Solvent tolerance mechanisms in Pseudomonas putida

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CHAPTER 1

Aim and scope of this thesis

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Pseudomonas putida S12 as a solvent-tolerant bacterial strain

Organic solvents are important in biocatalysis as precursors of various high-value chemicals, as end products and as in-situ product extractant (1). However, the usage of organic solvents in biocatalysis is challenging due to their toxicity to microbial cells. Organic solvents can cause the disruption of the bacterial cell membrane and lipid bilayer. Interestingly, some microorganisms can tolerate and assimilate toxic organic solvents in high concentrations. The first reported solvent-tolerant microorganism was *Pseudomonas putida* IH-2000 that can survive in 50% (vol/vol) of toluene (2). Following this discovery, several other solvent-tolerant *P. putida* strains were identified such as strain DOT-T1E and S12 (3, 4).

Pseudomonas putida is a diderm (Gram-negative), rod-shape bacterium belonging to the class Gamma proteobacteria and family Pseudomonadaceae. Several strains of *P. putida* were isolated from soil for utilizing organic solvent as its carbon source, for example strains mt-2 and DOT-T1E for toluene utilization and strain S12 for styrene utilization (3–5). Currently, *P. putida* S12 has been implemented for the production of high-value chemicals like phenol, p-hydroxybenzoate, and p-hydroxystyrene; all of which have solvent-like properties and therefore are generally toxic for most of microbial hosts (6–8). Moreover, this strain can also grow in a two-phase fermentation system using toxic organic solvents, thus making it excellent for industrial application.

Recently, whole genome sequencing has been performed on *P. putida* S12 (9). The genome of *P. putida* S12 consists of 5.8-mega base pairs chromosome (GeneBank Accession number CP009974) and a single copy of 583-kilo base pairs megaplasmid pTTS12 (GeneBank Accession number CP009975). A gene cluster encoding a solvent extrusion pump (*srpRSABC*) was revealed to be located on the plasmid pTTS12. However, the role of this megaplasmid in conferring solvent tolerance to *P. putida* S12 had not been described beyond the solvent extrusion pump SrpABC.

Several research questions are being addressed in this thesis. Initial efforts have been made to transfer genetic traits from solvent-tolerant bacteria to industrial production strains. Can such transfers be extended to more industrial strains? Moreover, will these engineered strains reach similar tolerance levels as native strains in combination with optimal production yields? In native solvent-tolerant strains, multiple efflux pumps operate simultaneously to prevent accumulation of organic solvents. However, simultaneous overexpression of multiple efflux pumps is disadvantageous in engineered strains due to membrane composition

changes and insertion machinery overload (10). How can expression levels be optimized for combinations of pumps operating simultaneously? Moreover, with many omics data becoming available to date, genetic traits responsible for solvent tolerance in different strains can be predicted. How to address the challenge of constructing a solvent tolerance model operating in different species?

Scope and outline of this thesis

This PhD project focuses mainly on the mechanism behind solvent tolerance of *P. putida* S12 and its genome stability. **Chapter 1** gives a general introduction on *P. putida* S12 as a model organism of solvent tolerance as well as its industrial relevance. Here, the gap of knowledge on the mechanisms of solvent tolerance and the outline of this thesis are described.

In **Chapter 2**, current knowledge and understanding of solvent-tolerant bacteria are described. Application and challenges in working with solvent-tolerant bacteria in biocatalysis are discussed. Furthermore, the emerging synthetic biology tools and advance in metabolic engineering regarding the solvent-tolerant bacteria are also discussed.

While *P. putida* S12 (ATCC 700801) is highly tolerant towards various organic solvents due to the presence of solvent extrusion pump (11, 12), it is unknown whether other parts of the megaplasmid plays a role in the solvent tolerance. **Chapter 3** and **Chapter 4** focus on the role of the megaplasmid in solvent tolerance. In **Chapter 3**, comparative analysis revealed that megaplasmid pTTS12 belongs to the incompatibility P-2 (IncP-2) plasmid group. Heavy-metal resistance is the main characteristic of the IncP-2 plasmid group and indeed, pTTS12 contains tellurite, chromate, and mercury resistance cassettes. In addition to the heavy-metal resistance cassettes, plasmid pTTS12 contains the solvent extrusion pump SrpABC, phenylpropionate and styrene-phenylacetate degradation pathways. Further observations on the modular functional build-up of these gene clusters in pTTS12 are described in **Chapter 3**.

In **Chapter 4**, a novel toxin-antitoxin (TA) SlvT-SlvA module is characterized. This TA module was found to be upregulated in the presence of toluene from previous transcriptomic data (13). SlvT-SlvA belongs to the COG5654-COG5642 TA family. Like other members of the COG5654 toxin family, SlvT can deplete cellular NAD^+, rendering the cells to stop growing in the absence of its antitoxin SlvA. The role of the SlvT-SlvA toxin-antitoxin pair in solvent tolerance and maintaining plasmid stability are discussed in **Chapter 4**.

In **Chapter 5**, the intrinsic solvent tolerance of *P. putida* S12 is addressed. Plasmid pTTS12 plays an important role in conferring solvent tolerance to *P. putida* S12. In the absence of this plasmid, *P. putida* S12 lost its ability to survive high concentration of toluene. Adaptive laboratory evolution (ALE) experiments were performed on the plasmid-cured *P. putida* S12 enabling growth on increasing concentrations of toluene. Eventually, evolved strains were able to grow on a high toluene concentration (10% (vol/vol)) even in the absence of megaplasmid pTTS12. Whole-genome and RNA sequencing analysis revealed the genetic interplay which allowed restoration of solvent tolerance in the plasmid-cured strains. Reverse engineering of the key mutations found in ALE experiment successfully reinstate the solvent tolerance trait in plasmid-cured *P. putida* S12, thus confirming intrinsic solvent tolerance of *P. putida* S12.

In **Chapter 6**, an **A**raC **f**amily transcriptional **r**egulator (proposed name Afr) encoded on RPPX_14685 locus is characterized. This locus carried a point mutation which leads to amino acid substitution of threonine (pos. 53) to proline, at its putative effector binding domain. This mutation occurred in all of the independent replicates of plasmid-curing experiments using intercalating agent, mitomycin C. Here, the genes regulated by Afr were identified and the role of Afr in enabling plasmid-curing and the recovery of solvent tolerance in ALE-derived strains are discussed.

Chapter 7 summarizes the interplay of the various mechanisms that contribute to the solvent tolerance in *P. putida* S12. Further research questions that need to be tackled and the trade-offs of the solvent tolerance trait are discussed.

In summary, the work described in this thesis illustrates the solvent tolerance mechanism in an industrially relevant strain of *Pseudomonas*; *P. putida* S12. One of the key challenges in the production of valuable chemicals using microbial cell factory is the unpredictability of yield due to product/intermediate toxicity. Therefore, biocatalysis of high-value chemicals require a robust microbial host, like solvent-tolerant bacteria. A profound analysis and understanding of solvent-tolerant mechanisms is therefore needed. This work may contribute in understanding the genetic and physiological interplay to confer and implement solvent tolerance traits in bacteria.

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