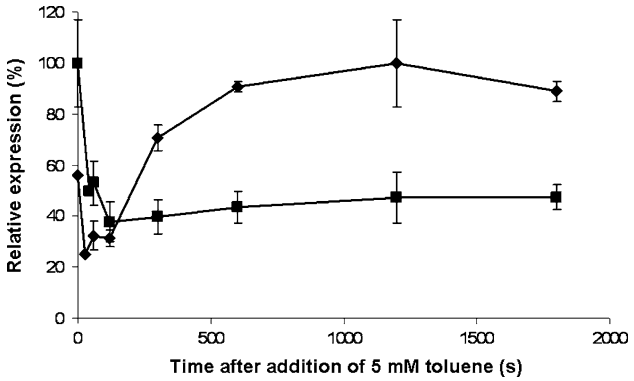

Fig. 2 Relative expression in percentage of the maximum expression of: (*squares*) *trgI* and (*diamonds*) *srpB* after addition of 5 mM toluene to an exponentially growing culture of *P. putida* S12, as determined by qPCR. Values are the average of duplicate experiments (*error bars* represent deviation 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

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 salt medium on glucose or fructose. As the functioning of transport proteins (Bernal et al. 2007) and membrane protein topology (Bogdanov et al. 2002) 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.

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 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-

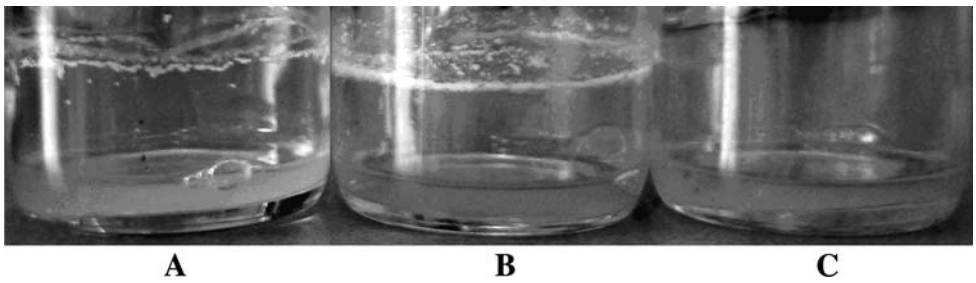

Fig. 3 **a** *Pseudomonas putida* S12, **b** *P. putida* S12ptrgI, and **c** *P. putida* S12ΔtrgI after 24 h of culturing in the presence of 3 mM toluene. Debris of dead cells is deposited onto the wall of the bottles in the cultures of *P. putida* S12 and *P. putida* S12ptrgI

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 (Heipieper and de Bont 1994): 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.

Table 5 MIC's (mg/L; see "Materials and methods") after 1 day of exposure to various antibiotics of *P. putida* S12, *P. putida* S12ΔtrgI and *P. putida* S12ptrgI

	P. putida S12	*P. putida* S12ΔtrgI	*P. putida* S12ptrgI
Ampicillin	500	1,000	ND
Carbenicillin	500	1,000	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	2,000	1,000	2,000
Gentamycin	7.8	3.9	ND
Kanamycin	3.9	3.9	3.9

Values are the average of two experiments. *ND* not determined because strain possesses tetracyclin or β-lactam resistance gene

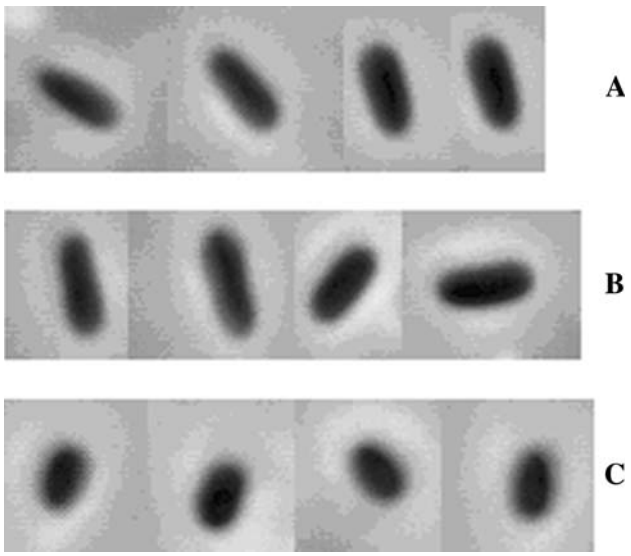

Fig. 4 Typical cells from stationary-phase cultures in the absence of toluene of **a** *P. putida* S12, **b** *P. putida* S12ptrgI, **c** *P. putida* S12ΔtrgI. Phase-contrast, ×100 magnification, Leitz Aristoplan microscope, Leica DC500 camera

Discussion

In a previous study, the solvent tolerance responses of *P. putida* S12 were studied on the proteomic level (Volkers et al. 2006). 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 (Hegde et al. 2003), 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: (1) responses that can be directly connected to (known) solvent tolerance mechanisms; (2) responses related to the increased energy demand brought about by the solvent; (3) 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* (Boer et al. 2003; Fujita et al. 2004) and *E. coli* (Hua et al. 2004). 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. (2005) 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. (2001) that SrpS and SrpR are repressors of *srpABC*. However, if SrpR, in analogy to the *ttgGHI* regulator TtgV in *P. putida* DOT-T1E (Fujita et al. 2004) (96% identical to SrpR), 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)) (Mostertz et al. 2004), 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 (Neumann et al. 2005), 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 (Isken et al. 1999; Volkers et al. 2006): 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 tenfold 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 channelled 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 (Bogdanov et al. 2002; Bernal et al. 2007).

In agreement with 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 (Volkers et al. 2006). 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 (Kieboom et al. 2001; Segura et al. 2001; Dominguez-Cuevas et al. 2006). 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 (Kieboom et al. 2001; Ramos et al. 2002). 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 down-regulation 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 min (Neumann et al. 2005), 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 2 h after toluene exposure (Kieboom et al. 1998b). 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 altered cell morphology and 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 (Hartmans et al. 1989; Segura et al. 2005) 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.

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