A novel toxin-antitoxin module SlvT–SlvA regulates megaplasmid stability and incites solvent tolerance in *Pseudomonas putida* S12

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Abstract

*Pseudomonas putida* S12 is highly tolerant towards organic solvents in saturating concentrations, rendering this microorganism suitable for the industrial production of various aromatic compounds. Previous studies revealed that *P. putida* S12 contains a single-copy 583 kbp megaplasmid pTTS12. pTTS12 encodes several important operons and gene clusters facilitating *P. putida* S12 to survive and grow in the presence of toxic compounds or other environmental stresses. We wished to revisit and further scrutinize the role of pTTS12 in conferring solvent tolerance. To this end, we cured the megaplasmid from *P. putida* S12 and conclusively confirmed that the SrpABC efflux pump is the major determinant to solvent tolerance on the megaplasmid pTTS12. In addition, we identified a novel toxin-antitoxin module (proposed gene names *slvT* and *slvA* respectively) encoded on pTTS12 which contributes to the solvent tolerant phenotype and is important in conferring stability to the megaplasmid. Chromosomal introduction of the *srp* operon in combination with *slvAT* gene pair created a solvent tolerance phenotype in non-solvent tolerant strains such as *P. putida* KT2440, *E. coli* TG1, and *E. coli* BL21(DE3).
Importance

Sustainable alternatives for high-value chemicals can be achieved by using renewable feedstocks in bacterial biocatalysis. However, during bioproduction of such chemicals and biopolymers, aromatic compounds that function as products, substrates or intermediates in the production process may exert toxicity to microbial host cells and limit the production yield. Therefore, solvent-tolerance is a highly preferable trait for microbial hosts in the biobased production of aromatic chemicals and biopolymers. In this study, we revisit the essential role of megaplasmid pTTS12 from solvent-tolerant P. putida S12 for molecular adaptation to organic solvent. In addition to the solvent extrusion pump (SrpABC), we identified a novel toxin-antitoxin module (SlvAT) which contributes to short term tolerance in moderate solvent concentrations as well as to stability of pTTS12. These two gene clusters were successfully expressed in non-solvent tolerant strains of P. putida and E. coli strains to confer and enhance solvent tolerance.
39 Introduction

One of the main challenges in the production of aromatic compounds is chemical stress caused by the added substrates, pathway intermediates, or products. These chemicals, often exhibiting characteristics of organic solvents, are toxic to microbial hosts and may negatively impact product yields. They adhere to the cell membranes, alter membrane permeability, and cause membrane damage (1, 2). *Pseudomonas putida* S12 exhibits exceptional solvent tolerance characteristics, enabling this strain to withstand toxic organic solvents in saturating concentrations (3, 4). Consequently, a growing list of valuable compounds has successfully been produced using *P. putida* S12 as a biocatalyst by exploiting its solvent tolerance (5–9).

Following the completion of its full genome sequence and subsequent transcriptome and proteome analyses, several genes have been identified that may play important roles in controlling and maintaining solvent tolerance of *P. putida* S12 (10–12). As previously reported, an important solvent tolerance trait of *P. putida* S12 is conferred through the RND-family efflux pump, SrpABC, which actively removes organic solvent molecules from the cells (13, 14). Initial attempts to heterologously express the SrpABC efflux pump in *E. coli* enabled instigation of solvent-tolerance and production of 1-naphtol (15, 16). Importantly, the SrpABC efflux pump is encoded on the megaplasmid pTTS12 of *P. putida* S12 (12).

The 583 kbp megaplasmid pTTS12 is a stable single-copy plasmid specific to *P. putida* S12 (12). It encodes several important operons and gene clusters enabling *P. putida* S12 to tolerate, resist and survive the presence of various toxic compounds or otherwise harsh environmental conditions. Several examples include the presence of a complete styrene degradation pathway gene cluster, the RND efflux pump specialized for organic solvents (SrpABC) and several gene clusters conferring heavy metal resistance (12, 17, 18). In addition, through analysis using TADB2.0, a toxin-antitoxin database (19, 20), pTTS12 is predicted to contain three toxin-antitoxin modules. Toxin-antitoxin modules recently have been recognized as important determinants of resistance towards various stress conditions, like nutritional stress and exposure to sublethal concentration of chemical stressor.
Toxin-antitoxin modules identified in pTTS12 consist of an uncharacterized RPPX_26255 - RPPX_26260 system and two identical copies of a VapBC system (23). RPPX_26255 and RPPX_26260 belong to a newly characterized type II toxin-antitoxin pair COG5654-COG5642. While toxin-antitoxin systems are known to preserve plasmid stability through post-segregational killing of plasmid-free daughter cells (24), RPPX_26255-RPPX_26260 was also previously shown to be upregulated during organic solvent exposure suggesting its role in solvent tolerance (11).

In this paper, we further address the role of pTTS12 in conferring solvent tolerance of *P. putida* S12. Curing pTTS12 from its host strain might cause a reduction in solvent tolerance, while complementation of the *srp* operon into the cured strain may fully or partially restore solvent tolerance. Furthermore, we wished to identify additional genes or gene clusters encoded on pTTS12 and putative mechanisms that might also play a role in conferring solvent tolerance to *P. putida* and non-solvent tolerant *E. coli*. 

(21, 22).
Results

Megaplasmid pTTS12 is essential for solvent tolerance in *P. putida* S12

To further analyze the role of the megaplasmid of *P. putida* S12 in solvent tolerance, pTTS12 was removed from *P. putida* S12 using mitomycin C. This method was selected due to its reported effectiveness in removing plasmids from *Pseudomonas* sp. (28), although previous attempts regarded plasmids that were significantly smaller in size than pTTS12 (29). After treatment with mitomycin C (10-50 mg L\(^{-1}\)), liquid cultures were plated on M9 minimal media supplemented with indole to select for plasmid-cured colonies. Megaplasmid pTTS12 encodes two key enzymes: Styrene monoxygenase (SMO) and Styrene oxide isomerase (SOI) that are responsible for the formation of indigo coloration from indole. This conversion results in indigo coloration in spot assays for wild-type *P. putida* S12 whereas white colonies are formed in the absence of megaplasmid pTTS12. With the removal of pTTS12, loss of indigo coloration and hence, of indigo conversion was observed in all three plasmid-cured strains and the negative control *P. putida* KT2440 (Figure 1A).

With mitomycin C concentration of 30 mg L\(^{-1}\), 2.4% (3 out of 122) of the obtained colonies appeared to be completely cured from the megaplasmid, underscoring the high genetic stability of the plasmid. No colonies survived the addition of 40 and 50 mg L\(^{-1}\) of mitomycin C, whereas all the colonies that survived the addition of 10 and 20 mg L\(^{-1}\) of mitomycin C retained the megaplasmid. Three independent colonies cured from the megaplasmid were isolated as *P. putida* S12-6, *P. putida* S12-10, and *P. putida* S12-22. Complete loss of the megaplasmid was further confirmed by phenotypic analysis (Figure 1), and by full genome sequencing. Several operons involved in heavy metal resistance were previously reported in the pTTS12 (12). The *terZABCD* operon contributes to tellurite resistance in wild-type *P. putida* S12 with minimum inhibitory concentration (MIC) as high as 200 mg L\(^{-1}\) (Figure 1B). In the megaplasmid-cured strains, severe reduction of tellurite resistance was observed, decreasing the potassium tellurite MIC to 50 mg L\(^{-1}\) (Figure 1B).

Genomic DNA sequencing confirmed complete loss of pTTS12 from *P. putida* strains S12-6, S12-10, and S12-22 without any plasmid-derived fragment being inserted within the chromosome.
and genomic alterations by mitomycin C treatment were minimal. Complementation of pTTS12 into the plasmid-cured *P. putida* S12 strains restored the indole-indigo transformation and high tellurite resistance to the similar level with wild-type strain (Figure S1). Repeated megaplasmid curing experiments indicated that *P. putida* S12 can survive the addition of 30 mg L\(^{-1}\) Mitomycin C with the frequency of 2.48 (± 0.58) x 10\(^{-8}\). Among these survivors, only 2% colony population lost the megaplasmid, confirming the genetic stability of pTSS12. In addition, attempts to cure the plasmid by introducing double-strand breaks as described by Wynands and colleagues (30) were not successful due to the pTTS12 stability.

Growth comparison in solid and liquid culture in the presence of toluene was performed to analyze the effect of megaplasmid curing in constituting solvent tolerance trait of *P. putida* S12. In contrast with wild-type *P. putida* S12, the plasmid-cured strains were unable to grow under toluene atmosphere (data not shown). In liquid LB medium, plasmid-cured *P. putida* S12 strains were able to tolerate 0.15% v/v toluene, whereas the wild-type *P. putida* S12 could grow in the presence of 0.30% v/v toluene (Figure 2). In the megaplasmid-complemented *P. putida* S12-C strains, solvent tolerance was restored to the wild-type level (Figure S1-D). Hence, absence of megaplasmid pTTS12 caused a significant reduction of solvent tolerance in *P. putida* S12.

The SrpABC efflux pump and gene pair RPPX_26255-26260 are the main constituents of solvent tolerance encoded on pTTS12

The significant reduction of solvent-tolerance in plasmid-cured *P. putida* S12 underscored the important role of megaplasmid pTTS12 in solvent-tolerance. Besides encoding the efflux pump SrpABC enabling efficient intermembrane solvent removal (12, 13), pTTS12 encodes more than 600 genes and hence, may contain additional genes involved in solvent tolerance. Two adjacent hypothetical genes, RPPX_26255 and RPPX_26260, were previously reported to be upregulated in a transcriptomic study as a short-term response to toluene addition (11). We propose to name RPPX_26255-26260 gene pair as ‘slv’ due to its elevated expression in the presence of solvent. In a
first attempt to identify additional potential solvent tolerance regions of pTTS12, we deleted the
srpABC genes (Δsrp), RPPX_26255-26260 genes (Δslv), and the combination of both gene clusters
(Δsrp Δslv) from pTTS12 in wild-type P. putida S12.

All strains were compared for growth under increasing toluene concentrations in liquid LB
medium (Figure 2). In the presence of low concentrations of toluene (0.10% v/v), all strains showed
similar growth. With the addition of 0.15% v/v toluene, S12 Δslv, S12 Δsrp and S12 Δsrp Δslv
exhibited slower growth and reached a lower OD_{600nm} compared to the wild-type S12 strain. S12 Δslv
and S12 Δsrp achieved a higher OD_{600nm} in batch growth compared to S12 ΔpTTS12 and S12 Δsrp Δslv
due to the presence of SrpABC efflux pump or RPPX_26255-26260 gene pair.

Interestingly, S12 Δsrp Δslv (still containing pTSS12) exhibit diminished growth compared to
S12 ΔpTTS12. This may be an indication of megaplasmid burden in the absence of essential genes for
solvent tolerance. With 0.20% and 0.30% v/v toluene added to the medium, S12 Δsrp, S12 Δsrp Δslv,
and S12 ΔpTTS12 were unable to grow while the wild-type S12 and S12 Δslv were able to grow
although S12 Δslv reached a clearly lower OD_{600nm}. Taken together, these results demonstrate an
important role for both the SrpABC efflux pump and the slv gene pair in conferring solvent tolerance.
We chose P. putida S12-6 for further experiments representing megaplasmid-cured P. putida S12.

Solvent tolerance can be exerted by ectopic expression of SrpABC efflux pump and slv gene pair in
Gram-negative bacteria

Functionality of the srp operon and slv gene pair was explored in the model Gram-negative non-
solvent tolerant strains, P. putida KT2440, E. coli TG1 and E. coli BL21(DE3). We complemented
srpRSABC (srp operon), slv gene pair, and a combination of both gene clusters into P. putida S12-6, P.
putida KT2440, E. coli TG1, and E. coli BL21(DE3) using mini-Tn7 transposition. These strains were
chosen due to their common application as model industrial strains while lacking solvent-tolerance.
P. putida KT2440 is another robust microbial host for metabolic engineering due to its adaptation
towards physicochemical stresses, however contrary to P. putida S12, this strain is not solvent
tolerant (36). *E. coli* BL21(DE3), derived from strain B, is the common *E. coli* lab strain optimized for protein production due to its lacking Lon and OmpT proteases and encoding T7 RNA polymerase (37). *E. coli* TG1 was previously reported to successfully produce 1-naphtol with the expression of SrpABC (15, 16) and therefore this strain was included in this study as a comparison.

Chromosomal introduction of *slv* into S12-6 and KT2440, improved growth of the resulting strains at 0.15% v/v toluene compared to S12-6 and KT2440 (Figure 3). The introduction of *srp* or a combination of *slv* and *srp* enables S12-6 and KT2440 to grow in the presence of 0.30% v/v toluene. In KT2440, the introduction of both *slv* and *srp* resulted in a faster growth in the presence of 0.30% v/v toluene compared to the addition of only *srp* (Figure 3B). Interestingly, the growth of S12-6 *srp,slv* and S12.6 *srp* are better in comparison with S12 wild-type (Figure 3A). The observed faster growth of S12-6 *srp,slv* and S12.6 *srp* may be due to more efficient growth in the presence of toluene supported by a chromosomally introduced *srp* operon, compared to its original megaplasmid localization. Indeed, replication of this large megaplasmid is likely to require additional maintenance energy. To corroborate this, we complemented the megaplasmid lacking the solvent pump, pTT12 (Tc<sup>4</sup>::srpABC) into *P. putida* S12-6 *srp* resulting in the strain *P. putida* S12-9. Indeed, *P. putida* S12-9 showed further reduced growth in the presence of 0.20 and 0.30% toluene (Figure S2), indicating the metabolic burden of carrying the megaplasmid. We conclude that the SrpABC efflux pump can be regarded as the major contributor to solvent tolerance from pTT12. The *slv* gene pair appears to promote tolerance of *P. putida* S12 at least under moderate solvent concentrations.

The intrinsic solvent tolerance of *E. coli* strains was observed to be clearly lower than that of *P. putida* (Figure 4). The wild-type *E. coli* strains were able to withstand a maximum 0.10% v/v toluene, whereas plasmid-cured *P. putida* S12-6 and *P. putida* KT2440 were able to grow in the presence of 0.15% v/v toluene. With the introduction of *slv* and *srp* in both *E. coli* strains, solvent tolerance was increased up to 0.15% and 0.20% v/v toluene respectively (Figure 4). A combination of *slv* and *srp* also increased tolerance to 0.20% v/v toluene while showing a better growth than
chromosomal introduction of just srp. However, none of these strains were able to grow in the presence of 0.30% v/v toluene.

qPCR analysis of SrpABC expression (Table S1) in P. putida S12, P. putida KT2440, E. coli TG1, and E. coli BL21(DE3) confirmed that srpA, srpB, and srpC were expressed at basal levels in all strains. In the presence of 0.10% toluene, the expression of srpA, srpB, and srpC was clearly upregulated in all strains. Thus, the lower solvent tolerance conferred by introducing SrpABC efflux pump in E. coli strains was not due to lower expression of the srp genes. Analysis of the codon adaptation index (CAI) (http://genomes.urv.es/CAIcalc/) (31) showed that for both the P. putida and E. coli strains the CAI values of the srp operon are suboptimal, clearly below 0.8 to 1.0 (Table S2). Interestingly, the CAI values were higher for E. coli (0.664) than for P. putida (0.465) predicting a better protein translation efficiency of the srp operon in E. coli. Hence, reduced translation efficiency is not likely to be the cause of lower performance of srp operon in E. coli strains for generating solvent tolerance. Overall, our results indicate that in addition to the solvent efflux pump, P. putida S12 and P. putida KT2440 are intrinsically more robust compared to E. coli TG1 and E. coli BL21(DE3) in the presence of toluene.

slv gene pair constitutes a novel toxin-antitoxin system

BLASTp analysis was initiated to further characterize RPPX_26255 and RPPX_26260. This indicated that RPPX_26260 and RPPX_26255 likely represent a novel toxin-antitoxin (TA) system. Through a database search on TADB2.0 (19, 20), we found that RPPX_26260 is a toxin of COG5654 family typically encoding a RES domain-containing protein, having a conserved Arginine (R) – Glutamine (E) – Serine (S) motif providing a putative active site and RPPX_26255 is an antitoxin of COG5642 family. Based on its involvement in solvent tolerance, we propose naming the toxin-encoding RPPX_26260 as slvT and the antitoxin-encoding RPPX_26255 as slvA.

Makarova and colleague identified putative toxin-antitoxin pairs through genome mining of reference sequences in NCBI database (32). They identified 169 pairs of the COG5654-COG5642 TA
system from the reference sequences. Here, we constructed a phylogenetic tree of the COG5654-
COG5642 TA system including SlvA (AJA16859.1) and SlvT (AJA16860.1) as shown in Figures 5A and
6A. SlvA and SlvT cluster together with other plasmid-borne toxin-antitoxin from Burkholderia
vietnamensis G4, Methylibium petroleiphilum PM1, Rhodospirillum rubrum ATCC 11170, Xanthobacter autotrophicus Py2, Sinorhizobium meliloti 1021, Sinorhizobium medicae WSM419, and
Gloeobacter violaceus PCC7421. Multiple alignments of SlvAT against these toxin-antitoxin of
COG5654-COG5642 TA system are shown in Figures 5B and 6B.

Of the 169 TA pairs of the COG5654-COG5642 TA system, three TA pairs have recently been
characterized: ParST from Sphingobium sp. YBL2 (AJR25281.1, AJR25280.1)(26), PP_2433-2434 from
P. putida KT2440 (NP_744581.1, NP_744582.1)(27), and MbcAT from Mycobacterium tuberculosis
H37Rv (NP_216506.1, NP216505.1)(25) as indicated by bold text and asterisks in Figure 5A and 6A.

3D-model prediction of SlvT and SlvA protein using the I-TASSER suite for protein structure and
function prediction (33), indicated that SlvT and SlvA showed highest structural similarity to the
MbcAT system from Mycobacterium tuberculosis (Figure 5C and 6C) which is reported to be
expressed during stress condition (25). Amino acid conservation between SlvAT and these few
caracterized toxin-antitoxin pairs is relatively low, as they do not belong to the same clade (Figure
5A and 6A). However, 100% conservation is clearly observed on the putative active side residues:
arginine (R) 35, tyrosine (Y) 45, and glutamine (E) 56 and only 75% consensus is shown on serine (S)
133 residue (Figure S3).

According to the model with highest TM score, SlvT is predicted to consist of four beta
sheets and four alpha-helices. As such, SlvT shows structural similarity with diphtheria toxin which
functions as ADP-ribosyl transferase enzyme. Diphtheria toxin can degrade NAD+ into nicotinamide
and ADP ribose (34). A similar function was recently identified for COG5654-family toxins from P.
putida KT2440, M. tuberculosis, and Sphingobium sp (25–27).
slvT toxin causes cell growth arrest by depleting cellular NAD⁺

To prove that slvAT presents a pair of toxin and antitoxin, slvA and slvT were cloned separately in pUK21 (lac-inducible promoter) and pBAD18 (ara-inducible promoter), respectively. The two constructs were cloned into E. coli BL21(DE3). Growth of the resulting strains was monitored during conditional expression of the slvA and slvT genes (Figure 7A). At the mid-log growth phase, a final concentration of 0.8% arabinose was added to the culture (*), inducing expression of slvT. After 2 hours of induction, growth of this strain ceased while the uninduced control culture continued to grow. Upon addition of 2 mM IPTG (**), growth of the slvT-induced culture was immediately restored, reaching a similar OD₆₀₀nm as the uninduced culture.

Bacterial cell division was further studied by flow cytometer-analyses during the expression of slvT and slvA. After approximately 6 hours of growth (indicated by grey arrow on Figure 7A), samples were taken from control, arabinose, and arabinose + IPTG induced liquid culture. Cell morphology was analyzed by light microscopy and DNA content of the individual cells in the culture were measured using flow cytometer with SYBR green II staining (Figure 7B). Indeed, absence of dividing cells and lower DNA content were observed during the induction of only slvT toxin with arabinose (Figure 7B). Subsequent addition of IPTG to induce slvA expression was shown to restore cell division and an upshift of DNA content similar to that of control strain (Figure 7B). While the expression of slvT was not observed to be lethal to bacterial strain, this experiment showed that the expression of slvT toxin stalled DNA replication and subsequently cell division. The induction of slvA subsequently restored bacterial DNA replication and cell division.

To corroborate a putative target of SlvT, concentrations of NAD⁺ were measured during the induction experiment (Figure 7C). Before the addition of arabinose to induce slvT (orange arrow on Figure 7A), NAD⁺ was measured and compared to the strain harboring empty pUK21 and pBAD18 (Figure 7B). On average, at this time point NAD⁺ level is similar between the slvAT bearing strain and the control strain. NAD⁺ was measured again after arabinose induction when growth of the induced strain has diminished (blue arrow on Figure 7A). At this time point, the measured NAD⁺ was 32%
(±14.47) of control strain. After the induction of slvA, NAD⁺ was immediately restored to a level of 77% (±9.97) compared to the control strain. Thus, induction of slvT caused depletion of NAD⁺, while induction of slvA immediately increased NAD⁺ level, indicating that slvAT is a pair of toxin-antitoxin which controls its toxicity through NAD⁺ depletion.

slvAT regulates megaplasmid pTTS12 stability

In addition to its role in solvent tolerance, localization of the slvAT pair on megaplasmid pTTS12 may have implications for plasmid stability. pTTS12 is a very stable megaplasmid that cannot be spontaneously cured from P. putida S12 and cannot be removed by introducing double strand breaks (see above). We deleted slvT and slvAT from the megaplasmid to study their impact in pTTS12 stability. With the deletion of slvT and slvAT, the survival rate during treatment with mitomycin C improved significantly reaching 1.01 (± 0.17) x 10⁻⁴ and 1.25 (± 0.81) x 10⁻⁴ respectively while the wild-type S12 had a survival rate of 2.48 (± 0.58) x 10⁻⁴.

We determined the curing rate of pTTS12 from the surviving colonies. In wild-type S12, the curing rate was 2% (see also above) while in ΔslvT and ΔslvAT curing rate increased to 41.3% (± 4.1%) and 79.3% (± 10%) respectively, underscoring an important role for slvAT in megaplasmid stability.

We attempted to cure megaplasmid by introducing double strand break (DSB) as previously described on P. taiwanensis VLB120 (30, 35). This indeed was not possible in wild-type S12 and ΔslvT, however ΔslvAT now showed plasmid curing by DSB resulting in a curing rate of 34.3% (± 16.4%).

Since ΔslvT and ΔslvAT may compromise megaplasmid stability, we now performed megaplasmid stability tests by growing S12 and KT2440 harboring pSW-2 (negative control) (35), pTTS12 (positive control), pTTS12 ΔslvT, and pTTS12 ΔslvAT in LB medium with 10 passages (± 10 generations/passage step) as shown on Figure 8. Both KT2440 and S12 easily lost the negative control plasmid pSW-2 (Figure 8). Plasmid pTTS12 was not lost during this test confirming that pTTS12 is indeed a stable plasmid. Furthermore, the ΔslvT strains also did not show loss of...
megaplasmid. Interestingly, the ∆slvAT strains spontaneously lost the megaplasmid, confirming that the slvAT module is not only important to promote solvent tolerance but also determines megaplasmid stability in P. putida S12 and KT2440.
Discussion

Revisiting the role of pTTS12 and SrpABC efflux pump in solvent tolerance

In this study we conclusively confirm the role of SrpABC efflux pump encoded on pTTS12 and identify a novel toxin-antitoxin module playing an additional role in conveying solvent tolerance to this microbial host (Figure 9). Notably, megaplasmids may cause a metabolic burden to their host strains and they can be a source of genetic instability (11). Our results show that indeed pTTS12 imposed a metabolic burden in the presence of organic solvent (Figure S2). This plasmid is very large in size and contains a large number of genes that are not related to solvent tolerance. Hence, it may be interesting for biotechnological purposes to reduce plasmid size and consequently, metabolic burden. In addition, a streamlined and minimal genome size is desirable to reduce host interference and genome complexity (12, 13).

We investigated the heterologous expression of the SrpABC efflux pump in strains of both P. putida and E. coli, which successfully enhanced their solvent tolerance in these strains (Figure 3 and Figure 4). Previous reports on the implementation of SrpABC in whole-cell biocatalysis successfully increased the production of 1-naphtol in E. coli TG1 (15, 16). Production was still higher using P. putida S12 as this strain could better cope with substrate (naphthalene) toxicity while both P. putida S12 and E. coli TG1 showed similar tolerance to the product, 1-naphtol (16). In our experiments, the E. coli strains clearly showed a smaller increase in toluene tolerance than the P. putida strains although srpABC was expressed at a basal level and upregulated in the presence of 0.10% v/v. These results indicate that besides having an efficient solvent efflux pump, P. putida S12 and P. putida KT2440 are inherently more robust in the presence of toluene and, presumably, other organic solvents compared to E. coli TG1 and E. coli BL21(DE3). The absence of cis-trans isomerase (cti) resulting in the inability to switch from cis- to trans-fatty acid in E. coli (38) may contribute to this difference in solvent-tolerance. Additionally, P. putida typically has a high NAD(P)H regeneration capacity (39, 40) which can contribute to the maintenance of proton motive force during solvent
extrusion by RND efflux pump. Further detailed investigation is required to reveal the exact basis for its intrinsic robustness.

Identification of the novel antitoxin-toxin module SlvAT

In *P. putida* S12, deletion of srpABC genes still resulted in higher solvent-tolerance compared to the pTTS12-cured strains (Figure 2, panel 3). This indicated that within pTTS12 there were other gene(s) which may play a role in solvent-tolerance. Two genes of unknown function were upregulated in transcriptome analysis of toluene-shocked *P. putida*, RPPX_26255 and RPPX_26260, suggesting a putative role in solvent tolerance (11). Here, we confirmed this finding and demonstrated that these genes together form a novel toxin-antitoxin module (Figure 7). SlvT exerts toxicity by degradation of NAD⁺, like other toxins of the COG5654-family, and expression of antitoxin SlvA immediately restored NAD⁺ levels. Depletion of NAD⁺ interfered with DNA replication and caused arrest of cell division similar to another recently described COG5654-COG5642 family toxin-antitoxin pair (27). Indeed, the SlvAT toxin-antitoxin module was shown to be important for the stability of pTTS12 (Figure 8).

Based on TADB2.0 analysis, pTTS12 encodes three TA pairs: SlvAT and two identical copies of VapBC. VapBC was first identified from a virulence plasmid of *Salmonella* and known to prevent the loss of plasmid during nutrient-limiting condition (41). A previous report showed that VapBC can stabilize/retain ≥90% of pUC plasmid in *E. coli* within 300 hours of growth (42), similar to our result although demonstrated in a much smaller plasmid and under the control of the lac operon. Serendipitous plasmid loss due to double strand break was reported in pSTY which carries two identical copies of VapBC (30). Here, we observed a similar phenomenon in pTTS12 ΔslvAT. Hence, in the absence of SlvAT, two copies of VapBC were not sufficient to prevent plasmid loss of pTTS12 on rich media without selection pressure and by double strand break, indicating a major role for SlvAT.

A putative role of toxin-antitoxin module SlvAT in solvent tolerance
Toxin-antitoxin modules are known to be important in antibiotic persistent strains as a trigger to enter and exit the dormant state, causing the cell to become unaffected by the antibiotic (42). Among *Pseudomonas* species, several toxin-antitoxin modules are reported to be involved in survival strategies, such as stress response, biofilm formation, and antimicrobial persistence (27, 43–45). Previous transcriptomic studies reported upregulation of the *slvAT* locus as a response towards toluene addition and its expression at 10-30 minutes after toluene addition (11). In this paper, we show that SlvAT improves solvent tolerance in *P. putida* and *E. coli* strains independent of pTTS12 or SrpABC efflux pump. We hypothesize that SlvAT plays a role as a rapid response towards toluene addition. Activation of SlvT toxin may halt bacterial growth and this allows physiological adaptation and adjustments to take place (e.g. expression of extrusion pumps and membrane compaction) before resuming its growth and cell division in the presence of toxic organic solvent. It is interesting to note that *P. putida* S12 and KT2440 both encode another COG5654-COG5642 family toxin-antitoxin pair in their chromosome (locus tag RPPX_19375-19380 and PP2433-2434, respectively). In *P. putida* S12, this TA module is not being induced during solvent stress rendering it unlikely to play a role in solvent tolerance.

**The putative regulation mechanism of toxin-antitoxin module SlvAT in *P. putida* S12**

Expression of the *slvAT* locus with its native promoter region seemed to exert a similar physiological effect in solvent tolerance both in *E. coli* and *P. putida* (Figure 3 & Figure 4). Typically, toxin-antitoxin can regulate its own expression by antitoxin binding to the promoter region (46). Unstable antitoxin is encoded upstream of the stable toxin, giving a transcriptional advantage for production of antitoxin (47). While this paper presents the role of SlvAT module as a response to solvent stress, this toxin-antitoxin module may play a role in the response to various other stresses since pTTS12 itself encodes several modules involved in different stress response. It is interesting to further study whether organic solvent directly induce the expression of *slvAT* locus or intermediate signalling pathways are required. Several type II toxin-antitoxin modules are known to be regulated.
by proteases, such as Lon and Clp (48). These proteases degrade antitoxin protein, promoting toxin
activity, and thus upregulate the expression of toxin-antitoxin locus. Indeed, our preliminary
transcriptomic data show upregulation of specific protease encoding loci after toluene addition.
These may constitute putative regulatory proteases to the SlvAT module. Future research on the
dynamics of slvAT locus regulation is required to reveal the details of the control mechanisms
operating in vivo.

General summary
In summary, our experiments confirmed that the SrpABC efflux pump is the major
contributor of solvent tolerance on the megaplasmid pTTS12 which can be transferred to other non-
solvent tolerant host microbes. In addition, the megaplasmid carries a novel toxin-antitoxin system
SlvAT (RPPX_26255 and RPPX_26260) which promotes rapid solvent tolerance in P. putida S12 and is
important for maintaining the plasmid stability of pTTS12. Chromosomal introduction of the
srpRSABC operon genes in combination with slvAT confers a clear solvent tolerance phenotype in
other industrial strains previously lacking this phenotype such as P. putida KT2440, E. coli TG1, and E.
coli BL21(DE3). Taken together, both SrpABC and SlvAT constitute suitable candidate loci for
exchange with various microbial hosts to increase tolerance towards toxic compounds.
Materials and Methods

Strains and culture conditions

Strains and plasmids used in this paper are listed in Table 1. All *P. putida* strains were grown in Lysogeny Broth (LB) at 30 °C with 200 rpm shaking. *E. coli* strains were cultivated in LB at 37 °C with 250 rpm. For solid cultivation, 1.5% (w/v) agar was added to LB. M9 minimal medium was supplemented with 2 mg L⁻¹ MgSO₄ and 0.2% of citrate as sole carbon source (43). Toluene atmosphere growth was evaluated on solid LB media in a glass plate incubated in an exicator with toluene supplied through the gas phase at 30 °C. Solvent tolerance analysis was performed by growing *P. putida* S12 strains in LB starting from OD₆₀₀ 0.1 in Boston bottles with Mininert bottle caps. When required, gentamicin (25 mg L⁻¹), ampicillin (100 mg L⁻¹), kanamycin (50 mg L⁻¹), indole (100 g L⁻¹), potassium tellurite (6.75-200 mg L⁻¹), arabinose (0.8% m/v), and IPTG (2 mM) were added to the media.
DNA and RNA methods

All PCRs were performed using Phusion polymerase (Thermo Fisher) according to the manufacturer’s manual. Primers used in this paper (Table 2) were procured from Sigma-Aldrich. PCR products were checked by gel electrophoresis on 1% (w/v) TBE agarose containing 5 µg mL⁻¹ ethidium bromide (110V, 0.5x TBE running buffer). For RT-qPCR analysis, RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s manual. The obtained RNA samples were immediately reverse transcribed using iScript™ cDNA synthesis kit (BioRad) and cDNA may be stored at -20 °C prior to qPCR analysis. qPCR was performed using iTaq™ Universal SYBR Green Supermix (BioRad) on CFX96 Touch™ Real-Time PCR Detection System (BioRad). The genome sequence of *P. putida* S12 ∆pTTS12 was analysed using Illumina HiSeq (GenomeScan BV, The Netherlands) and assembled according to the existing complete genome sequence (Accession no. CP009974 and CP009975) (12). These sequence data have been submitted to the SRA database under accession number PRJNA602416.

Curing and complementation of megaplasmid pTTS12 from *P. putida* S12

*P. putida* S12 was grown in LB to reach early exponential phase (± 3 hours or OD₆₀₀nm 0.4-0.6). Subsequently, mitomycin C was added to the liquid LB culture to a final concentration of 10, 20, 30, 40, or 50 µg/ml. These cultures were grown for 24 hours and plated on M9 minimal media supplemented with indole to select for the absence of megaplasmid. Loss of megaplasmid was confirmed by loss of other phenotypes connected with the megaplasmid such as MIC reduction of potassium tellurite and solvent sensitivity under toluene atmosphere, as well as through genomic DNA sequencing. Complementation of megaplasmid pTTS12 was performed using bi-parental mating between *P. putida* S12-1 (pTTS12 Km⁸) and plasmid-cured strains *P. putida* S12 ∆pTTS12 (Gm⁸ :: Tn7) and followed by selection on LB agar supplemented with kanamycin and gentamicin.
Plasmid cloning

Deletion of srpABC, slvT, and slvAT genes was performed using homologous recombination between free-ended DNA sequences that are generated by cleavage at unique I-SceI sites (35). Two homologous recombination sites were chosen downstream (TS-1) and upstream (TS-2) of the target genes. TS-1 and TS-2 fragments were obtained by performing PCR using primers listed in Table S1. Constructs were verified by DNA sequencing. Mating was performed as described by Wynands and colleagues (30). Deletion of srpABC, slvT, and slvAT was verified by PCR and Sanger sequencing (Macrogen B.V., Amsterdam).

Introduction of the complete srp operon (srpRSABC) and slvAT was accomplished using the mini-Tn7 delivery vector backbone of pBG35 developed by Zobel and colleagues (44). The DNA fragments were obtained by PCR using primer pairs listed on Table 2 and ligated into pBG35 plasmid at PacI and XbaI restriction site. This construct generated a Tn7 transposon segment in pBG35 containing gentamicin resistance marker and srp operon with Tn7 recognition sites flanking on 5’ and 3’ sides of the segment. Restriction analysis followed by DNA sequencing (Macrogen, The Netherlands) were performed to confirm the correct pBG-srp, pBG-slv, and pBG-srp-slv construct. The resulting construct was cloned in E. coli WM3064 and introduced into P. putida or E. coli strains with the help of E. coli WM3064 pTnS-1. Integration of construct into Tn7 transposon segment was confirmed by gentamicin resistance, PCR, and the ability of the resulting transformants to withstand and grow under toluene atmosphere.

Toxin-antitoxin assay

Bacterial growth during toxin-antitoxin assay was observed in LB media supplemented with 100 mg L⁻¹ ampicillin and 50 mg L⁻¹ kanamycin. Starting cultures were inoculated from 1:100 dilution of overnight culture (OD₆₀₀ = 0.1) into a microtiter plate (96 well) and bacterial growth was measured using Tecan Spark™ 10M. To induce toxin and antitoxin, a total concentration of 0.8% m/v arabinose and 2 mM IPTG were added to the culture respectively. Cell morphology was observed using light microscope (Zeiss Axiolab 5) at 100x magnification. A final concentration of 2.5x SYBR Green I
(10000x stock, New England Biolabs) was applied to visualize DNA, followed by two times washing with 1x phosphate buffer saline (PBS), and analyzed using a Guava® easyCyte Single Sample Flow Cytometer (Millipore). At indicated time points, NAD⁺ levels were measured using NAD/NADH-Glo™ assay kit (Promega) according to the manufacturer’s manual. The percentage of NAD⁺ level was calculated by dividing the measured luminescence of tested strains with that of the control strains at the same timepoints. RPPX_26255 and RPPX_26260 was modelled using I-TASSER server (33) and visualized using PyMol (version 2.3.1). Phylogenetic trees of toxin-antitoxin module derived from COG5654-COG5642 family were constructed using MEGA (version 10.0.5) as a maximum likelihood tree with 100 bootstrap and visualized using iTOL webserver (https://itol.embl.de) (45).
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Table 1. Strains and plasmids used in this paper

| Strain               | Characteristics                                                                 | References |
|----------------------|---------------------------------------------------------------------------------|------------|
| *P. putida* S12      | Wild-type *P. putida* S12 (ATCC 700801), harboring megaplasmid pTTS12          | (3)        |
| *P. putida* S12-1    | *P. putida* S12, harboring megaplasmid pTTS12 with Km" marker                  | This paper |
| *P. putida* S12-6/ 10/ S12-22 | ΔpTTS12, harboring megaplasmid pTTS12 with Km" marker                  | This paper |
| *P. putida* S12-9    | ΔpTTS12, Gm" srpABC::Tn7, complemented with megaplasmid pTTS12 (Tc"::srpABC)  | This paper |
| *P. putida* S12-C    | *P. putida* ΔpTTS12 (S12-6/ S12-10/ S12-22), harboring megaplasmid pTTS12    | This paper |
| *P. putida* KT2440   | Derived from wild-type *P. putida* mt-2, ΔpWW0                                  | (46)       |
| E. coli HB101        | recB pro leu hsdR SmR                                                         | (47)       |
| E. coli BL21(DE3)    | E. coli B, F" ompT gal dcm lon hsdS (r6 m9) λ(DE3) [lac lacUV5-77p07 ind1 sam7 nin5] (malB)k=1(λ) | (37)       |
| E. coli DH5α λpir    | sup E44, ΔlacUV5 (ΔlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1, λpir phage lysogen | (48)       |
| E. coli TG1          | E. coli K-12, glv44 thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5(rχ mχ) F' [traD36 proAB" lacO lacZΔM15] | Lucigen    |
| E. coli WM3064       | thrB1004 pro thi rpsL hsdS lacZΔM15 RP4-1360 Δ(araBAD)567 ΔapA1341::erm pir | William Metcalf |

**Plasmid**

| Plasmid               | Characteristics                                                                 | References |
|-----------------------|---------------------------------------------------------------------------------|------------|
| pRK2013               | RK2-Tra', RK2-Mob', Km", ori ColE1                                             | (49)       |
| pEMG                  | Km", Ap", ori R6K, lacZa MCS flanked by two I-sceI sites                       | (35)       |
| pEMG-ΔsrpABC          | pEMG plasmid for constructing *P. putida* S12 ΔsrpABC                           | This paper |
| pEMG-ΔslvAT           | pEMG plasmid for constructing *P. putida* S12 ΔslvAT                           | This paper |
| pEMG-ΔsrpΔslvT        | pEMG plasmid for constructing *P. putida* S12 ΔsrpΔslvT                         | This paper |
| pSW-2                 | Gm", ori RK2, xyfS, Pm -> I-sceI                                               | (35)       |
| pBG35                 | Km", Gm", ori R6K, pBG-derived                                                 | (44)       |
| pBG-srp               | Km", Gm", ori R6K, pBG-derived, contains srp operon (RPXX_27995-27965)         | This paper |
| pBG-slv               | Km", Gm", ori R6K, pBG-derived, contains slv gene pair (RPXX_26255-26260)      | This paper |
| pBG-srp-slv           | Km", Gm", ori R6K, pBG-derived, contains slv gene pair (RPXX_26255-26260) and srp operon (RPXX_27995-27965) | This paper |
| pBAD18-slvT           | Ap", ara operon, contains slvT (RPXX_26260)                                    | This paper |
| Vector | Description | Source |
|--------|-------------|--------|
| pUK21-slvA | Km<sup>+</sup>, lac operon, contains slvA (RPPX_26255) | This paper |
| pTnS-1 | Ap<sup>+</sup>, ori R6K, TnSABC+D operon | (50) |
Table 2. Primers used in this paper

| Primers | Sequences (5’-3’) | Restriction sites | PCR templates | Description |
|---------|------------------|------------------|---------------|-------------|
| TS1-srp-for | TATCGTGATCCGTTGTGGAAGCGCTAAATGA | KpnI | pTTS12 | Construction of pEMG-ΔsrpABC |
| TS1-srp-rev | CAGCCGCGCCGCGGATGGAAAGCTGCAAGGAG | NotI | pTTS12 | Construction of pEMG-ΔsrpABC |
| TS2-srp-for | CCGAACGCGCCGCGCCGAGCTGCTGAGGAGTACC | NotI | pTTS12 | Construction of pEMG-ΔsrpABC |
| TS2-srp-rev | TACGTCTAGAGCGGATGGTCAAGCCTACC | XbaI | pTTS12 | Construction of pEMG-ΔsrpABC |
| srpO_F | TGCAATTGATGGATCAGATGGCAGGAG | EcoRI | pTTS12 | Construction of pBGrp |
| srpO_R | TGCTCTAGATGGGAGATCAGGAG | XbaI | pTTS12 | Construction of pBGrp |
| slv_F | ATGCTTAATTAACTTTTTCGCTACAGACTCAGG | PacI | pTTS12 | Construction of pBGrp |
| slv_R | AGCGGGAATTCGCTGAGGAG | EcoRI | pTTS12 | Construction of pBGrp |
| slvA_F | AGAGAGCTCACATAGTAGTGCATAGGAG | SacI | pTTS12 | Construction of pUK21-slvA |
| slvA_R | GTCTAGACTCGATCCAGATGTAG | XbaI | pTTS12 | Construction of pUK21-slvA |
| slvT_F | GTCTAGATGGGAGATCAGGAG | XbaI | pTTS12 | Construction of pBAD18-slvT |
| slvT_R | GGAAGAGCTGATGGTAGAAGCTGACAGTACC | Sacl | pTTS12 | Construction of pBAD18-slvT |
| TS1_slv_F | TGCTGGAATTCCTTTTTCGCTACACGG | EcoRI | pTTS12 | Construction of pEMG-ΔslvAT |
| TS1_slv_R | GCAACTGATGCATAGGAGATCAGGAG | Sacl | pTTS12 | Construction of pEMG-ΔslvAT |
| TS2_slv_F | GCGCTGATGGGAGATCAGGAG | XbaI | pTTS12 | Construction of pEMG-ΔslvAT |
| TS2_slv_R | GCCAGGATCCGAGATCCAGCATAACGGGAGGAG | KpnI | pTTS12 | Construction of pEMG-ΔslvAT |
| TS1_slvT_F | GCAATAGGCTGAGGAGATCAGGAG | PacI | pTTS12 | Construction of pEMG-ΔslvT |
| TS1_slvT_R | TGCTGGAATTCCTTTTTCGCTACACGG | EcoRI | pTTS12 | Construction of pEMG-ΔslvT |
| TS2_slvT_F | CTACATCTGGGAGCTGAGGAGATCAGGAG | HindIII | pTTS12 | Construction of pEMG-ΔslvT |
| eco_gyrB_F | CGATAATTGGTTGCAACACCAGAT | - | gyrB | qPCR, reference gene |
| eco_gyrB_R | GAATTCCTCTCTCCGGAGACCAA | - | gyrB | qPCR, reference gene |
| eco_rpoB_F | AACAGGAGCTGAGGAG | - | rpoB | qPCR, reference gene |
| eco_rpoB_R | GCTTTAAACCCGCGATATACCT | - | rpoB | qPCR, reference gene |
| ppu_gyrB_F | GCTGTGACGAGATGTTTTCGTC | - | gyrB | qPCR, reference gene |
| ppu_gyrB_R | GCAGTTGGTGGATGCTGACT | - | gyrB | qPCR, reference gene |
| ppu_rpoB_F | GACAAAGATGTGCGAAAGAAG | - | rpoB | qPCR, reference gene |
| ppu_rpoB_R | GAAAGGATGCTGCAGGAT | - | rpoB | qPCR, reference gene |
| srpA_F | CTGGGAAATTTCTATCATGTC | - | srpA | qPCR, target gene |
| srpA_R | AAAGCTCTTGGTCTGCAAAGA | - | srpA | qPCR, target gene |
| srpB_F | TACATCAGGAGAAGACCGA | - | srpB | qPCR, target gene |
| srpB_R | GTGAGGGCTTGGTTCATCAG | - | srpB | qPCR, target gene |
| srpC_F | GCCATATGATGCTGCAGAG | - | srpC | qPCR, target gene |
| srpC_R | ATCCAAAGGATTTTGCAAAAA | - | srpC | qPCR, target gene |
Figure 1. Curing of the megaplasmid pTTS12 from *P. putida* S12.

A. Activity of styrene monooxygenase (SMO) and styrene oxide isomerase (SOI) for indigo formation from indole in *P. putida* strains. Enzyme activity was lost in the megaplasmid-cured strains S12 ΔpTTS12 (white colonies). Indole (100 mg L⁻¹) was supplemented in M9 minimum media.

B. K₂TeO₃ resistance of *P. putida* strains on lysogeny broth (LB) agar. Tellurite resistance was reduced in the megaplasmid-cured strains S12 ΔpTTS12 (MIC 50 mg L⁻¹).

Figure 2. Megaplasmid pTTS12 determines the solvent tolerance trait of *P. putida* S12.

Solvent tolerance analysis was performed on wild-type *P. putida* S12, *P. putida* S12 ΔpTTS12 (strains S12-6, S12-10, and S12-22), *P. putida* S12 Δsrp, *P. putida* S12 Δslv, and *P. putida* S12 Δsrp Δslv growing in liquid LB media with 0, 0.10, 0.15, 0.20 and 0.30% v/v toluene. The removal of the megaplasmid pTTS12 clearly caused a significant reduction in the solvent tolerance of *P. putida* S12 ΔpTTS12. Deletion of srpABC (Δsrp), RPPX_26255-26260 (Δslv), and the combination of these gene clusters (Δsrp Δslv) resulted in a lower solvent tolerance. This figure displays the mean of three biological replicates and error bars indicate standard deviation. The range of y-axis are different in the first panel (0 - 5), second panel (0-3) and third to fifth panels (0 – 1.5).

Figure 3. Chromosomal introduction of srp and slv gene clusters increased solvent tolerance in *P. putida* strains.

Solvent tolerance analysis of the strains with chromosomal introduction of srp operon (srpRSABC), slv gene pair (RPPX_26255-26260) and the combination of these gene clusters into *P. putida* S12.
ΔpTTS12 (represented by strain S12-6) (A) and wild-type *P. putida* KT2440 (B) in liquid LB with 0, 0.10, 0.15, 0.20 and 0.30% v/v of toluene. Wild-type *P. putida* S12 was taken as a solvent tolerant control strain. This figure displays the mean of three independent replicates and error bars indicate standard deviation. The range of y-axis are different in the first panel (0 - 6), second panel (0-3) and third to fifth panels (0 – 1.5).

**Figure 4.** Chromosomal introduction of *srp* and *slv* gene clusters increased solvent tolerance in *E. coli* strains.

Solvent tolerance analysis of the strains with chromosomal introduction of *srp* operon (*srpRSABC*), *slv* gene pair (RPPX_26255-26260) and the combination of these gene clusters into *E. coli* BL21(DE3) (A) and *E. coli* TG1 (B) in liquid LB with 0, 0.10, 0.15, 0.20 and 0.30% v/v of toluene. This figure displays the mean of three independent replicates and error bars indicate standard deviation. The range of y-axis are different in the first panel (0 - 6), second panel (0-3) and third to fifth panels (0 – 1.5).

**Figure 5.** Bioinformatics analysis of SlvT as a member of COG5654 toxin family

A. Phylogenetic tree (neighbour joining tree with 100 bootstrap) of COG5654 family toxin from reference sequences identified by Makarova and colleagues (29). Different colours correspond to the different toxin-antitoxin module clades. Asterisks (*) and bold text indicate the characterized toxin proteins: ParT from *Sphingobium sp*. YBL2 (AJR25280.1), PP_2434 from *P. putida* KT2440 (NP_744582.1), MbcT from *Mycobacterium tuberculosis* H37Rv (NP_216505.1), and SlvT from *P. putida* S12 (AJA16860.1).
B. Multiple sequence alignment of the COG5654 toxin SlvT from *P. putida* S12 with several putative COG5654 family toxin protein which belong in the same clade. Putative active site residues are indicated by black arrows.

C. Protein structure modelling of SlvT using I-TASSER server (30) which exhibits high structural similarity with MbcT from *Mycobacterium tuberculosis* H37Rv. Shown are the close up of putative active site of SlvT toxin (Arg-35, Tyr-45, Glu-56, and Ser-133).

**Figure 6. Bioinformatics analysis of SlvA as a member of COG5642 toxin family**

A. Phylogenetic tree (neighbour joining tree with 100 bootstrap) of COG5642 family toxin from reference sequences identified by Makarova and colleagues (29). Different colours correspond to the different toxin-antitoxin module clades. Asterisks (*) and bold text indicate the characterized toxin proteins: ParS from *Sphingobium sp.* YBL2 (AJR25281.1), PP_2433 from *P. putida* KT2440 (NP_744581.1), MbcA from *Mycobacterium tuberculosis* H37Rv (NP_216506.1), and SlvA from *P. putida* S12 (AJA16859.1).

B. Multiple sequence alignment of the COG5654 toxin SlvA from *P. putida* S12 with several putative COG5642 family toxin protein which belong in the same clade. Putative active site residues are indicated by orange and black arrows.

C. Protein structure modelling of SlvA using I-TASSER server (30) which exhibits high structural similarity with MbcA from *Mycobacterium tuberculosis* H37Rv. Shown are the close up of antitoxin putative C-terminal binding site to block SlvT toxin active site (Ala-127, Gly-128, Ala-129, Gln-130, and Gly-131).

**Figure 7. Heterologous expression of SlvAT in *E. coli* BL21(DE3)**

A. Growth curves of *E. coli* BL21(DE3) harbouring pBAD18-slvt and pUK21-slva showing growth reduction after the induction of toxin by a total concentration of 0.8% arabinose (*) and growth
restoration after antitoxin induction by a total concentration of 2 mM IPTG (**). Samples were taken at the time points indicated by coloured arrows for cellular NAD⁺ measurement.

B. Flow cytometry analysis of DNA content and cell morphology visualization on *E. coli* BL21(DE3) during *slvT* and *slvAT* expression. Median value of green fluorescence representing DNA content during *slvT* expression (118.202), *slvAT* expression (236.056), and control (208.406) are indicated by a, b, and c respectively. Samples were taken at the time point indicated by grey arrow on Figure 7A.

C. Cellular NAD⁺ measurement during the expression of toxin-antitoxin module. Induction of toxin *SlvT* caused a reduction in cellular NAD⁺ level to 32.32% (±14.47%) of the control strain, while the expression of SlvA restored cellular NAD⁺ level to 77.27% (±9.97%) of the control strain.

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**Figure 8.** SlvAT is important for pTTS12 maintenance in *P. putida*.

pTTS12 (variant with Km<sup>R</sup>) maintenance in *P. putida* S12 and *P. putida* KT2440 growing in LB liquid medium without antibiotic selection for 10 passages (± 10 generations per passage). pSW-2 was taken as negative control for plasmid stability in *P. putida*. This experiment was performed with three biological replicates and error bars represent standard deviation.

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**Figure 9.** Schematic representation of the gene clusters involved in solvent tolerance from megaplasmid pTTS12.

SrpABC efflux pump is the major contributor of solvent tolerance trait from the megaplasmid pTTS12. This efflux pump is able to efficiently extrude solvents from membrane lipid bilayer. A COG5654-COG5642 family toxin-antitoxin module (*SlvT* and *SlvA* respectively) promoted the growth of *P. putida* S12 in the presence of moderate solvent concentration and stabilized pTTS12 plasmid. In the absence of SlvA, SlvT causes toxicity by conferring cellular NAD⁺ depletion and subsequently halt DNA replication and cell division.
srpC srpB srpA srpS srpR slvA slvT

COG5654 family

Toxin

COG5642 family

Antitoxin

Toxin-antitoxin module

Solvent efflux pump

Tolerant to high solvent concentration

Tolerant to moderate solvent concentration

Solvent concentration

Low

High

Plasmid stabilization

Nicotinamide

ADP-ribose

COG5642 family antitoxin

COG5642 family toxin

NAD^+

COG5654 toxin

Toxin-antitoxin module