

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 up-regulates 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.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank[™]/EBI Data Bank with accession number(s) AF292393.

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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 reversion 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 mutators (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 reversion 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^{−4}. 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

deal with structural changes of the (outer) membranes (19–23) 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 transition into a solvent-tolerant genetic variant.

In this study we show that the insertion sequence *ISS12* is responsible for the emergence of this genetic variant. The dynamics of transposition and the underlying mechanism of solvent tolerance is uncovered, and the possible strategy of *P. putida* S12 to employ *ISS12* as a mutator element to maintain a subpopulation of genetic variants preconditioned for extremely adverse conditions is discussed.

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, growing 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). *P. putida* S12 (pKRZ-*srp*) and *P. putida* S12PT (pKRZ-*srp*) are the respective transformants of *P. putida* S12 and *P. putida* S12PT that carry the pKRZ-*srp* promoter-probe plasmid. This plasmid contains the promoterless *lacZ* gene downstream of the *srp* promoter (24). *P. putida* JK1CAM is a chloramphenicol-resistant mutant of *P. putida* S12 from which insertion sequence *ISS12* was first isolated by PCR¹ amplification. *E. coli* strain DH5 α (*supE44* Δ *lacU169* (ϕ 80 *lacZ*AM15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*) was used for amplification of recombinant plasmids according to standard methods (35).

LB broth (35) was used as complete medium. Solid media contained 2% of agar. Ampicillin (50 μ g/ml) was added to maintain all plasmids in *E. coli*. Kanamycin (50 μ g/ml) was added to maintain plasmid pKRZ-*srp* in *P. putida* strains, and gentamicin (10 μ g/ml) was added to maintain plasmids pJWB1, pJWsrpS, and pJWsrpRS in *P. putida* strains. *E. coli* and *P. putida* strains were grown at 37 °C and 30 °C, respectively.

Incubations—Incubations in the presence of toluene were carried out in airtight Boston bottles equipped with Mininert valves (Phase Separations) in a horizontally shaking water bath at 30 °C. The survival frequency in the presence of toluene 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 suitable dilutions in 0.9% (w/v) saline on 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 QIAEXII 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. 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 (Roche Molecular 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 reactions (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 BLAST server (39). Plasmids pGEM-7Zf(+) and pGEM-T Easy (Promega) were used as the cloning vector for genomic DNA and PCR-amplified DNA, respectively. Plasmid pJWB1 is an *E. coli*-*Pseudomonas* shuttle vector obtained after replacement of the *lacZ* α portion of pUCP22 (40) by the multiple cloning site from pGEM7-Zf(+) (Promega) flanked by a restriction site of *Sfi*I and *Not*I. pJWB1 was used for cloning *srpS* and *srpRS* as follows: DNA fragments containing the *srpS* and *srpRS* genes, including the 5'-noncoding region, were obtained after PCR amplification of these regions from total *P. putida* S12 DNA using primers 3 and 4 (*srpS*) and 3 and 5 (*srpRS*). Both PCR-amplified fragments were flanked by a restriction site of *Kpn*I and *Not*I and were ligated in pJWB1 cut with *Kpn*I and *Not*I to give rise to plasmids pJWsrpS and pJWsrpRS. Plasmid DNA was introduced in *P. putida* strains or *E. coli* DH5 α by electroporation (41) using a Gene Pulser (Bio-Rad Laboratories).

PCR primers (from 5' to 3'), with relevant restriction sites underlined are: 1, CCCGGGTCAAGGAGGTGACTCATG; 2, CGCGGCCGCG-ATAATTCGCCACTTCAGTTCC; 3, GCGGGTACCGACGCGGGGCTATTGCTGAATCG; 4, GCGGCGGCCGCTAGGGAGCTTTCTTCGACGC; and 5, GCGGCGGCCGCTCACTCGAAGGATTGACTTGC.

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*.² 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 GenBank™/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 IS21 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-

¹ The abbreviations used are: PCR, polymerase chain reaction; cfu, colony-forming unit(s); kb, kilobase(s); IS, insertion sequence; bp, base pair(s).

² J. Wery, unpublished data.

IR-L1

IR-L2

TCGCGATTCCACG	TGACTCGGACACCCATTCCACGCGCATCCGGACAGTGATT	CCACGCTGATCCGGACAC	TCATTCCACGAGCATCCGGACACCGACT	100
CCACGGTCATTCCGGACACTTTT	⁻³⁵ TGGCAGG	CAGCCACGAGGATTTATTCACTACCATCGATCTCTTTTTCGAAGCAGAG	^{RBS} AGGTCGTTGTGGAGCGTTT	200
ATCCATGCGTAAATCCGAGAGGTGTACGCTCAAGTTGACTGCGGCTGTCCGTGCGCAAGATCGCCCGCAGCCTGGGCATTGGCCACAGCAGTGCC				300
GGTGATTACCTCTGCCGTTT	GCCGCCAGCGGCCTCACCTGGCCCTGTTCGTTGTCCGATGCCGAGTTGGAGCAGCAACTGTTCCCGCCGGCCCCGGCGG			400
TTGCCAGTGAGAAGCGGCCTTTACCCGATTGGGCATGGGTGCATGCCGAAGTGC				500
GAGCCAGCCTCAGGGCTTTCAGTACAGCTGGTCTGTGAGCACTACCGAGCCTGGCAGGGCAAGTTGGACGTGGTGATGCGTCAGGAGCACCGCTCGGC				600
GAGAAGCTGTTGCTGCACTATGCCGGCCAGACGGTGCCGGTTATCGATCGCCACAGCGCGAGATCCGCCAGGCGCAGGTGTTGCTGCGGGTGCTCGGGC				700
CGTCCAGCTACACCTTCGCCAAGCCACCTGGTCGAGCAGCTGCCGGACTGGCTAGGCTCCCATGCCCGCTGCTTCGCCTTCCTCGGCGCGTGCCGGA				800
GATCGTGGTGCCGGACAACCTGCGCAGCGCGGTGAGCAAGAGTACCCTACGAGCCGACATCAACCCAGCTACCGCGATCTGGCCGAGCACTATGGC				900
GTGGCGGTGGTGCCGGCGCGGGCAGCAAAACCGCGCACAAGGCCAAGGCCAAGTCCGCGTGCAAGTGGTTCGAGCGTTGGATCCTCGCCGACTGAGGA				1000
ATCGGCAGTTCTTCTCCCTGGATGAAGTCAACACGGCCATCGCCGGGCTGCTGGAGCGGCTCAACCAACGCCCGTTCAAGAAGCTGCCGGGCTCCCGGCA				1100
GTCGGCCTTCGACAGCCTGGATCGTCCGGCGCTGCGCCCCCTGCCGGAGCAACCCTACGCTACGCGGAGTGAAGAAGCGCGGGTGACATCGACTAC				1200
CACGTCGAGGTGATGGGCATTACTACTCGGTGCGGTATCAACTGGTGAAGAAGCAGCTGGAGGTGCGCCTGACGGCGCGCACCGTCGAGTTTTTCCACG				1300
CCAACAGCGAGTGGCCAGCCACCTGCGCTCAATGCACAAGGGCAGGCACAGCAGCGAGCGGACATGCCCAAGGCCATCGCGAGCATGCCGAGTG				1400
GACGCCGCAACGGCTGATCCGCTGGGCGGAGCAGACCGGGCCGAACACCGCCGGCGTGATCCGGCACATCCTCGAACGGCGCATCCATCCGAGCAGGGC				1500
TACCGGGCCTGCCTGGGCATCTGCGCCTGGGTAAACCCACGGTGAGGCGCGTCTGGAGTTGGCCTGCGCTGCGCCATCAGCCTCGGCACGTGCAGCT				1600
ACAAGAGCCTCGAATCGATCCTGCGCCAGGGGCTGGAAAACCTGCCGTAGCTCAAAACCAACCTGCCGTGCTGCCGACGACACGCCAACCTGCGCGG				1700
ATCCGCCTACTACCACTGACCCCAAGGAATCCACCATGCTGCCCCATCCGACCCTGGACAAGCTGCAAAACCTGCGCCTGCACGGCATGCTCAAGGGCGC				1800
TGAATGAACAAGTAAACCCCGGACATCGACAGCCTGAGCTTGAAGAAGCGCTCGGCCTGCTGGTCGACCGGAGCTGACCGAACGCGATGACAAGCG				1900
CCTGAGCAGCCGCTGCGCCAGGCCCCGGCTCAAGCACAAACGCTGCCTCGAAGACATCGACTACCGCAGCCCGCGCGGACTGGATAAGGCGCTGATCCTG				2000
CAACTGAGCAGTGGTCAGTGGCTGCGCGACGGCCTCAACCTGATCATCGGCGGCCCCACCGGTGTCCGTTAAACCTGGCTGGCCTGCGCCCTGGCCCCAC				2100
AGGCCTGCCGGGAGGGCTACAGCGTGCCTACCTGCGCCTGCCACGTTTGTGGAAGAACTGGGTCTGGCCCATGGCGACGGCCGCTTCGCCAAGCTGAT				2200
GAGCAGCTACGCCAAGACCGACCTGCTGATCCTCGACGACTGGGGCTGGCCCCGTTACCGGCGAGCAACGGCGCGACATGCTGGAGCTACTGGACGAC				2300
CGTTACGGCCAGCGCTCGACATCGTACCAGCAGATGCCGTTGGACAAGTGGCACGAAGTATCGGCGATCCGACCTGGCCGATGCCATCCTCGACC				2400
GCCTGGTGACAACGCTTATCGGATCAATCTGAAGGGTGAATCAATGCGCAACGGACGAGAA	⁻³⁵ TTGACG		⁻¹⁰ TAACAT	2500
CCCTGCGTGCCTGCGCTCCGACTGCCTGTCCGAATGAGCGTGGAACCA	GTGTCGGATCAGCGTGG		CGCTGGAATCCGCA	2598

TABLE I
Identities and similarities of the putative proteins encoded by *orf1* and *orf2* from ISS12 with homologous proteins from most related insertion sequences

	Identity	Similarity	Organism	Accession number
	%			
Orf1 ISS12				
Orf1 IS1491	92	94	<i>P. alcaligenes</i>	U84154
Y4UI	47	65	<i>Rhizobium</i> sp.	Z68203
Orf1 IS408	47	66	<i>B. cepacia</i>	L09108
Orf1 IS1162	47	64	<i>P. fluorescens</i>	X79443
Y4BL	48	63	<i>Rhizobium</i> sp.	AE000066
Orf1 IS1474	48	62	<i>P. alcaligenes</i>	U67315
Orf2 ISS12				
Orf2 IS1491	82	82	<i>P. alcaligenes</i>	U84154
Orf2 IS1474	56	70	<i>P. alcaligenes</i>	U67315
Y4PL	54	70	<i>Rhizobium</i> sp.	AE000090
Orf2 IS408	53	68	<i>B. cepacia</i>	L09108
Y4UH	50	63	<i>Rhizobium</i> sp.	Z68203
Orf2 IS1162	55	66	<i>P. fluorescens</i>	X79443

quences. 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 (A_{600}) 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 *Kpn*I, 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

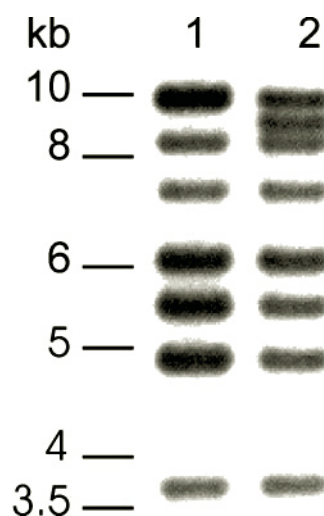


FIG. 2. Analysis of total DNA from *P. putida* S12 growing before and after a 1% (v/v) toluene shock. Both DNAs were isolated from the culture, digested with *Kpn*I and analyzed by Southern hybridization with a 0.7-kb DNA fragment from ISS12 as the probe. Lanes 1 and 2 show the hybridization patterns of *P. putida* S12 before and after the toluene shock, respectively.

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 *Kpn*I (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.

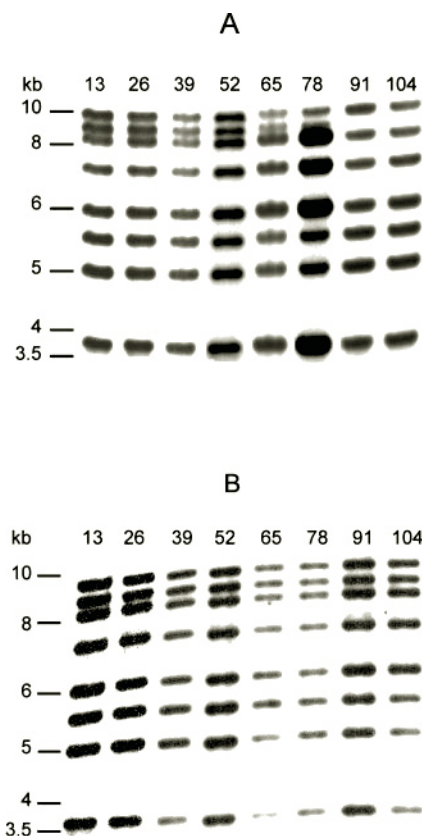


FIG. 3. Genetic stability of *P. putida* S12PT. Southern analysis of total DNA obtained from cultures inoculated with an aliquot of *P. putida* S12PT population growing after a solvent shock (A) or a pure *P. putida* S12PT colony (B). Both cultures were submitted to prolonged cultivation in the absence of toluene by repeated transfer into fresh medium. Total DNA was isolated at several instances during cultivation, digested with *Kpn*I, and hybridized with a 0.7-kb DNA fragment from ISS12 as the probe. Numbers above the lanes indicate generations of growth after inoculation. Arrows indicate the extra ISS12 insertion.

the extra ISS12 insertion could no longer be detected and the hybridization pattern resembled that of wild-type *P. putida* S12. In addition, the tolerance for a toluene shock decreased to wild-type levels after 104 generations (data not shown).

In the second approach we started the growth experiment with a pure *P. putida* S12PT clone (genetically homogeneous) and monitored the presence of the extra ISS12 copy during ~104 generations of cultivation (Fig. 3B). Now we found that the extra copy remained present in the population and the tolerance for a toluene shock remained high (data not shown). This result indicated that the extra ISS12 insertion is not readily lost from the genome, suggesting that the disappearance of the *P. putida* S12PT genotype in the first approach was due to out-competition by wild-type *P. putida* S12 cells that most probably were initially present as a minority in the starting culture.

It was further investigated if wild-type *P. putida* S12 has a selective advantage over *P. putida* S12PT in competition experiments. To facilitate discrimination between the different strains *P. putida* S12 containing a kanamycin marker, *P. putida* JK1 (25), was employed as the reference. Exponentially growing cells of both *P. putida* JK1 and *P. putida* S12PT were mixed and allowed to grow for 100 generations. Both the starting and end ratios of the two different strains were determined by plating suitable dilutions of the mixtures on LB agar with and without kanamycin and comparing the cfu counts on both plates. *P. putida* JK1 and *P. putida* S12PT were mixed to a starting ratio of 1 (*P. putida* JK1:*P. putida* S12PT, 1:1). In this

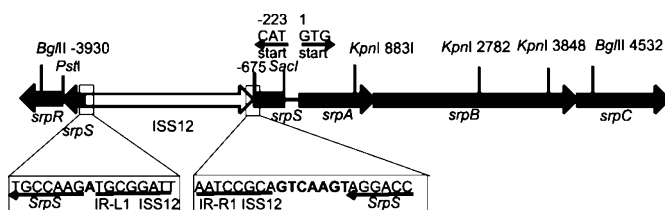


FIG. 4. Schematic representation of insertion of ISS12 in the *srpS* gene in *P. putida* S12PT. Nucleotide sequences adjacent to the left and right terminal IR-L1 and IR-R1 are boxed.

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 *Bgl*II-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 *Pst*I and *Sac*I, 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 pKRZ-*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 pKRZ-*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

TABLE II

Induction of β -galactosidase expression in *P. putida* S12 (pKRZ-srp) and *P. putida* S12PT (pKRZ-srp) cultured in the presence or absence of toluene

<i>P. putida</i> strain	β -Galactosidase activity ^a	
	–toluene	+toluene
	Miller units	
S12(pKRZ-srp)	1.6 (\pm 0.1)	16.6 (\pm 0.2)
S12PT(pKRZ-srp)	11.2 (\pm 2.6)	17.2 (\pm 2.6)

^a *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.

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 that 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.

Complementation of *P. putida* S12PT—The increased level of solvent pump-promoter activity in *P. putida* S12PT suggested that the most plausible explanation for toluene tolerance of this strain is the interruption of *srpS*. This would imply that complementation with the intact regulatory region would result in an increased sensitivity. To prove this, *P. putida* S12PT was transformed with plasmids pJWsrpS and pJWsrpRS, containing *srpS* and *srpRS*, respectively. The latter plasmid was also tested, because transcription of *srpR* is also likely to be impeded by the ISS12 insertion as *srpS* and *R* share a mutual upstream nontranslated region. The tolerance of transformants for a sudden shock of toluene was compared with *P. putida* S12PT transformed with empty plasmid pJWB1 (Table III). It was shown that sensitivity was dramatically increased in *P. putida* S12PT only if complemented with both *srpR* and *S*.

DISCUSSION

Insertion sequences have been shown to be important for genomic plasticity of certain bacteria. Over 500 bacterial insertion sequences have been characterized at the nucleotide sequence level (14), and in the literature many examples of insertion sequences can be found that are involved in activation of neighboring genes, inactivation of genes by interruption, and rearrangement of their hosts genome (12–14). However, the role of these elements in genetic adaptation and the dynamics

TABLE III

Survival of different *P. putida* strains after sudden addition of 1% (v/v) toluene

<i>P. putida</i> strain ^a	Survival frequency
	%
S12	0.004
S12PT	5
S12PT(pJWB1)	5
S12PT(pJWsrpS)	5
S12PT(pJWsrpRS)	0.0005

^a 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 *SrpABC* 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

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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