

Solvent tolerance mechanisms in Pseudomonas putida

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Summary

Bacterial biocatalysis constitutes a sustainable alternative for high-value chemicals production by enabling the utilization of renewable feedstocks. However, biobased production of aromatic compounds and biopolymers requires a specialized microbial cell factory. Microbial hosts may experience cell toxicity caused by the solvent-like compounds that emerge as products, substrates or intermediates during the production process. Therefore, solvent tolerance is an essential trait for the microbial hosts used in biobased production of aromatic chemicals and biopolymers. The work described in this thesis focused on identifying and characterizing genes/gene clusters which are involved in conferring solvent tolerance trait in bacteria.

Pseudomonas putida S12 is inherently solvent-tolerant and, therefore, constitutes a promising platform for biobased production of aromatic compounds and biopolymers (overview in **chapter 2**). The genome of *P. putida* S12 consists of a 5.8-Mbp chromosome and a 580-kbp megaplasmid pTTS12. In **chapter 3**, systematic analysis of the genes encoded on pTTS12 revealed that a large fraction of these genes is involved in stress response, increasing survival under harsh conditions like heavy metal and solvent stress. Comparative analysis of pTTS12 provides a thorough insight into its structural and functional build-up. This plasmid is highly stable and carries a complex arrangement of transposable elements containing heavy metal resistance clusters and several aromatic degradation pathways.

In **chapter 4**, we revisit the essential role of pTTS12 for molecular adaptation to solvent stress in *P. putida* S12. In addition to the solvent extrusion pump (SrpABC), we identified a novel toxin-antitoxin module (SIvAT) which contributes to the short-term tolerance in moderate solvent concentrations and to the stability of pTTS12. Indeed, toxin-antitoxin modules have been reported to be involved in survival strategies, such as stress response, biofilm formation, and antimicrobial persistence in *Pseudomonas*. SIvT toxin expression causes NAD⁺ degradation which leads to the arrest of cell division. The expression of SIvA antitoxin immediately restores NAD⁺ levels. This toxin-antitoxin module may act as an immediate response towards solvent stress by stalling cell growth, allowing the bacteria to quickly adapt to their environment. The solvent tolerance gene clusters from pTTS12: *slvAT* and *srp* operon, were successfully expressed in the non-solvent-tolerant strains of *P. putida* and *Escherichia coli* strains to confer and enhance their solvent tolerance.

Removal of pTTS12 caused a significant reduction in the solvent tolerance of *P. putida* S12. In **chapter 5**, we succeeded in restoring solvent tolerance in the plasmid-cured *P. putida* S12 using adaptive laboratory evolution (ALE). We further investigate the intrinsic solvent tolerance of *P. putida* S12 (in the absence of pTTS12). Whole genome sequencing identified several single nucleotide polymorphisms (SNPs) and a mobile element insertion, which allow the solvent-adapted strains to grow in the presence of 10% (vol/vol) toluene. Mutations were identified on an RND efflux pump regulator *arpR*, resulting in a constitutive upregulation of the multifunctional efflux pump ArpABC. Single nucleotide polymorphisms (SNPs) were also found in the intergenic regions and subunits of ATP synthase, RNA polymerase subunit β ', the global two-component regulatory system (*gacA/gacS*), and a putative AraC family transcriptional regulator *afr* loci. Transcriptome analysis further revealed a constitutive down-regulation of proton-influx dependent activities in ALE-derived strains; such as flagellar assembly, F0F1 ATP synthase, and membrane transport proteins.

Our experiments pointed to the importance of the *afr* locus in enabling the plasmid curing and recovery of solvent tolerance. However, the role of this putative transcriptional regulator remained elusive. In **chapter 6**, the role of Afr was further characterized. Transcriptional analysis (RNA-seq) and confirmatory RT-qPCR experiments indicated that Afr positively regulates at least 32 loci. These genes/gene clusters putatively encode for membrane transporters, porins, and dehydrogenases; including the MexEF-OprN multidrug efflux pump known to be involved in active export of several antibiotics. Moreover, the mutation and truncation of Afr changed the antibiotic resistance profile, underscoring the central role of Afr as a stress-response regulator in *P. putida* S12.

The work described in this thesis further enhanced our knowledge and insight into the mechanisms operating in *P. putida* to establish solvent tolerance. Adaptability of *P. putida* S12 is dependent on the ability to cope with the high energy demand of solvent stress. The inherent metabolic flexibility of *P. putida* S12 has partly been developed through horizontally transferred traits, such as aromatic degradation pathways and solvent extrusion pumps.