An Insertion Sequence Prepares *Pseudomonas putida* S12 for Severe Solvent Stress*

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The novel insertion sequence ISS12 plays a key role in the tolerance of *Pseudomonas putida* S12 to sudden toluene stress. Under normal culturing conditions the *P. putida* S12 genome contained seven copies of ISS12. However, a *P. putida* S12 population growing to high cell density after sudden addition of a separate phase of toluene carried eight copies. The survival frequency of cells in this variant *P. putida* S12 population was 1000 times higher than in "normal" *P. putida* S12 populations. Analysis of the nucleotide sequence flanking the extra ISS12 insertion revealed integration into the srpS gene. srpS forms a gene cluster with srpR and both are putative regulators of the solvent resistance pump SrpABC. SrpABC makes a major contribution to solvent tolerance in *P. putida* S12 and is induced by toluene. The basal level of srp promoter activity in the *P. putida* S12 variant was seven times higher than in wild-type *P. putida* S12. Introduction of the intact srpRS gene cluster in the variant resulted in a dramatic decrease of survival frequency after a toluene shock. These findings strongly suggest that interruption of srpS by ISS12 upregulates expression of the solvent pump, enabling the bacterium to tolerate sudden exposure to lethal concentrations of toxic solvents. We propose that *P. putida* S12 employs ISS12 as a mutator element to generate diverse mutations to swiftly adapt when confronted with severe adverse conditions.

It has long been recognized that DNA in living organisms is not a static entity. The process of continuous mutation of DNA enables adaptation to changing environments, and it is a prerequisite for evolution. It was generally accepted that this mutation and subsequent arising of variant organisms is a spontaneous process that generates a pool of genetically different individuals in a population under nonselective conditions, from which under selective conditions the individual(s) with beneficial mutation(s) will originate.

In 1943 Luria and Delbrück (1) were the first to experimentally study the origin of phage-resistant *Escherichia coli* mutants that arose from a sensitive population if plated in the presence of phage. They concluded, in favor of the spontaneous or growth-dependent mutation hypothesis, that the mutation to phage resistance was already generated in the population prior to exposure to the phage. Work by Cairns et al. (2) did not support this conclusion, providing evidence that mutation could also originate from a more “directed” process, which occurred after cells were put on selective plates. This type of mutation was named adaptive (3) or stationary phase mutation (4) and is observed in non- or slow growing populations of bacteria and yeasts (5) subjected to nonlethal stress.

Adaptive mutation is generally screened for by reversal of specific mutations in genes that bring about an auxotrophy. It was shown that adaptive mutation is a stress-inducible mechanism that involves transient genome-wide hypermutation of a subpopulation of cells, so-called mutants (6–9). Mutator strains are thought to be DNA-mismatch repair-deficient strains (10) that generate mutations at high frequency (11), creating diversity in a population and thereby increasing the chance for survival under unfavorable conditions.

Most investigations that study spontaneous and adaptive mutations employ reversal systems and thus exclude a possible role of insertion sequence elements. However, these elements could provide an important mechanism for swift genetic adaptation, because they move through the genome (in-)activating genes and introducing genomic rearrangements (12–14).

In 1983 Chao et al. (15) provided the first evidence that transposable elements may act as mutator genes conferring evolutionary advantage under chemostat culturing conditions. In competition experiments using an *E. coli* strain with and without transposon Tn10, it was found that the Tn10 strains win, if present at a starting ratio above 10<sup>−8</sup>. Additionally, it was found that the winning Tn10 strains had a transposition to a new, undetermined site. It was concluded that Tn10 conferred advantage by increasing the mutation rate of the host bacterium. More recently it was shown that insertion sequence elements play an important role in genetic adaptation of *E. coli* under starving conditions (16).

Here we address the role of a newly discovered insertion sequence in genetic adaptation to sudden lethal solvent stress. It resides naturally in the genome of the solvent-tolerant bacterium *Pseudomonas putida* S12.

Solvent-tolerant bacteria are quite extraordinary organisms able to grow in the presence of a separate phase of solvents like toluene. In normal bacteria these solvents accumulate within a few minutes (17) in the membranes of cells to concentrations that destabilize the lipid order and bilayer stability (18), thus destroying structural and functional properties. In solvent-tolerant bacteria two major adaptational responses have been found that counterbalance these effects. The first mechanisms...
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... that bring about a less permeable barrier to solvents. The second mechanism is an extrusion system that transports solvents from the inner membrane out of the cell (17, 24–30). In P. putida S12, the srpABC genes encode such a solvent efflux pump responsible for the extrusion of uncharged lipophilic compounds like toluene (25).

Both adaptational responses occur after exposure to nonlethal inducing amounts of toluene (pre-adaptation). In this way, all cells in the population are prepared to survive even a separate phase of the solvent (1% v/v). This solvent-tolerant phenotype is rapidly lost when incubated in the absence of toluene. If toluene (1% v/v) is added shock-wise to cells that are not pre-adapted, then lysis of cells occurs. Surprisingly, a few cells in the population were consistently found to survive such a shock (26, 31–33). These surviving individuals eventually grew to a high density in the presence of a separate phase of the solvent. Contrary to pre-adapted cells, such a population maintained its toluene-tolerant phenotype after prolonged incubation without toluene (32), suggesting a tolerant variant into a solvent-tolerant variant gene.

In this study we show that the insertion sequence ISS12 is the wild-type strain and P. putida S12PT is a variant of P. putida S12, grown after sudden addition of 1% (v/v) toluene that carries one extra copy of insertion sequence ISS12 in srpS. P. putida JK1 is derived from P. putida S12 by transposon mutagenesis and carries a kanamycin resistance cassette in its genomic DNA in a stable fashion (25).

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, and Growth of Strains—P. putida S12 (34) is the wild-type strain and P. putida S12PT is a variant of P. putida S12, grown after sudden addition of 1% (v/v) toluene that carries one extra copy of insertion sequence ISS12 in srpS. P. putida JK1 is derived from P. putida S12 by transposon mutagenesis and carries a kanamycin resistance cassette in its genomic DNA in a stable fashion (25).

Incubations—Incubations in the presence of toluene were carried out in air tight Boston bottles equipped with Minivent valves (Plastic Separations) in a horizontally shaking water bath at 30 °C. The survival time of the population was determined by measuring the amount of colony-forming units (cfu), before and after incubating cells, that were in the early exponential growth phase (an optical density of 0.5 cm−1 at 600 nm), for 0.5 h in the presence of a separate phase of toluene (1% v/v). The cell viability was determined by plating 0.1-mL samples onto LB agar plates. The agar plates were incubated for 20 h.

For induction experiments 3 mm toluene was added to cultures in the early exponential phase (an optical density of 0.3 cm−1 at 600 nm). Cells were allowed to grow to an optical density of 1.5 cm−1 at 600 nm. Subsequently, β-galactosidase activity was determined by the method of Miller (36), using chloroform and sodium dodecyl sulfate to permeabilize the cells.

DNA Techniques, Plasmid Construction, and PCR Primers—Total genomic DNA from P. putida strains was prepared by the hexadecyl trimethyl ammonium bromide (CTAB) procedure (37). Insert sequences were isolated from 0.7% agarose gels using the QIAEX II gel extraction kit (Qiagen). DNA digestions and ligations were carried out using enzymes purchased from Life Technologies and applied according to the supplier's recommendations. PCR amplification reactions were run according to the manufacturer's protocol, using primers 1 and 2 (see "PCR primers"). Sequencing of purified double-stranded plasmid DNA was accomplished using AmpliTaq FS DNA fluorescent dye terminator reagents (PerkinElmer Life Sciences) in a Gene Amp PCR system 9600 (PerkinElmer Life Sciences). Sequencing products were detected using an Applied Biosystems 373A stretch-automated DNA sequencer (Applied Biosystems Inc.). Nucleotide and protein sequence analysis was carried out with the National Center for Biotechnology Information database (35). Plasmid DNA was isolated by the alkaline-sodium dodecyl sulfate lysis method of Birnboim and Doly (38). For DNA hybridizations, total DNA of different P. putida strains was digested, separated by agarose gel electrophoresis, and transferred to nylon filters according to standard protocols (35). Hybridizations were done using the nonradioactive DIG DNA labeling and detection kit (Boehringer Mannheim Biochemicals) according to the manufacturer's recommendations. PCR reaction for amplifying the DNA region in P. putida JK1CAM containing insertion sequence ISS12 was performed using Pwo DNA polymerase (Roche Molecular Biochemicals) with high fidelity DNA synthesis. The DNA amplification reaction was set up according to the manufacturer's protocol, using primers 1 and 2 (see "PCR primers"). Sequencing of purified double-stranded plasmid DNA was accomplished using AmpliTaq FS DNA fluorescent dye terminator reagents (PerkinElmer Life Sciences) in a Gene Amp PCR system 9600 (PerkinElmer Life Sciences). Sequencing products were detected using an Applied Biosystems 373A stretch-automated DNA sequencer (Applied Biosystems Inc.). Nucleotide and protein sequence analysis was carried out with the National Center for Biotechnology Information database (35). Plasmid DNA was isolated by the alkaline-sodium dodecyl sulfate lysis method of Birnboim and Doly (38).

RESULTS

Characterization of ISS12—ISS12 was isolated from a chloramphenicol-resistant P. putida S12 mutant, JK1CAM, that was shown to carry a 2.5-kb interruption of the gene coding for the solvent-pump porin, srpC. A 4-kb DNA fragment containing the srpC gene with the interrupting DNA was generated by PCR amplification with primers designed on both ends of srpC.

The nucleotide sequence analysis of the interrupting DNA revealed a 2598-bp sequence with typical characteristics of an insertion sequence (IS) element (Fig. 1). This sequence has been submitted to the GenBankTM/EBI database (accession number AF292393). The element was delimited by two perfect matching inverted repeats of 14 bp (IR-L1/IR-R1) and 18 bp (IR-L2/IR-R2). Furthermore, two open reading frames were found, orf1 and orf2, that putatively encode proteins of 509 amino acids (58,175 Da) and 251 amino acids (28,528 Da), respectively. These amino acid sequences have extensive homology with those deduced from orf1 and orf2 of IS1491, isolated from Pseudomonas alcaligenes NCIB 9867 (Table I) (42). Less, but significant homology is observed with other IS elements that, like IS1491, belong to the IS1 family of insertion sequences. In addition, structural characteristics of ISS12 also reveal relationship with IS21 (Fig. 1) (14, 43). The reading frame of orf2 is located in a relative reading phase of −1 compared with orf1, and both orfs are separated by only 17 bp. Furthermore, orf1 reveals two motifs typical of insertion se-

The abbreviations used are: PCR, polymerase chain reaction; cfu, colony-forming units; kb, kilobase(s); IS, insertion sequence; bp, base pair(s).

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sequences. The N terminus contains 25 amino acids that have high probability of forming a helix-turn-helix configuration capable of DNA binding and a so-called DDE triad. DDE motifs are found in the catalytic domains of transposases of many bacterial elements and integrases of retroviruses (45). The putative protein encoded by orf2 contains well conserved potential nucleoside triphosphate binding domains A and B (45).

Distribution of ISS12 in a P. putida S12 Population and the Effect of a Solvent Shock—The distribution of ISS12 over the genome of P. putida S12 was determined, and the effect of a toluene shock hereon was studied. For this purpose, chromosomal DNA was isolated from an aliquot of P. putida S12 cells cultured in LB to an optical density at 600 nm (A600) of 0.5. Subsequently, 1% (v/v) toluene was added to the remainder of the culture, killing approximately 99.99% of the cells within 30 min. After 24–48 h, a culture had grown up and total DNA was isolated from this population, designated as P. putida S12PT (post-toluene). Both DNAs were digested with KpnI, which does not cut in the DNA of ISS12. After separation by agarose gel electrophoresis, the DNAs were transferred to nylon filters and hybridized with an internal 650-bp DNA probe from ISS12 (Fig. 2). Lane 1 shows the hybridization pattern of DNA from P. putida S12 cells before the toluene shock. Here seven distinct hybridizing DNA fragment ranging from 3.5 to 10 kb are visible, indicating that at least seven copies of ISS12 are dispersed over the genome of P. putida S12. In lane 2 the result with P. putida S12PT DNA is shown. Here an extra hybridizing DNA fragment of approximately 9 kb is visible, indicating transposition of ISS12.

This result strongly suggested that individuals of a P. putida S12 population that carry an extra copy of ISS12 in a particular location in the genome are more tolerant of a toluene shock.

To investigate this, exponentially growing unadapted P. putida S12 cells were exposed for 30 min to 1% toluene and subsequently an aliquot was spread on LB agar. Only 0.004% of the plated cells had survived to form colonies. DNA was isolated from 15 individual colonies and analyzed for the distribution of ISS12 as described for P. putida S12 and S12PT. The hybridization pattern of 1 DNA resembled that of the wild-type P. putida S12, whereas the pattern of 14 DNAs was identical to P. putida S12PT DNA (data not shown).

We further tested the correlation between transposition and solvent tolerance by comparing the survival frequency of both P. putida S12 and S12PT after sudden addition of 1% (v/v) toluene. It was found that approximately 0.004% of the P. putida S12 and 5% of the P. putida S12PT cells survived the shock, a 1000-fold increase of survival frequency.

Phenotypic and Genotypic Dynamics of the P. putida S12PT Population—The stability of both genotype and phenotype of a P. putida S12PT population was tested during prolonged cultivation in LB medium in two different ways. In the first approach the growth experiment was started with an inoculum from P. putida S12PT that had emerged from wild-type P. putida S12 after a toluene shock. This P. putida S12PT population was possibly not genetically homogeneous, because there was a chance that wild-type cells were also present. DNA was isolated from the culture after 13, 26, 39, 52, 65, 78, 91, and 104 generations of growth after inoculation. Southern hybridization on these DNAs was performed with an ISS12-derived probe after digestion with KpnI (Fig. 3A). After 91 generations

Fig. 1. Nucleotide sequence of ISS12 from P. putida S12. The deduced amino acid sequences of the encoded proteins are shown below the nucleotide sequence. Left and right inverted repeat sequences (IR-L, IR-R) are boxed, putative ribosome-binding sites (RBS), are underlined, putative RNA polymerase-binding sites (~35 and ~10 boxes) are in boldface and indicated with an arrow, termination codons are indicated with an asterisk, the Orf1 N-terminal amino acids, which form a potential helix-turn-helix motif, are underlined by a single line, the conserved DDE catalytic triad in Orf1 is indicated with dots, and the nucleoside triphosphate binding domains A and B in Orf2 are underlined by double lines.
mixture P. putida JK1 had overgrown P. putida S12PT, because no significant differences were found in cfu counts between both types of plates. To obtain a more accurate measure of the competition advantage of P. putida JK1, a starting ratio of 0.001 (P. putida JK1:P. putida S12PT, 1:1000) was chosen. Here it was found that the share of P. putida JK1 in the cell mixture had increased 50-fold. In a control experiment a mixture of P. putida S12 and P. putida JK1 was also tested. Here both strains were mixed to a starting ratio of 1. The end ratio showed that the share of P. putida JK1 had declined by a factor 7, indicating a competitive advantage of the wild-type P. putida S12 over the reference strain.

These results clearly indicate that P. putida S12 has a competitive advantage over P. putida S12PT under these nonselective conditions.

Nucleotide Sequence Analysis of the Region Adjacent to the Extra ISS12 Copy in P. putida S12PT—The exact location of integration of the extra ISS12 copy in P. putida S12PT was determined (Fig. 4). For this purpose this copy was isolated from BglII-digested total DNA from P. putida S12PT on a 9-kb DNA fragment and cloned. The nucleotide sequence of the DNA-region flanking ISS12 was determined using primers specific for either end of ISS12. Screening for similar nucleotide sequences in the GenBank™/EBI database revealed a 100% match with the gene srpS (GenBank™/EBI accession number AF061937), which is located 223 bp upstream of the genes for the solvent resistance pump srpABC. srpS forms a gene cluster, srpRS, with the downstream-located srpR (Fig. 4). Sequence similarity studies revealed that both genes putatively encode regulatory proteins involved in control of srpABC expression (not shown).

Two nucleotides adjacent to the left inverted repeat IR-L and six nucleotides adjacent to IR-R could not be assigned to either ISS12 or srpS.

To verify that the extra insertion of ISS12 in the 14 toluene shock-surviving individuals was also confined to this gene, Southern analysis was performed on their DNAs. The DNAs were digested with PstI and SacI, which cut at either end of the srpS gene (Fig. 4) and do not cut ISS12. Hybridization with an internal DNA probe from ISS12 yielded a 3.4-kb hybridizing band, which was absent in wild-type P. putida S12 (result not shown), reflecting integration of the insertion element (2.6 kb) in srpS (0.8 kb).

srpABC Promoter Activity in P. putida S12PT—We anticipated that the direct cause for increased solvent tolerance in P. putida S12PT was the disruption of the putative pump regulator srpS. To support this hypothesis, we introduced promoter probe vector pKZR-srp (24) into P. putida S12 and S12PT. In this plasmid, the promoter region of srpABC is fused to the promoterless lacZ gene. Both strains were cultivated either in the presence or absence of 3 mM toluene to the late exponential phase. We chose to use 3 mM toluene, because it was shown previously that this amount induced the expression of the lacZ gene in pKZR-srp significantly, without affecting the growth of P. putida S12 (24). It was shown that β-galactosidase activity in P. putida S12PT transformant was approximately 7-fold...
higher than in the _P. putida_ S12 transformant in the absence of toluene. If grown in the presence of toluene, β-galactosidase activity had increased 10-fold in the _P. putida_ S12 transformant and 1.5-fold in _P. putida_ S12PT transformant reaching comparable levels in both strains (Table II). These results show that the basal level of _srp_ promoter activity is markedly higher in _P. putida_ S12PT and can only be matched in the wild-type strain after toluene induction. This finding suggests the tolerance of _P. putida_ S12 PT for a sudden toluene shock is based on constitutive, relatively high expression of the solvent pump and that, the other way around, the sensitivity of the wild-type strain is due to a lack of pump in the membrane at the instant of exposure. To test this hypothesis, the survival frequency of toluene-induced _P. putida_ S12 was determined. Cells were grown to an _A_600 of 0.5 in the presence of 3 mM toluene and subsequently diluted 1 to 5 into medium with 5 mM toluene. At an _A_600 of 0.5, 1% (v/v) toluene was added and dilutions of the culture were spread on LB agar. Approximately 5% of the cells survived to form colonies, which is comparable to the survival frequency observed with “uninduced” _P. putida_ S12PT. The DNAs of 15 surviving colonies were analyzed for distribution of _ISS12_. It was found that none contained an extra insertion (data not shown) and that toluene tolerance was lost within 10 generations of nonselective growth.

**Table II**

| _P. putida_ strain | β-Galactosidase activity<sup>a</sup> |
|--------------------|-------------------------------------|
|                    | _-toluene_ | _+toluene_ |
| _S12_ (pKRZ-srp)   | 1.6 (±0.1) | 16.6 (±0.2) |
| _S12PT (pKRZ-srp)  | 11.2 (±2.6) | 17.2 (±2.6) |

<sup>a</sup> _P. putida_ strains transformed with promoter-probe plasmid pKRZ-srp (in parentheses) were grown to the midexponential phase (an optical density of 0.3 at 600 nm) in LB broth. At this time point both cultures were divided in two and allowed to grow either in the absence (−) or presence (+) of 3 mM toluene to the late exponential phase (an optical density of 1.5 at 600 nm). Subsequently, β-galactosidase was determined by the method of Miller (36). Standard deviations are within parentheses.

**Table III**

| _P. putida_ strain<sup>b</sup> | Survival frequency<sup>c</sup> % |
|--------------------------------|-------------------------------|
| _S12_                          | 0.004                         |
| _S12PT_                        | 5                             |
| _S12PT (pJWB1)                 | 5                             |
| _S12PT (pJWsrpS)               | 5                             |
| _S12PT (pJWsrpRS)              | 0.0005                        |

<sup>b</sup> In parentheses are the names of the introduced plasmids.

of transposition in changing environments have hardly been studied.

We show here that _P. putida_ S12 under sudden lethal conditions is able to produce a genetic variant, by means of ISS12, enabling the bacterium to survive. In the variant _P. putida_ S12PT, the underlying mechanism of toluene shock survival appears to be the up-regulation of the _srpABC_ genes. This would imply that the region in which ISS12 is inserted is involved in repression of _srpABC_. It could still be argued that the structural change of the DNA upstream of the _srpABC_ genes resulting from the ISS12 insertion also influences the expression accounting for (part of) the toluene tolerance. However, complementation with intact _srpRS_ genes showed a dramatic decrease of toluene tolerance, proving that structural factors did not play an important role. Because complementation with _srpS_ alone did not result in a decreased tolerance, we propose that both _srpR_ and _S_ are needed for effective repression of the _srpABC_ genes. It goes without saying that more detailed experiments concerning these genes and possibly other are needed for a complete picture of the regulation of the solvent pump genes.

We showed that in _P. putida_ S12PT, activation of the _srp_ promoter is up-regulated in the absence of toxic solvents and we hypothesize for this reason that the high survival frequency of this variant is due to the fact that it is already prepared to deal with sudden solvent stress. Isken and De Bont (17) measured accumulation of 14C-labeled toluene in the membrane of _P. putida_ S12. Using 4 mM of the solvent, which is below the saturating concentration (6 mM) but toxic to the cells, they found maximum accumulation in the membrane within 10 min of incubation. More recently, Kieboom _et al_. (24) found that maximum activation of the _srp_ promoter in _P. putida_ S12 by toluene occurred 200 min after addition of the solvent. After ~60 min, activation reached 50% of the maximum. These findings indicate that a lack of time to engage the principal defense mechanism against sudden solvent stress is the main reason that over 99.99% of a _P. putida_ S12 population is killed upon a toluene shock. The solvent shock tolerant nature of this species lies in the presence of the extra copy of _ISS12_ in the _srpS_ gene in a small minority of its population.

It is highly unlikely that transposition of _ISS12_ occurs in response to the toluene shock. This is simply because the toluene will have had its killing effect before the _SrxAB_ pump is in operation. Indeed, we found that pre-adaptation to nonlethal amounts of toluene led to a high survival frequency of _P. putida_ S12 after subsequent addition of 1% of the solvent, without _ISS12_ transposition. From the above it follows that the variant, to survive, must be present before the toluene shock. Thus, under normal conditions _P. putida_ S12 always maintains a subpopulation, accounting for at least 0.004% of the whole population, that carries _ISS12_ inserted in _srpS_.

From our results it also becomes clear that mutation by _ISS12_ is a more important mechanism than other growth-dependent mutations, like point mutations, frameshifts, deletions, or others, that could lead to inactivation of _srpS_ and...
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subsequent toluene shock survival. This suggests that ISS12 is a mutator element that is employed by the bacterium to maintain subpopulations of preconditioned cells.

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