Regulation of solvent tolerance in *Pseudomonas putida* S12 mediated by mobile elements

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Summary

Organic solvent-tolerant bacteria are outstanding and versatile hosts for the bio-based production of a broad range of generally toxic aromatic compounds. The energetically costly solvent tolerance mechanisms are subject to multiple levels of regulation, involving among other mobile genetic elements. The genome of the solvent-tolerant *Pseudomonas putida* S12 contains many such mobile elements that play a major role in the regulation and adaptation to various stress conditions, including the regulation of expression of the solvent efflux pump SrpABC. We recently sequenced the genome of *P. putida* S12. Detailed annotation identified a threefold higher copy number of the mobile element ISS12 in contrast to earlier observations. In this study, we describe the mobile genetic elements and elaborate on the role of ISS12 in the establishment and maintenance of solvent tolerance in *P. putida*. We identified three different variants of ISS12 of which a single variant exhibits a high translocation rate. One copy of this variant caused a loss of solvent tolerance in the sequenced strain by disruption of *srpA*. Solvent tolerance could be restored by applying selective pressure, leading to a clean excision of the mobile element.

Introduction

*P. putida* S12

*Pseudomonas putida* S12 is a Gram-negative bacterium that was isolated on styrene as a sole carbon source (Hartmans *et al.*, 1990). It has the exceptional ability to tolerate a wide variety of toxic organic solvents in concentrations that are lethal to most microorganisms (Isken and de Bont, 1998). *P. putida* S12 is able to metabolize organic solvents such as styrene or 1-octanol and is also highly resistant towards non-metabolized organic solvents such as toluene. This ability is extremely beneficial for the production of fine chemicals and their removal using two-phase water-organic solvent fermentation systems (Wierckx *et al.*, 2005; Heipieper *et al.*, 2007; Verhoef *et al.*, 2009). As such, *P. putida* S12 is a promising platform for the production of aromatic compounds (Nijkamp *et al.*, 2005; Wierckx *et al.*, 2005; Tiso *et al.*, 2014; Loeschcke and Thies, 2015).

Solvents and their effects on bacterial membranes

Organic solvents of a log $P_{OW}$ of 1–4 are destructive for bacterial lipid membranes (Heipieper *et al.*, 2007). These solvents accumulate in lipid membranes, causing an increase in membrane fluidity and a decrease in bilayer stability (Sikkema *et al.*, 1994; Weber *et al.*, 1994). Loss in bilayer stability contributes to a passive flux of protons across the membranes and affects the ATP synthesis by the proton motive force PMF. In addition, the membrane protein interactions are altered due to this unstable lipid bilayer (Sikkema *et al.*, 1994). To compensate for these adverse conditions, solvent-tolerant *P. putida* strains adapt to these conditions by changes in their inner- and outermembranes and by the activation of an active efflux pump for organic solvents (Heipieper and de Bont, 1994; Weber *et al.*, 1994; Ramos *et al.*, 1997; Isken and de Bont, 1998; Ramos *et al.*, 2002; Ramos *et al.*, 2015). The solvent efflux pump SrpABC in *P. putida* S12 is exclusively activated by solvent stress (Kieboom *et al.*, 1998b; Kieboom and de Bont, 2001). Highly similar efflux pumps have also been identified in other solvent-tolerant strains, such as TtgGHI in *P. putida* DOT-T1E (Segura *et al.*, 2003).
Insertion sequence-mediated regulation

Insertion sequences are the smallest transposable elements in prokaryotes with extensive effects on genome plasticity (Casacuberta and González, 2013). They have strong relevance regarding gene (in)activation and thus play an important role in adaptive responses of microorganisms to environmental changes and challenges. Phenotypic variations within a population have been recognized as traits that enable survival or adaptation to specific environments (van der Woude and Bäumler, 2004). These variations have been mainly studied in the context of host-pathogen interactions, although in *P. putida* and *P. brassicacearum* phase, variation is also involved in the production of exo-enzymes and secondary metabolites (Chabeaud et al., 2001; van den Broek et al., 2003). Transposition of insertion sequences has been associated with phase variation, affecting the cell surface morphology in *N. meningitides* and *S. epidermidis* (Ziebuhr et al., 1997; Hammerschmidt et al., 1996). Examples of insertion sequences that cause a specific phenotype in *P. putida* S12 are the ISS12 and the ISPpu21. Both insertion sequences were found to be present in the *srpS* repressor gene of the solvent efflux pump SrpABC in a subpopulation of *P. putida* S12, thus increasing the constitutive expression of the solvent resistance pump and consequently the survival upon solvent stress (Wery et al., 2001; Sun and Dennis, 2009).

Insertion sequences in the *P. putida* S12 genome

The complete genome of *P. putida* S12 has been recently sequenced and was found to consist of a circular chromosome (5799 Kbp) and a megaplasmid 584 Kbps (Kuepper et al., 2015). The chromosome has an average GC content of 61.8%, and the megaplasmid has a 57.8% GC content [accession no: CP009974 (chromosome), CP009975 (plasmid pTTS12)]. Surprisingly, this genome showed a high number of one specific insertion sequence, ISS12, while in the previous studies, fewer ISS12 copy numbers were observed in *P. putida* S12 using Southern blot analysis (Wery et al., 2001; Volkers et al., 2010).

Investigating the high occurrence of ISS12 mobile element in the recently sequenced *P. putida* S12 genome (here after referred to as *P. putida* S12/A) led to an interesting observation. To our surprise, the *P. putida* S12/A could not tolerate a second phase of styrene or sudden solvent shock of toluene as reported for the original strain. In this study, the role of insertion sequences (specifically ISS12) in the regulation of solvent tolerance was investigated. We compared the *P. putida* S12/A to its original stocks of *P. putida* S12 currently available at TNO (the Netherlands) and the ATCC collection (USA).

Results and discussion

Mobile elements in *P. putida* S12

Detailed annotation of mobile elements was performed on the sequence of *P. putida* S12, to confirm the high occurrence of mobile elements in the *P. putida* S12/A. An overview of characterized mobile elements in *P. putida* S12/A is summarized in Table 1. This strain contains five Tn3-like and two Tn7-like transposable elements. However, the majority (~55 copies) of mobile elements are characterized as insertion sequences that belong to different families, such as IS3, ISS and IS21. These sequences showed highest DNA similarity to other insertion sequences of *Pseudomonas* strains, using BLAST search and comparison with the ISFinder database (Sigui et al., 2005). The IS3, IS66 and IS110 family of insertion sequences (~11 copies) were observed exclusively on the chromosome, while IS256, IS110-1S1111, IS5-IS427 and Tn3-like (~eight copies) were only observed on the megaplasmid. The remaining insertion sequences, ISS12 and ISPpu21, were observed on both the chromosome and the megaplasmid.

We focused on ISS12 insertion sequences, because the translocation of this element has been linked to resistance and adaptation of *P. putida* towards solvents (Wery et al., 2001; Volkers et al., 2010). ISS12 can translocate into the repressor *srpS* of the *srpABC* operon, which bestows upon *P. putida* S12 its characteristic solvent tolerance (Kieboom et al., 1998; Kieboom and de Bont, 2001; Volkers et al., 2015). ISS12-mediated disruption of *srpS* leads to constitutive expression of *srpABC*, which enables survival of a small subpopulation upon a sudden solvent shock (Wery et al., 2001). Additional translocations of ISS12 were observed after adaptation of *P. putida* S12 to high concentrations of benzene (Volkers et al., 2010). *P. putida* S12/A showed a threefold higher copy number of the insertion sequence ISS12 in contrast to earlier observations based on Southern blot data (Wery et al., 2001; Volkers et al., 2010).

ISS12 genetic diversity and organization

With 33 copies, ISS12 elements comprise half of the total insertion sequences in the *P. putida* S12/A analysis. BLAST search reveals several matches with 70–99% DNA sequence identity to other insertion sequences in *Pseudomonas* strains (Fig. S1). These include other *P. putida* strains as well as *P. pseudoalcaligenes*, *P. aeruginosa*, *P. mandelii* and *P. stutzeri*. Other insertion sequences were observed with < 70% similarity, mainly in *Marinobacter*, *Burkholderia* and *Polaromonas* species.
ISS12 consists of 2596 bp and is one of the largest insertion sequences. It contains two open-reading frames (ORFs), encoding an integrase that enables the transposition and an ATPase AAA helper protein (Fig. 1). The ORFs are flanked by ±150-bp sequence containing inverted repeat regions (Fig. 1A). The majority of ISS12 elements (28 copies) have a 100% similarity to the previously described sequence of ISS12 (accession number AF292393, Wery et al., 2001). Within ISS12, two putative regulatory sequences were proposed, one at the upstream of transposase (Pup) and one at the downstream of the ATPase AAA encoding gene (Pdown) (Wery et al., 2001; indicated in Fig. 1B). A putative SoxR binding motif (Palma et al., 2005) lies just downstream of Pup, 25 base pairs after the ATG start site of transposase encoding gene (indicated in Fig. 1A and Fig. S2). The Pup, Pdown including SoxR motif (Pup,soxR) and Pdown promoter sequences were cloned in a Tn7 promoter-probe vector (Zobel et al., 2015) and integrated into attTn7 site in P. putida S12. The analysed promoter activity of Pup and Pup, soxR was similar to the background autofluorescence signal of the negative control S12 WT (Fig. 1C). Thus, surprisingly, these putative upstream promoters are inactive under the tested conditions, indicating that the transposase must be activated either by upstream regulatory elements, or that some other form of transcriptional regulation is imposed (Reimmann et al., 1989). The downstream promoter Pdown has a relatively low activity, which is approximately sixfold weaker than Pem7 in P. putida S12 (Fig. 1C), a synthetic promoter of average strength (Zobel et al., 2015). No effect was observed on promoter activities under oxidative stress conditions using 0.25 and 0.5 mM H2O2 (Fig. S3), indicating that the putative SoxR binding motif is not involved.

We identified two other variants of the insertion sequence after alignment of all ISS12 insertion sequences. According to our analysis, all ISS12 variants were already present in previous sequences of P. putida S12 and are not the result of mutations during transposition in S12/A strain. These variants were named ISS12_C and ISS12_D with 79% and 76% identity to ISS12 respectively (Table 2 and Fig. 1). The ISS12 sequence (at position 5324675–5327253 on CP009974) that previously was annotated as ISS12_B had 100% identity to ISS12_C sequence. This became apparent after cloning all ISS12 variants of P. putida S12 in pJET1.2 and sequencing (Fig. S4). This was probably caused during the assembly process at that specific region due to high occurrence of this insertion sequence. The ISS12_C variant has four copies in the P. putida S12/A genome, and the reading frames of the two encoded genes and start/stop codons are retained. The encoded proteins of the ISS12_C variant show an 84% and 85% identity at the amino acid level to the integrase and ATPaseAAA encoding genes of ISS12. The inverted repeats (IR) are different although there are still several candidate IRs present in this ISS12 variant (Fig. 1B). Inverted repeat regions at the flanks of insertion sequences play an important role in their translocation efficiency. They often contain the transposase binding site as well as inverted repeats for DNA secondary structures required for transposition (Reimmann et al., 1989; Berger and Haas, 2001). The ISS12_C is also an active insertion sequence, which was demonstrated by two new translocation events into the chromosome of P. putida S12/A. It has highest similarity to the ISP14 with 98% identity based on the amino acid sequence of two ORFs. The ISS12_D variant is only present in a single copy and has a frameshift in the open-reading frames of the integrase encoding genes, whereas the ATPaseAAA gene is retained normally. The encoded ATPaseAAA gene of the ISS12_D variant shows a 76% identity at the amino acid level to the ATPaseAAA in ISS12. This variant also shows

Table 1. Mobilome of P. putida S12.

| Name | Family group | Homologous sequences | Identity | Copy number | Location |
|------|--------------|----------------------|----------|-------------|----------|
| IS66 | IS66         | ISPu15               | 83%      | 2           | Chromosome |
| ISPu21 | IS5         | ISPu21               | 100%     | 4           | Chromosome/plasmid |
| IS5 | IS5          | ISPu22               | 92%      | 1           | Chromosome |
| ISS110 | ISS110      | ISPu9                | 85%      | 4           | Chromosome |
| IS256 | IS256       | ISXax1               | 79%      | 1           | Plasmid |
| IS3-IS150 | IS3-IS150 | ISPv8                | 69%      | 5           | Chromosome |
| Tn3-like | Tn3      | ISXc4                | 68%      | 2           | Plasmid |
| Tn3-like | Tn3      | TnAs1                | 92%      | 2           | Plasmid |
| Tn3-like | Tn7      | Tn7-like             | 100%     | 2           | Chromosome/plasmid |
| Tn3-like | Tn3      | TnAs3                | 77%      | 1           | Plasmid |
| IS110-IS1111 | IS110-IS1111 | ISAzv2_aa1          | 75%      | 1           | Plasmid |
| IS5-IS427 | IS5-IS427 | ISPv8                | 77%      | 1           | Plasmid |
| ISS12 | IS21        | ISS12 ISPu7           | 100%     | 28          | Chromosome/Plasmid |
| ISS12_C | IS21     | ISPv14               | 98%      | 4           | Chromosome/Plasmid |
| ISS12_D | IS21     | ISPv14               | 85%      | 1           | Chromosome/Plasmid |
| Unidentified | | | | | Chromosome/plasmid |

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candidates for alternative inverted repeats, and it has an additional 50 bp at the 5’ prime region (Fig. S2). The single occurrence of this ISS12 variant indicated that it might be a silent insertion sequence due to an early stop codon in ORF1 and a larger repeat region at its left flank compared to other ISS12 variants. Similar as ISS12_C, ISS12_D has the highest (~85% based on amino acid sequence) similarity to the ISPsy14.

**Fig. 1.** Insertion sequence ISS12 and its variants in *P. putida* S12.

A. The ISS12 sequence contains two open-reading frames (ORFs) and two inverted repeats at the flanks (IR). After insertion in a DNA sequence, a directed repeat (DR) is observed at the flanks of ISS12 that has been created during the insertion.

B. Self-plot of ISS12-variants without ORF1 and ORF2; the black asterisks (*) on red line represent the location of ORFs. The self-plot shows different variations in inverted repeats (green) and directed repeats (blue) within the ISS12-variants.

C. Activity of ISS12-putative promoter sequences integrated in *P. putida* S12 using Tn7 probe vector. The promoter activity was calculated based on GFP expression of promoter-GFP constructs for the $P_{\text{up}}$, $P_{\text{up,soxR}}$, $P_{\text{down}}$ (indicated in A) and compared to background fluorescence signal of *P. putida* S12 and the positive control integrated BG13 promoter ($P_{\text{em7-BCD2}}$). The mean of triplicates is shown in the graph, and error bars indicate standard deviation. ***indicates $p$-value < 0.001 with unpaired student $t$-test.
variants ORF 1a ORF 2a IR1 IR2 DR
ISS12pared to AF292393.
P. putida assembly data (Fig. S5). The ISS12 elements that were unique to the S12/A strain showed clear gaps in the re-coverage at those specifi
creating the assembly process, we observed complete sequences, 23 are unique to the sequence duplications, the upstream and downstream presence in the other three location of the insertion sequences and their absence or
be observed clearly by a successful alignment of reads regions in the backbone. The presence of ISS12 in re-
avoid assembly of reads that would match to multiple backbone. The assembly parameters were set as such to
mutants (Nijkamp et al., 2005) and two unpublished (Table 3). The older shotgun sequencing (accession number: AYKV00000001) and two unpublished sequences of phenol- and cinnamic acid-producing mutants (Nijkamp et al., 2005 and Wierckx et al., 2005) were re-assembled using the complete sequence as the backbone. The assembly parameters were set as such to avoid assembly of reads that would match to multiple regions in the backbone. The presence of ISS12 in re-assembled data of previous sequenced S12 strains could be observed clearly by a successful alignment of reads at the 5' and 3' flanks of insertion elements, while ISS12s unique to the S12/A strain showed clear gaps in the re-assembly data (Fig. S5). The ISS12 elements that were not present in previously published sequences of P. putida S12 were removed from the backbone, and by repeating the assembly process, we observed complete coverage at those specific positions. Of 33 insertion sequences, 23 are unique to the P. putida S12/A sequenced in 2015. Table 3 gives an overview of the location of the insertion sequences and their absence or presence in the other three P. putida S12 genome sequences. To validate the sequence analysis on the presence and absence of the ISS12 in the original P. putida S12 (freshly acquired from ATCC, 700801), we performed PCRs on 33 regions in both the original and the recently sequenced strains. Ten sequence regions of ISS12 showed a PCR product of 3 kb in the original strain, while the remaining regions showed a PCR product of 400 bp and thus no ISS12 insertions, as expected (Table 3). In the recently sequenced strain, all PCR products showed the expected 3-kb product of ISS12.

To investigate insertion specificity of ISS12 and the sequence duplications, the upstream and downstream sequences of transposition sites and the typical directed repeats created after insertion were extracted and aligned. In line with other insertion sequences of the IS21 family, no conserved consensus sequence was found after alignment of upstream and downstream sequences of ISS12 that would indicate a preference site of insertion for ISS12 (Berger and Haas, 2001; Arias-Palomo and Berger, 2015). At the initial target site, ±7 bp was duplicated after transposition resulting in a directed repeat at both ends of the ISS12 sequence (Fig. 1A). The majority of transpositions was characterized by a 7-bp repeat length (24 sites; Table 3). Both 6- and 8-bp lengths for these directed repeats were observed at four sites, and 5-bp length repeat was only observed at one single site (Table 3).

**ISS12 insertion sites and disrupted genes**

To investigate the possible effects of the insertions in the genome, the surrounding sequences of the 33 ISS12 elements were analysed. Based on this analysis, 25 insertion sites resulted in disruption of coding regions, while the remaining insertions (eight sites) do not disrupt a known ORF. In total, 14 genes were clearly disrupted on the chromosome and 11 on the megaplasmid. Besides insertions in the ORFs, insertions in regions that alter rates of for example of transcription-like promoter regions can alter the function of genes as well and may cause a phenotype. The disrupted and neighbouring genes for each insertion in the sequence are documented in Table 3. Based on this analysis, a striking effect of ISS12 on the phenotype was expected for the insertion in srpA. This gene encodes the inner membrane protein of the efflux pump, which is crucial for resistance to solvents (Kieboom et al., 1998a; Wery et al., 2001; Volkers et al., 2015).

In general, insertion sequences have been demonstrated to play an important role in the adaptation towards environmental stresses. They are able to regulate gene and operon expression by diverse molecular mechanisms and to increase the rate of adaptive mutations (Stoebe and Dorman, 2010; Casacuberta and González, 2013). In few cases, insertion and excision of insertion sequences have been demonstrated to regulate phenotypical changes (Ziebuhr et al., 1997; Hammerschmidt et al., 1996; van der Woude and Bäumler, 2004). An insertion sequence of the IS21 family has also been associated with an increased expression of the MexAB OprM efflux system in P. aeruginosa (Boutoille et al., 2004). In agreement with a role in the tolerance towards high solvent stress in P. putida S12 (Wery et al., 2001; Sun and Dennis, 2009; Volkers et al., 2010), transposition of insertion sequences has been associated with adaptation towards high physiological stress in E. coli, such as osmolarity (Stoebel et al., 2009), metal-limiting conditions (Chou et al., 2009) and nutrient limiting conditions (Gaffé et al., 2011).
### Table 3. Analysis of ISS12 insertion sites in *P. putida* S12/A.

| Located on | Variant | Position | Orientation | Presence in previous sequences | Directed Repeat (sequence) | Directed Repeat (length) | ISS12_location |
|------------|---------|----------|-------------|--------------------------------|----------------------------|-------------------------|----------------|
| Chromosome | ISS12   | 5798528-2596 | Reverse | No | GGAAGTG | 7 | Integrase – Peptidase S24 |
| Chromosome | ISS12   | 1303416-1306017 | Forward | No | TCTGTG | 6 | Membrane protein (UPF0114 family) – cation transporter |
| Chromosome | ISS12   | 1343201-1345781 | Forward | No | CACTACC | 7 | Putative Holin – putative integrase |
| Chromosome | ISS12   | 1508754-1511356 | Forward | No | CGCGGAC | 7 | Major facilitator transporter (MFS) |
| Chromosome | ISS12   | 1901500-1904102 | Reverse | Yes | TTCTTGG | 7 | ABC transporter |
| Chromosome | ISS12   | 2092282-2094884 | Forward | Yes | GCAAACAC | 7 | Copper resistance gene |
| Chromosome | ISS12   | 2509647-2512248 | Forward | No | CAACAT | 6 | Hypothetical protein |
| Chromosome | ISS12   | 2747110-2749712 | Reverse | No | GCTAGTG | 7 | Amidohydrolase, Sarl regulator |
| Chromosome | ISS12   | 2756345-2758947 | Reverse | No | GACGGTT | 7 | Benzoylformate decarboxylase |
| Chromosome | ISS12   | 2767364-2769967 | Reverse | No | CCTGTATG | 8 | Serine – tRNA ligase |
| Chromosome | ISS12   | 2913093-2915695 | Reverse | No | CATGAC | 7 | Hypothetical (First gene in Type IV secretion operon) |
| Chromosome | ISS12   | 3348765-3489368 | Forward | No | CACAGGA | 8 | PrfB RsmZ-like tRNA |
| Chromosome | ISS12   | 3503916-3506518 | Reverse | No | CGAGGTG | 7 | TRNA 2-thiocytidine biosynthesis protein |
| Chromosome | ISS12   | 4425759-4428360 | Forward | No | GGGGGG | 6 | Glutaminase |
| Chromosome | ISS12   | 4894007-4896609 | Reverse | Yes | CGCAAGT | 7 | Glycosyl hydrolase family 32 |
| Chromosome | ISS12   | 4905864-4908466 | Forward | No | AGCTCGA | 7 | Hypothetical protein |
| Plasmid    | ISS12_C | 4954933-4957427 | Forward | Yes | ATGACA | 6 | Hypothetical |
| Plasmid    | ISS12   | 4963941-4966543 | Forward | Yes | GACAACG | 7 | SH3 domain-containing protein |
| Plasmid    | ISS12   | 5273052-5275654 | Forward | No | CATAGGC | 7 | GntR transcriptional regulator – Transporter |
| Chromosome | ISS12   | 5346755-5350052 | Forward | No | GGCGTG | 7 | Transposase |
| Chromosome | ISS12   | 5641776-5644378 | Forward | Yes | CTGATG | 7 | Major facilitator transporter (MFS) |
| Plasmid    | ISS12_C | 53788-540369 | Reverse | Yes | GCCCTGGC | 8 | Transposase – Hypothetical protein |
| Plasmid    | ISS12   | 80378-82980 | Forward | No | TCTACAC | 7 | Tellurium resistance-like protein |
| Plasmid    | ISS12   | 2753710-2756312 | Forward | No | CCGCT | 5 | Hypothetical protein |
| Plasmid    | ISS12   | 2756345-2758947 | Reverse | No | GACGGTT | 7 | Carbon storage regulator |
| Plasmid    | ISS12   | 312315-313508 | Reverse | No | ACCACAG | 7 | Solvent resistance pump (SrPA) |
| Plasmid    | ISS12   | 399104-401706 | Forward | No | GCCACAG | 7 | Hypothetical protein |
| Plasmid    | ISS12   | 436378-4626280 | Reverse | No | CTCCTTG | 7 | Hypothetical protein |
| Plasmid    | ISS12   | 488389-490991 | Forward | No | CGCATGGC | 8 | Hypothetical protein |

**Notes:**
- **a.** Insertions in a gene.
- **b.** Insertion between two genes.
- **c.** The insertion at the promoter regions of listed genes.

We hypothesize that the multiple translocations events of ISS12 in *P. putida* S12/A may have been caused by physiological stress or unintended selection on rich media. As the strain was kept in cryostorage, the translocations likely occurred during transfers from one laboratory to another, which happened at least three times after submission to the ATCC culture collection. This highlights the importance of proper strain shipment and maintenance for the reproducibility of experiments in different laboratories. The analysis of insertion sites indicated translocation into two major facilitator transporters, one ABC transporter, one efflux transporter and one regulator that likely affects a transporter. Reducing the required energy for these transporters would be favourable for growth. In addition, tellurium resistance, copper resistance and benzoylformate decarboxylase genes are not required under growth conditions in rich media, and under these growth conditions, it could also decrease the required energy for cell maintenance.

**ISS12 transposition and *P. putida* S12 revertants**

As expected from disruption of *srpA* by ISS12, *P. putida* S12/A did not show any colonies after 1–20% v/v toluene shock for 30 min, whereas a large number of colonies were obtained with the control *P. putida* S12-ATTC strain (schematically visualized in Fig. 2). To investigate the role of ISS12 on solvent resistance in *P. putida* S12/A, selective pressure experiments in toluene were performed (Fig. 2A). Overnight cultures of *P. putida* S12/A were grown to mid-exponential phase (OD_{600} ≈ 0.7); different dilutions were plated on LB agar and incubated inside and outside an exsiccator with a saturated toluene atmosphere at 30°C. Surprisingly,
under these conditions, a small number of colonies appeared after 2 days on the plates under the toluene atmosphere (Fig. 2A). The ratio of colonies on the plates incubated inside and outside of the exsiccator was 1:107 respectively. Colony PCRs on srpA using primers pr29F and pr29R showed the absence of ISS12 in srpA in all the colonies (19 of 19) selected from plates incubated under toluene atmosphere, while colonies selected from plates outside of the exsiccator retained the ISS12 element in this gene (Fig. 2B). From these PCR products, eight samples were sequenced together with 2 PCR products from P. putida S12 as a control, using primers pr29F and pr29R. The alignment of these sequences around the ISS12 insertion site in srpA showed a 100% identity to the control, and the sequence of srpA was completely restored (Fig. S6A). To confirm that these colonies are the result of a genuine reversion, from six colonies gDNA was isolated and the presence of all other 33 ISS12-elements was verified by PCR. Except for the ISS12 in srpA, no consistent translocation from other sites was observed in these six revertants (Table S2). As disruption of srpS gene is also known to increases solvent tolerance in P. putida S12 (Wery et al., 2001; Sun and Dennis, 2009), PCRs were also performed on srpS in revertant strains (Fig. S6B). The amplified products of srpS was the size of an intact gene, and no disruption by ISS12 was observed in P. putida S12/A nor in any of the revertant strains.

In line with the important role of the SrpABC efflux pump in solvent tolerance (Kieboom et al., 1998b), disruption of srpA by ISS12_A caused specific and dramatically reduced solvent tolerance in this strain. Loss of this specific insertion sequence under stress conditions restored srpA function and concomitantly, solvent tolerance. We observed a lower excision rate ($10^{-7}$) of ISS12 from srpA compared to early observations of insertion into srpS ($10^{-6}$; Wery et al., 2001). By nature, the excision rate must be lower than the insertion rate to maintain the insertion sequence. Possibly, insertion and excision require the activation of DNA replication, which might be physiologically unfavourable under severe stress conditions (Arias-Palomo and Berger, 2015; van der Woude and Bäumler, 2004; Berger and Haas, 2001). For the transposition as well as phase variation events, the physiological state of the bacterium seems to play an important role and often occurs during DNA replication (van der Woude and Bäumler, 2004). The mechanism for transposition in the IS21 family requires the transposase for the cut-and-paste action and the ATPase AAA for regulation of the insertion site. Both are encoded on these insertion sequences (Berger and Haas, 2001; Arias-Palomo and Berger, 2015). Although the molecular regulation mechanism of ISS12 was not the focus of this study, the first indication of a mechanism for the excision of ISS12 is likely enzymatic. Probably, by the same transposase that is involved in the insertion, because this family of insertion sequences replicate through a cut-and-paste mechanism (Berger and Haas, 2001; Skipper et al., 2013). Excision mediated by a homologous recombination event combined with a skipping of the DNA replication complex cannot be excluded, although we consider it less likely. This is due to the fact that for restoration of the efflux pump, only clean excision of ISS12 from srpA results in an intact gene product. This includes the removal of exactly 7-bp directed repeat. Hence, excision not resulting in intact gene product was not observed in revertants which survived toluene atmosphere, which might partly account for the two orders of magnitude lower excision rate compared to insertion rate of ISS12.

**Mobile elements and biotechnological applications**

Several studies show a change in the number of ISS12 elements in P. putida S12 under biotechnologically relevant conditions, for example, during adaptation to benzene (Volkers et al., 2010), under toluene shock (Wery et al., 2001), and in this work describing excision of ISS12 during toluene stress. The remaining ISS12 elements in P. putida S12/A were retained in the revertants, which indicates that the transposition at these sites does not affect the tolerance towards toluene. The present study provides further evidence for involvement of IS21 family of insertion sequences in environmental adaptation (Casacuberta and González, 2013), but also highlights a fluidity of bacterial genomes, which might be undesirable in a biotechnological context. Another insertion sequence, ISPpu21, is also found in srpS that regulates its expression in similar way as ISS12 (Sun and Dennis, 2009). These examples show a clear role of these insertion sequences in rapid adaptation to solvent stress. The exact trigger of transposition and the associated regulation of the transposase genes should play an important role in this context. Further analysis is required to investigate the molecular mechanism involved in the regulation of this large family of insertion sequences in beneficial adaptation to environmental changes and also the adverse effects due to genome instability in biotechnological applications.

**Experimental procedures**

**Bacterial strains**

*Pseudomonas putida* S12 was isolated at Wageningen University, the Netherlands, and deposited at (ATCC ATCC700801). For this study, the deposited *P. putida* S12 at ATCC and the wild-type strain present at TNO were acquired and compared to the recently sequenced
S12/A strain (CP009974 and CP009975). Permanent cultures were stored in glycerol stocks at −80°C.

Cultivation conditions

All strains were cultivated on Lysogeny broth (LB), containing 10 g/l tryptone, 5 g/l yeast extract and 5 g/l sodium chloride using shake flasks. For solvent containing media, experiments were performed in 250-ml tightly closed Boston bottles, containing 20-ml medium, in a horizontal shaker (Innova 4330, New Brunswick Scientific) at 200 rpm and 30°C. Growth was monitored by measuring the optical density at 600 nm (OD600) using a spectrophotometer (Ultrospec 2100 pro; Amersham Biosciences, Piscataway, New Jersey, USA).

For organic solvent adaptation, overnight cultures of P. putida S12 strains were diluted 1:50 in 50 ml of fresh LB medium and grown up to an OD600 of about 0.7. At this mid-log phase, 16 ml of culture was supplemented with 4 ml of toluene using tightly closed Boston bottles and incubated in horizontal shaker at 30°C for 30 min. As control, 16-ml culture was supplemented with 4 ml water and grown under same conditions. The tolerance of the strains to solvent shock was determined by spreading suitable dilutions or total cell content on LB plates and scoring for growth or no growth.

DNA methods

All PCRs for analysing the presence of insertion sequences were performed using Phire polymerase (ThermoFisher, Waltham, MA, USA), and for sequence analysis, Phusion polymerase (ThermoFisher, Waltham, MA, USA) was used according to the manufacturers’ instructions. The colonies from these plates were analysed in solvent shock experiments, and PCR was performed to confirm the presence or absence of insertion sequences.

For organic solvent shock, overnight cultures of P. putida S12 strains were diluted 1:50 in 50 ml of fresh LB medium and grown up to an OD600 of about 0.7. At this mid-log phase, 16 ml of culture was supplemented with 4 ml of toluene using tightly closed Boston bottles and incubated in horizontal shaker at 30°C for 30 min. As control, 16-ml culture was supplemented with 4 ml water and grown under same conditions. The tolerance of the strains to solvent shock was determined by spreading suitable dilutions or total cell content on LB plates and scoring for growth or no growth.

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manual. All primers were purchased from Sigma-Aldrich (St. Louis, MO, USA) and are listed in Table S1. All PCRs were analysed by size specific gel electrophoresis on 1% (w/v) TAE agarose gels containing 5 μl ethidium bromide per 100 ml agarose solution in an electric field (90V, 0.5x TAE running buffer). The PCR fragments were isolated from the agarose gel using the High Pure PCR Preparation Kit (ThermoFisher) and were either cloned into pJET1.2 (ThermoFisher) before sequencing or directly sequenced at Macrogen (Amsterdam, the Netherlands). P_up, P_up_soxR and P_down sequences were amplified by nested PCR using primers listed in Table S1 from gDNA of P. putida S12. These sequences were subsequently cloned into mini-Tn7 probe vector using Pacl and AvrII restriction sites (Zobel et al., 2015), and integration into the genome of S12 was verified by PCR. The promoter activities were calculated based on the GFP intensity measurements during the growth at the log phase using the biolector (m2p-labs), and induction of the promoters was done with H2O2 at early exponential phase.

Sequence analyses

All analysis was performed using GENEOUS SOFTWARE (BioMatters, Auckland, New Zealand), unless otherwise stated in the text. BLAST search of insertion sequence ISS12 was performed at the NCBI website (Altschul et al., 1990). A query coverage of >70% and an e-value >E−5 were used as cut-off. The selected sequences were aligned using MAFFT (Katoh and Standley, 2013) or Muscle (Edgar, 2004); and phylogenetic trees were created using default settings of FastTree Builder (Price et al., 2009) integrated within the Geneious software. Phylogenetic trees were created using the ‘maximum likelihood’ and pseudocounts. The trees were visualized as cladogram in increasing order, and distances were computed in ‘number of base substitutions per site’. The ISFinder (Sigueri et al., 2005) and ACLAME (Leplae et al., 2009) were used to identify additional insertion sequences. In addition, for the best matching identities, results were aligned using the available full sequences to get the percentage identity and to avoid information loss due to shorter homologous regions retained by a BLAST hit.

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Conflict of Interest

None declared.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Cladogram of similar insertion sequences to ISS12.
**Fig. S2.** Alignment of different ISS12 variants in *P. putida* S12.
**Fig. S3.** Activity of ISS12-putative promoter sequences under oxidative stress.

**Fig. S4.** Sequence alignment of cloned ISS12-variant B in *P. putida* S12/A.
**Fig. S5.** Presence or absence of ISS12 insertion sites in *P. putida* S12.
**Fig. S6.** Sequence alignment of *srpA* and PCR amplification of *srpS* in *P. putida* S12/A revertants.

**Table S1.** List of primers used in this study.
**Table S2.** PCR analysis of ISS12 elements in *P. putida* S12/A and the revertants after toluene stress.