Solvent tolerance mechanisms in Pseudomonas putida

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CHAPTER 7

Discussion

Pseudomonas putida as a robust microbial host for the production of high-value chemicals

The transition from a fossil-fuel to a bio-based economy is necessary to tackle the current environmental issues and ensure the sustainable economic growth. Current high demands in the production of value added products (e.g. food, energy, high-value chemicals) from renewable biological resources needs to be followed by system innovations in biotechnology fields. Recent developments in microbial physiology and metabolic engineering enable the production of biobased chemicals to become economically competitive with the petrochemical-based production (1–4).

Industrial microbial hosts need to fulfill a number of key performance indicators; for instance straightforward handling, reproducible production behavior, and more importantly, natural robustness to bioprocessing conditions (5). *Pseudomonas putida* meets these criteria due to its fast growth, high biomass yields, and low maintenance demands (6). This bacterium lacks a functional Embden-Meyerhof-Parnas (EMP) pathway and therefore, glycolysis occurs via Entner-Doudoroff (ED) pathway (7). Indeed, 90% of the consumed sugar is converted into gluconate and enters central carbon metabolism as 6-phosphogluconate (ED pathway) (8). However, 10% of the triose phosphates were found to be recycled back to form hexose phosphates, which evidently, merges ED, EMP, and pentose phosphate (PP) pathways into an EDEMP pathway (8). As the result, this bacterium exhibits an overflow metabolism which results in a surplus of ATP production and high NAD(P)H regeneration rates (8, 9). In addition, *P. putida* has been successfully engineered for efficient utilization of alternative carbon sources (e.g. D-xylose and L-arabinose), underlining its versatile metabolic constitution (10).

The remarkable solvent tolerance trait of *P. putida* S12 offers a greater degree of freedom in bioprocess development for aromatic chemicals (11). Therefore, *P. putida* S12 has been exploited for bioproduction of various aromatic chemicals, e.g. phenol, p-hydroxybenzoate, p-hydroxystyrene, and FDCA (4, 12–14). In this chapter, the genetic interplay of the advantageous solvent tolerance trait in *P. putida* S12 will be discussed.

Immediate and adaptive responses are required to circumvent solvent toxicity

Organic solvents ($LogP_{o/w}$ 1-4) may directly diffuse through the membrane or enter the cyto-

plasmic compartment through membrane porins and subsequently accumulate in bacterial membrane (Fig. 7.1). This accumulation leads to the increase of fluidity and disruption of the membrane. Damaged macromolecules and cellular components upon exposure to organic solvent may elicit a variety of cellular responses (15). Such responses can be divided into immediate and adaptive responses (15, 16). Immediate responses are a first line defence to solvent stress which are induced very rapidly or within minutes following the addition of organ-ic solvent. While such responses ensure the survival to solvent-shock, but they may not be sufficient to support long term growth in the presence of solvent. For this, adaptive responses ultimately take over after hours or generations of solvent exposure in bacterial cultures. In this chapter, the time frame of various cellular responses toward solvent stress in *P. putida* S12 will be addressed.

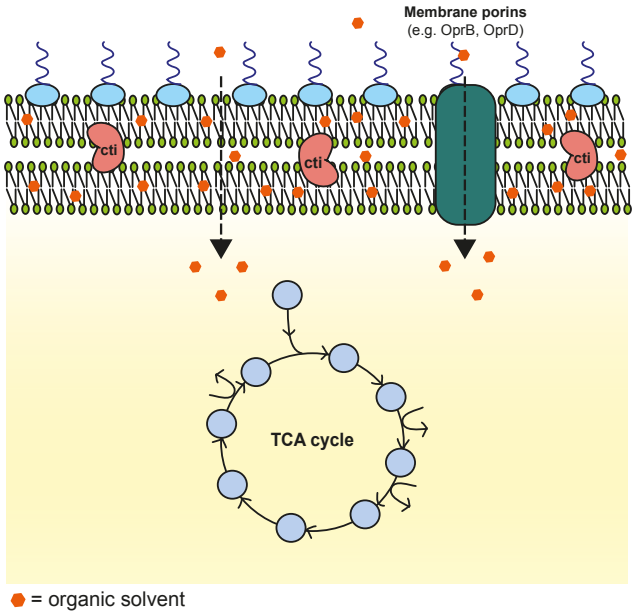

Fig. 7.1. Illustration of bacterial cells experiencing solvent stress.

Solvent tolerance mechanisms in *Pseudomonas putida* S12

Membrane lipid bilayer

Membrane compaction is important for the tolerance of *P. putida* S12 against organic solvents. By compacting the membrane, *P. putida* S12 reduces the internalization of organic solvent into the cells. As extensively discussed by Rühl and colleagues, trans-unsaturated

fatty acid concentrations were increased in *P. putida* S12 treated with 1-butanol, reducing the "kink" caused by the cis-conformation of unsaturated fatty acid (17). However, previous transcriptomic studies (18, 19) nor the transcriptomic data in this thesis (Chapter 5) did not show an increase in the expression of cis-trans isomerase (cti) during elevated temperature and solvent stress. Eberlein and colleagues argued that cti is constitutively expressed in a sufficient amount and its activity is controlled by the change of membrane fluidity as an immediate response to stress (20). A recent model of cti's mode of action by Eberlein and colleagues illustrate that in the presence of organic solvent, the membrane bilayer becomes more fluid and thus allow cti to access the membrane and change the cis-unsaturated fatty acid into its trans-conformation (Fig. 7.1, Ref. (20)). Introduction of cti into *E. coli*, a non-solvent-tolerant microbial host, significantly improved its solvent tolerance towards n-butanol (21).

In addition to cti activity, an increase of phosphatidylethanolamine (PE) and cardiolipin (CL) head groups was observed as a response to sudden 1-butanol addition at the expense of phosphatidylglycerol (PG) in *P. putida* S12 (17). However, variation of headgroup species was not observed when *P. putida* S12 was previously adapted to the presence of 1-butanol. Similar to cti activity, headgroup shift may play a role as an immediate response to stabilize bacterial membrane in the presence of organic solvent.

Bioenergetics and redox balance

During solvent stress, tricarboxylic acid (TCA) cycle components are upregulated, the NAD(P) H/NAD(P)$^+$ ratio is increased, but cell growth is typically reduced (Fig. 7.2, Ref. (16, 22–25)). Upregulation of the TCA cycle and subsequent increase of the NAD(P)H/NAD(P)$^+$ ratio enable the cells to cope with the demand of active solvent extrusion by the efflux pumps in maintaining proton motive force. When plasmid-cured *P. putida* S12 was adapted to high toluene concen-tration (ALE-derived strains), we observed the downregulation of F0F1 ATP synthase follow-ing the mutations found in the intergenic regions and subunits of this gene cluster (Chapter 5). Apparently, the demand of solvent extrusion pump requires the repression of other membrane proteins which are also energized by H^+ influx. On the other hand, respiratory proteins such as succinate dehydrogenase and cytochrome C oxidase were upregulated, presumably as an attempt to maintain redox balance and intracellular pH (Chapter 5, Ref. (24)). This remarkable metabolic flexibility allows *P. putida* S12 to survive the addition of high toluene concentration, even in the absence of its megaplasmid containing its main solvent pump.

Membrane proteins

P. putida S12, like other *P. putida* strains, contains multiple **r**esistance, **n**odulation, and cell-**d**ivision (RND) efflux pumps encoded on its chromosome and megaplasmid. Among these pumps, SrpABC, a homologue to TtgGHI of *P. putida* DOT-T1E and *Pseudomonas taiwanensis* VLB120, is the main solvent extrusion pump. SrpABC expression can be induced by organic solvents, which in turn will be extruded by this pump (26, 27). The *srpRSABC* operon is encoded on pTTS12 megaplasmid, along with a 3-phenylpropionate degradation gene cluster adjacent to it (Chapter 3). Identical arrangement of the *srpRSABC* operon and the 3-phenylpropionate degradation gene cluster was also found in *P. taiwanensis* VLB120, suggesting that these gene clusters were recently disseminated, presumably through horizontal gene transfer.

In *P. putida* S12, another RND efflux pump, ArpABC (homologue to TtgABC *P. putida* DOT-T1E and MexAB-OprM in *P. aeruginosa* (28, 29)), plays a role as a secondary extrusion pump to organic solvents. Notably, the role of this extrusion pump was initially unclear in *P. putida* S12 since megaplasmid removal or *srpABC* deletion would render the strain to be non-solvent-tolerant (Chapter 4) (30, 31). Kieboom and colleagues hypothesized that ArpABC may have a lower affinity towards organic solvent compared to SrpABC (31). Further analysis on the pumps efficiency and specific affinity to organic solvent however, remains of interest.

In the ALE-derived strains (Chapter 5), we observed that mutations occurred at the *arpR* locus which encodes for the repressor to ArpABC efflux pump. These mutations subsequently caused moderate upregulation of *arpBC* (*ttgBC*) loci. However, to achieve tolerance to a high toluene concentration, upregulation of this pump was not sufficient. Downregulation of other membrane proteins which are energized by H^+ influx, such as flagellar assembly, F0F1 ATP synthase, and transporters, is also appears necessary. Indeed, downregulation of membrane proteins, such as flagellar assembly gene clusters, was also reported by Molina and colleagues in their study regarding the response of *P. putida* strains towards solvent stress (22). This may be to accommodate the demand of ArpABC pump on proton motive force and due to the spatial restriction of the membrane surface in which these proteins are embedded.

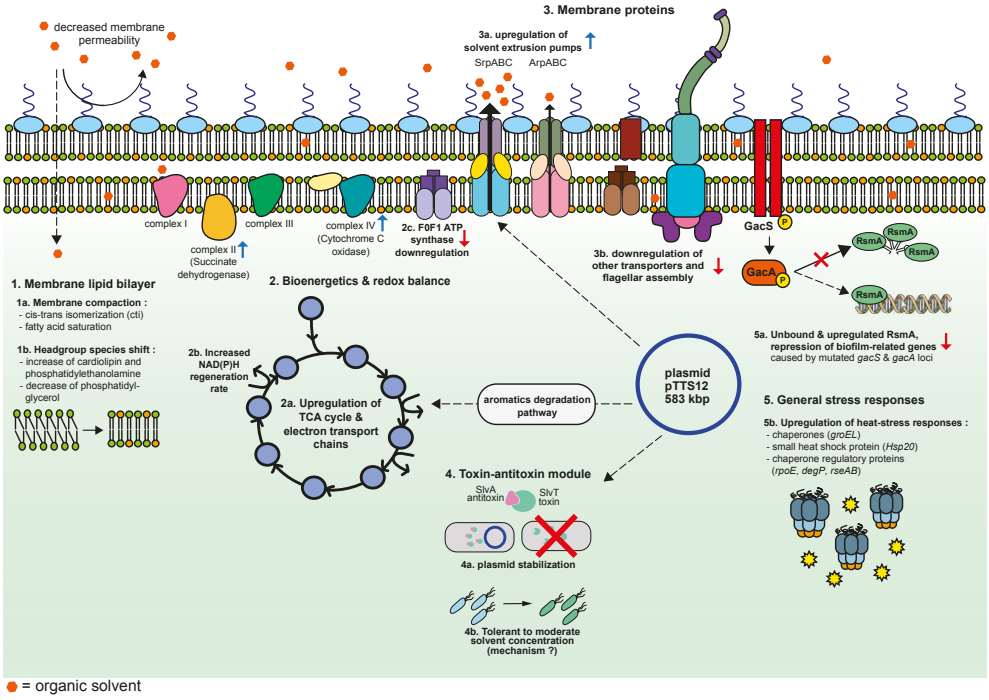

Fig. 7.2. Schematic representation of the solvent tolerance mechanism in *P. putida* S12

Toxin-antitoxin module

Bacterial toxin-antitoxins (TA) are ubiquitous systems that can be found encoded on bacterial chromosomes and plasmids; their role has been a controversial subject in recent years. TA modules have been reported to be important for the bacterial defence mechanisms, for instance in antibiotic resistance, bacterial persister formation, and more recently, as a predecessor to CRISPR-Cas systems as a phage defence system (32–35). However, Rosendahl and colleagues argued that most of the chromosomal TA systems do not demonstrate any clear benefit to their host and that they are maintained due to their low fitness cost (36).

In Chapter 4, a novel SlvT-SlvA TA module was identified to play a role in solvent stress response of *P. putida* S12. The polycistronic SlvT-SlvA TA mRNA was upregulated in transcriptomic data of *P. putida* S12 growing in the presence of toluene (Chapter 5 and Ref. (16)). Further scrutiny on this TA module revealed a role in stabilizing and maintaining the pTTS12 megaplasmid, especially during solvent stress when this plasmid may cause metabolic burden to *P. putida* S12 (Fig. 7.2). Moreover, chromosomal introduction of the SlvT-SlvA TA module in the plasmid-cured *P. putida* and *E. coli* strains slightly improved solvent

tolerance in these strains. This TA system is likely to act in the immediate response towards solvent stress by stalling cell growth. This would allow the bacteria to quickly adapt to its environment. Several proteases were found to be upregulated during solvent stress (16), that potentially may degrade SlvA antitoxin and subsequently release the SlvT toxin in its active form. Once active, SlvT toxin degrades NAD^+, thus affecting intracellular cofactor levels causing cell growth to be stalled.

General stress response and biofilm formation

One of the main stress responses in *P. putida* is the formation of biofilm. Biofilm formation occurs as a series of highly regulated steps: attachment, microcolony formation, maturation and dispersal (37). Reversible apical attachment of bacterial cells occurs to a surface upon initial contact, followed by irreversible lateral interaction. These attached cells rapidly multiplied into clonal microcolonies which then produce the biofilm matrix, consisting of exopolysaccharide (EPS), extracellular DNA and proteins (38). However, it was unclear whether biofilm formation is actually beneficial for constituting a high solvent tolerance phenotype. In addition, unintentional biofilm formation maybe disadvantageous in a fermenter set-up as previously described (39). Adherent cell layers in bioreactors may cause a negative effect on mass, energy and momentum transfer; creating an atypical econiche within the bioreactor; and inaccurate culture stoichiometric and kinetic parameters estimation (39).

The formation of biofilm in *P. putida* is regulated by the GacS/GacA two component system (37). Upon sensing the environmental signal, GacS becomes phosphorylated, which in turn, causes GacA to also become phosphorylated. GacA phosphorylation stimulates production of the small RNAs RsmZ and RsmY, which bind to the RsmA protein, releasing the repression of the biofilm matrix, quorum sensing signalling, and Type VI secretion system gene clusters (37). Both *gacS* and *gacA* loci were truncated or mutated in our ALE-derived strains with enhanced solvent tolerance (Chapter 5). In addition, RsmA was upregulated, probably unbound to RsmZ or RsmY, due to the disruption of *gacS* or mutated *gacA* loci. As the result, a significant reduction of biofilm formation was observed in our ALE-derived strains (Fig. 7.2). Reverse engineering of the key mutations revealed that after deletion of the *gacS* locus, solvent tolerance and growth parameters were generally improved (Chapter 5) while no biofilm formation occurred during solvent stress. It appears that in *P. putida* S12, it is essential to escape biofilm-forming tendency to adapt to high solvent concentration.

Several stress-related proteins were differentially expressed during solvent stress in *P. putida* S12 (Fig. 7.2). A previous chemostat-based proteomic study reported upregulation of heat shock protein GroEL caused by the addition of 5 mM toluene (24). In the ALE-derived strains, upregulation of another heat shock protein, Hsp20 (RPPX_17155), was observed (Chapter 5). Moreover, constitutive upregulation of the putative sigma factor E (RpoE), anti-sigma factor RseAB, and DegP, known to regulate chaperone proteins expression in *E. coli* (40), were observed in ALE-derived strains. Upregulation of several heat shock proteins and chaperones indicates the involvement of general stress responses, that are similar between heat and solvent stress. Heat stress primarily inactivate microbial activity through general protein unfolding. Thermophile bacteria respond to this stress by expressing chaperones and heat shock proteins to aid protein refolding (41–43). Similarly, solvent stress causes the release of membrane-bound proteins due to the membrane disruption (44). Thus, similar responses (e.g. chaperones and heat shock proteins upregulation) can be observed between thermophile and solvent-tolerant bacteria, aimed at refolding denatured proteins.

Regulating solvent tolerance mechanisms

Due to the high energy demand and potential toxicity of RND efflux pumps (45), such systems are typically tightly regulated. The SrpABC efflux pump is regulated by a pair of repressor and antirepressor, SrpS and SrpR respectively (46). The repression imposed by SrpS binding to the promoter region of srpABC operon can be subjugated by organic solvent binding to SrpS, or binding of SrpS to its antirepressor SrpR. The ArpABC efflux pump is regulated by a single repressor system, ArpR, which is a member of the TetR family regulators (31). Crystal structure analysis of TtgR (homologue of ArpR regulator in *P. putida* DOT-T1E) indicates the occurrence of two distinct binding sites within its large pocket (47). These binding sites allow the interaction between TtgR and antibiotics, solvents, or toxic plant secondary metabolites which are also extruded by the efflux pump. In addition to these repressor systems, mobile elements have been described to play an important role in regulating solvent efflux pumps. ISS12 and ISPpu21 were reported to disrupt the repressors of the *srpABC* and *arpABC* operons after prolonged exposure to organic solvent, thus enabling constitutive expression of the solvent extrusion pumps (Chapter 5, Ref. (48, 49)). On the other hand, prolonged storage in the absence of organic solvent was reported to cause ISS12 disruption of the *srpA* locus, rendering *P. putida* S12 to be less solvent-tolerant (50).

Other regulatory mechanisms have been described to be involved in the solvent stress response in *P. putida* S12. A putative modulator, TrgI, was found to be immediately repressed upon exposure to organic solvent (51). A knock-out of this gene causes a significant increase in solvent tolerance followed by differential expression of many genes, especially upregulation of the proteins related to the posttranslational modification, protein turnover, and molecular chaperones (16). In Chapter 5, an AraC family transcriptional regulator Afr, was found to be truncated in all of the ALE-derived strains. Afr positively regulates a number of membrane-bound proteins, including the MexEF-OprN antibiotic efflux pump (Chapter 6). Downregulation of TrgI and Afr regulatory proteins in *P. putida* S12 seemed to be important for the rewiring of metabolism during solvent stress.

Conclusion and future outlook

P. putida S12 circumvents the solvent stress through environmentally acquired mechanisms (e.g. solvent extrusion pumps and aromatic degradation pathway) in combination with inherent metabolic flexibility which allows this strain to adapt to the high energy demand of solvent extrusion pumps. While solvent extrusion pumps are indeed the main mechanisms to survive solvent stress, other accessory factors (e.g. membrane compaction, NAD(P)H regeneration rate, general stress responses) need to be taken into account to model and efficiently engineer the solvent tolerance trait in other non-solvent-tolerant microbial strains. Alternative mechanisms for cellular solvent removal, like outer membrane vesicle formation, have been described in other *P. putida* strains (52, 53) but requires further analysis in *P. putida* S12. Moreover, the works in this thesis indicate that there is a difference between SrpABC and ArpABC efflux pump in their ability to extrude solvent molecules. Further scrutiny on this difference; whether it is caused by pump specificity or affinity, membrane localization and other factors, may be beneficial for understanding and constructing a solvent-tolerant microbial cell factory.

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