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A Novel Insertion Sequence Derepresses Efflux Pump Expression and Preadapts *Pseudomonas putida* S12 for Extreme Solvent Stress[▽]

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A multidrug efflux pump, SrpABC, plays a key role in *Pseudomonas putida* S12 tolerance to toxic organic solvents. SrpRS are putative regulators of the SrpABC efflux pump encoded upstream of the *srpABC* structural genes, and previous studies suggest that SrpS is a repressor of SrpABC expression. An S12 isolate able to withstand extreme solvent stress carries a novel insertion sequence, IS*Ppu21*, interrupting *srpS*. This insertion preadapts S12 to extreme solvent conditions through constitutive SrpABC expression.

It has been noted that *Pseudomonas* species adapt to high concentrations of toxic solvents by using various mechanisms (22). An alteration of the cell envelope structure when the bacterium was exposed to organic solvents was observed previously (6). Weber and de Bont (23) detected an increase of *trans*-unsaturated fatty acid contents in cells grown with toluene. It was suggested that this isomerization of *cis*- into *trans*-unsaturated fatty acids plays an important role in solvent tolerance in bacteria (7, 17). A mutant lacking this *cis*-to-*trans* isomerization activity was sensitive to toluene (17). Pinkart et al. (16) observed a modification of lipopolysaccharide and an increase in total fatty acids in solvent-treated cells, in addition to an increase in the *trans*-unsaturated fatty acid content. Enhanced rigidity resulting from changes in cell membrane fatty acid and phospholipid composition was shown to exclude toluene from the cell membrane, and the removal of intracellular toluene by degradation contributes to the toluene tolerance phenotype of *Pseudomonas putida* DOT-T1 (18). Finally, Isken and de Bont were the first to demonstrate the presence of an active efflux system for toluene in *P. putida* S12 (8), and Kieboom et al. reported that this energy-dependent export mechanism is an efflux pump belonging to the resistance-nodulation-division efflux pump family (9). During the past decade, several other efflux systems have been discovered to be involved in bacterial tolerance to organic solvents (1, 13, 14, 19).

Bacterial efflux systems are energy consuming, and therefore, their expression must be tightly controlled under uninduced conditions. Because many multidrug efflux systems have a broad substrate range, it is suggested that they may even pump out useful metabolites from bacterial cells (12). For example, an *Escherichia coli* mutant strain lacking the repressor of the AcrAB-TolC efflux pump was shown to have a lower growth rate than the wild-type strain (15). Similarly, it was reported that the efflux pump-overproducing mutants of *Pseudomonas aeruginosa* and *P. putida* have reduced fitness compared with parental strains (20, 24). Thus, sustained overexpression of efflux pumps can be detrimental to cells.

The SrpABC efflux pump in *P. putida* S12, which is homologous to TtgGHI in *P. putida* DOT-T1E (19), was found to have two regulatory genes, *srpR* and *srpS*, situated upstream of and transcribed divergently from the structural genes *srpABC*. *srpS* encodes the putative pump repressor SrpS, while the function of *srpR*/SrpR is still undefined. In 2001, Wery et al. (24) discovered a 2.6-kb insertion sequence named IS*S12* that can insert itself into the repressor gene *srpS*, thus blocking *srpS* expression and thereby enhancing the expression of the SrpABC efflux pump. The authors proposed that *P. putida* S12 employs IS*S12* as a specific mutator element in order to generate mutations that allow the bacterium to swiftly adapt to extreme solvent conditions. Their results at least suggest that an insertion sequence is involved in the regulation of efflux pump expression. In this study, we identified and characterized a new *P. putida* S12 insertion sequence involved in the regulation of SrpABC expression.

Wery et al. (24) reported that 93% of the *P. putida* S12 cells that survived a 1% (vol/vol) toluene shock carried an extra copy of the 2.6-kb insertion sequence IS*S12* within the *srpS* gene. Our preliminary results showed much lower carriage of an insertion element in *srpS*. We therefore performed experiments in order to further understand the regulation of the SrpABC efflux pump. A single colony of *P. putida* S12 was used to inoculate 25 ml of Luria-Bertani (LB) broth, and the culture was grown overnight at 30°C. (Strains and plasmids used in this study are listed in Table 1.) One milliliter of the overnight culture was used to inoculate 50 ml of LB broth containing 1% (vol/vol) toluene. This shock dose of toluene killed most of the cells (approximately 99.99%) within 30 min. After shaking of the medium at 200 rpm and 30°C for 24 h, a new culture had grown up and total DNA was isolated from it. PCR was performed using a Mastercycler gradient thermocycler according to the recommendations of the manufacturer (Eppendorf, Hamburg, Germany). Total genomic DNA was isolated from *P. putida* strains by the hexadecyltrimethylammonium bromide procedure (2). Using primers 1 (TTGGAGGTGAATAC TGG) and 2 (TCGGTCTGCCTGGCTTCT), the region between which includes the previously reported insertion site of IS*S12*, we amplified the DNA sample by PCR (Fig. 1A). Interestingly, the PCR result showed an *srpS* band of the original size (data not shown), indicating that IS*S12* did not appear in

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
E. coli DH5α	F^- ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Invitrogen
P. putida S12	Wild type; *srpABC*$^+$ *srpR*$^+$ *srpS*$^+$	5
P. putida JK1	*srpB*::Tn*Mod*-KmO	4
P. putida S12TS	*srpS*::IS*Ppu21*	This study
P. putida S12DM	*srpS*::IS*Ppu21 srpB*::Tn*Mod*-KmO	This study
Plasmids		
pCR2.1-TOPO	TOPO TA cloning vector for direct insertion of PCR products and blue/white screening; Ap^r Km^r	Invitrogen
pJD101	Derived from BamHI digestion of *P. putida* JK1 chromosome; contains *srpR* through partial ORF of Tn*Mod*-KmO plasposon-mutated *srpB*	9
pJD102	Derived from PstI digestion of *P. putida* JK1 chromosome; contains partial ORF of Tn*Mod*-KmO plasposon-mutated *srpB* through *srpC*	9
pJD203	Constructed by digestion (with EcoRV and BamHI) and ligation of pJD101 and pJD102; contains partial ORF of Tn*Mod*-KmO plasposon-mutated *srpB* through *srpC*	This study
pBBR1MCS-2	Broad-host-range cloning vector; Km^r	11
pBBR1-2-*srpR*	Coding region of *srpR* cloned into pBBR1MCS-2 (using EcoRI and BamHI sites)	This study
pBBR1-2-*srpS*	Coding region of *srpS* cloned into pBBR1MCS-2 (using EcoRI and BamHI sites)	This study
pBBR1-2-*srpRS*	Coding regions of *srpR* and *srpS* cloned into pBBR1MCS-2 (using EcoRI and BamHI sites)	This study

this region. The more rigorous condition of a 20% (vol/vol) toluene shock was tested. Although the maximum aqueous solubility of toluene is exceeded at a 20% concentration, the toxic effect of toluene continues to increase with increasing toluene concentrations, due to increased frequency of cellular contact (25). However, even under these severe growth conditions, we did not find any insertion sequences within the region between primers 1 and 2 (Fig. 1B, lane 1). When a more inclusive primer pair, primers 3 (ACCACTCTGCCTCACT TCG) and 4 (ATCCAGGTCATCGCCAG), was used to amplify the DNA sample from cells that survived the 20% (vol/vol) toluene shock, the PCR result showed a band of 1.7 kb, much larger than the expected size of 537 bp (Fig. 1B, lane 2). DNA sequencing analysis identified a 1.2-kb insertion sequence, designated IS*Ppu21*, located in the region between primers 1 and 3. Automated DNA sequencing was performed using a DYEnamic ET kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). The newly isolated insertion sequence was determined to exhibit no homology to IS*S12*, and its insertion site in *srpS* is different from that of IS*S12*.

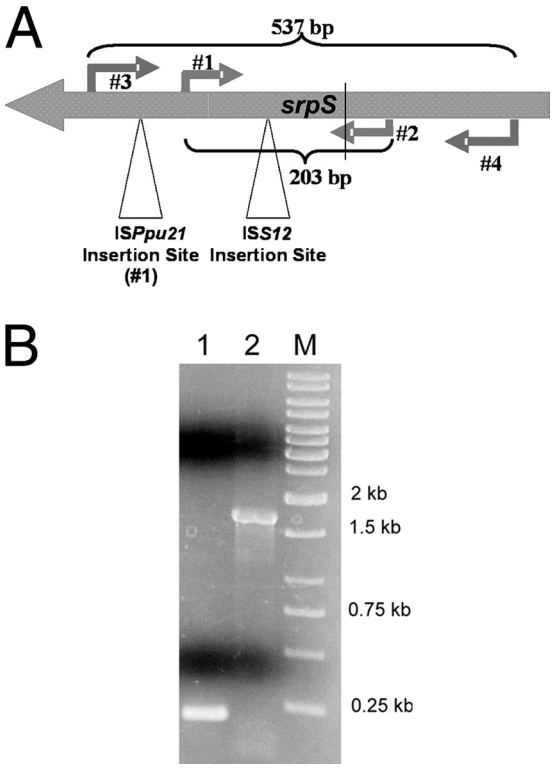

FIG. 1. Discovery of the new insertion sequence IS*Ppu21* within the *srpS* gene in *P. putida* S12 by PCR. (A) Locations of the PCR primers (1 through 4), sizes of the amplified regions, and insertion sites of IS*S12* and IS*Ppu21*. (B) The products resulting from the PCR were separated on a 0.7% (wt/vol) agarose gel. Lanes: 1, region amplified with primers 1 and 2; 2, region amplified with primers 3 and 4; M, molecular size markers.

Subsequently, we tested whether individual cells surviving the 20% (vol/vol) toluene shock carry an insertion sequence within the repressor protein gene *srpS*. To ensure that the cells used for total DNA extraction were genetically homogenous, an aliquot of the culture incubated with 20% (vol/vol) toluene for 24 h was spread onto LB agar plates and the plates were incubated at 30°C in a sealed glass chamber with saturated toluene vapor. Thirty-three separate cultures grown from different single colonies were used for total DNA extraction, and subsequently, DNA samples were PCR amplified with primers 8 (GAGCACTGGCAATCTAAC) and 9 (AGAGTCTACCA TTATGCGAT), which encompass the entire coding sequences of *srpR* and *srpS*. Among these samples, 22 (67%) carried no insertion sequence within the amplified region, 1 (3%) showed IS*S12* located in the same site discovered previously (24), and the other 10 samples (30%) were found to contain IS*Ppu21*. Further investigation by PCR analysis and DNA sequencing of the 10 samples that carried IS*Ppu21* showed two different insertion sites for IS*Ppu21* (Fig. 2): one (insertion site 1) located

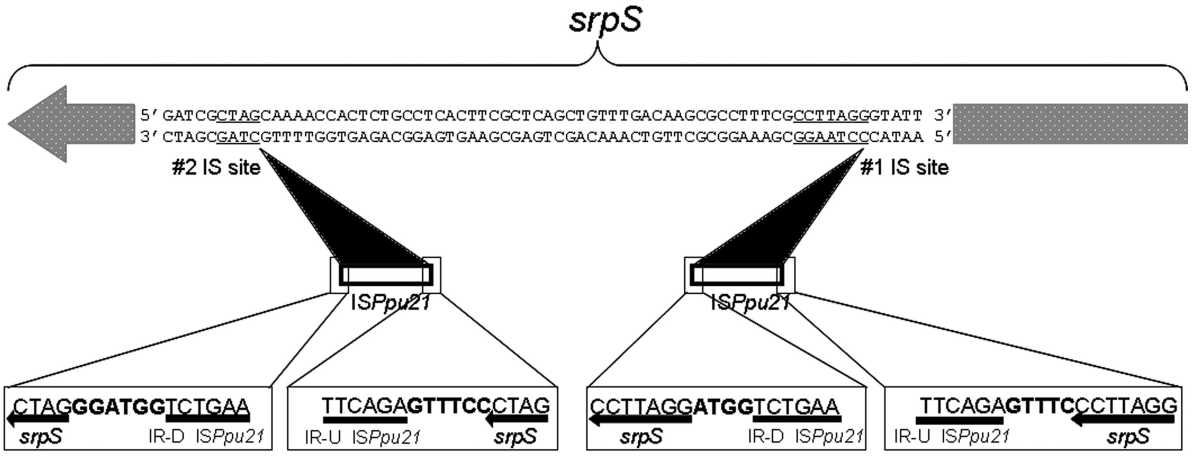

FIG. 2. Schematic diagram of the insertion (IS) sites of IS*Ppu21* in the *srpS* gene in *P. putida* S12TS. The two black triangles indicate the two different insertion points of IS*Ppu21* in *srpS*. The sequences of the upstream and downstream terminal inverted repeats (IR-U and IR-D), as well as the sequences adjacent to these terminal repeats, have been marked with boxes. The nucleotides not belonging to either *srpS* or IS*Ppu21* are shown in boldface.

between primers 1 and 3 (utilized in 6 of 10 samples) and the other (insertion site 2) located downstream of primer 3 but still within *srpS* (utilized in 4 of 10 samples). These results indicate that only approximately one-third of the cells that survive toluene shock carry an insertion sequence within the efflux pump repressor gene *srpS* and that the other two-thirds of the cells do not require an *srpS* insertion mutation to survive the high-dose toluene shock.

IS*Ppu21* is a 1,179-bp insertion sequence with a pair of 6-bp inverted repeats (TCTGAA) at its ends (Fig. 2). Interestingly, IS*Ppu21* elements introduced at insertion site 1 and insertion site 2 generate a pair of 7-bp and a pair of 4-bp target site duplication direct repeats, respectively. Only one open reading frame (ORF) was discovered in IS*Ppu21*, putatively encoding a protein of 326 amino acids. The nucleotide sequence of IS*Ppu21* has 100% identity to a region of the *P. aeruginosa* serotype O15-ATCC putative O antigen biosynthesis gene cluster (GenBank accession no. AF498406), and the ORF in this IS*Ppu21* orthologue is transcribed in the opposite direction from the *P. aeruginosa* chromosomal gene cluster. The putative product of the ORF in IS*Ppu21* has homology (identities from 61 to 93%) to transposase proteins belonging to the IS*5* family of transposases (Table 2).

We examined the distribution of the new insertion sequence IS*Ppu21* in the genomes of wild-type *P. putida* S12 and the solvent-adapted strain carrying IS*Ppu21* within *srpS*, designated *P. putida* S12TS. For this purpose, total DNA was isolated from both *P. putida* S12 and *P. putida* S12TS. The DNA samples were digested with either PstI or EcoRI, which do not digest within the IS*Ppu21* DNA sequence, and Southern hybridization analysis was performed using an internal 352-bp DNA probe made from IS*Ppu21* with primers 6 (TGCCGTC CTTGTTCTTG) and 7 (ATCGAGCCGCACTACCC). From each parental sample, there were three hybridizing DNA bands, indicating three copies of IS*Ppu21* distributed over the *P. putida* S12 genome (Fig. 3). One extra hybridizing DNA fragment appears in each sample from *P. putida* S12TS, suggesting that transposition of IS*Ppu21* occurs by a replicative mechanism.

TABLE 2. Amino acid sequence identities between the putative protein encoded by IS*Ppu21* and transposase proteins from different organisms

Protein	Identity (%)	Organism	Corresponding accession no.
IS*5* family transposase	93	*Pseudomonas resinovorans*	NP_758545
IS*Pssy* transposase	93	*Bordetella petrii*	YP_001632795
IS*1384* transposase	92	*P. putida*	AAC98743
IS*Pst5* transposase	92	*Pseudomonas stutzeri*	AAX14063
IS*5* family transposase	91	*P. aeruginosa*	ZP_00972095
IS*Psy2* transposase	90	*Pseudomonas syringae*	YP_275487
IS*4* family transposase	90	*Azotobacter vinelandii*	ZP_00415717
IS*5* family transposase	89	*Yersinia enterocolitica*	CAE46781
IS*1479* transposase	65	*Xanthomonas campestris*	YP_243093
IS*5* family transposase	62	*Xanthomonas oryzae*	YP_201728
IS*5* family transposase	62	*Achromobacter xylosoxidans*	YP_195864
IS*4* family transposase	62	*Burkholderia vietnamiensis*	YP_001109709
IS*BPH* transposase	61	*Achromobacter denitrificans*	NP_990893

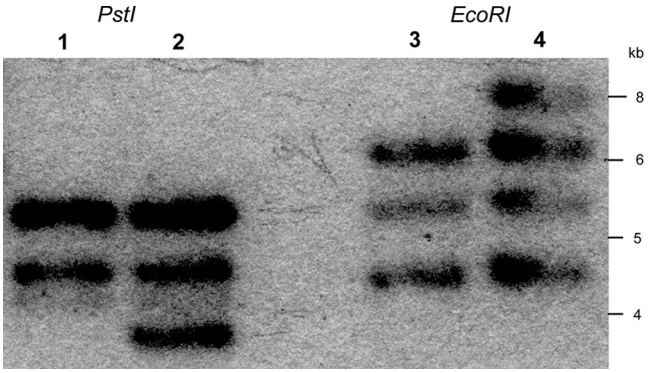

FIG. 3. Southern hybridization analysis of total DNA from *P. putida* S12 and *P. putida* S12TS. The DNA samples were digested with PstI (lanes 1 and 2) or EcoRI (lanes 3 and 4) and analyzed by Southern hybridization with a 352-bp DNA probe made from IS*Ppu21*. Lanes 1 and 3 show the results for wild-type *P. putida* S12; lanes 2 and 4 show the results for *P. putida* S12TS.

TABLE 3. Survival frequencies for different *P. putida* strains in response to a 20% (vol/vol) toluene shock[a]

P. putida strain[b]	Survival frequency (%)
S12	0.0003 ± 0.0002
S12TS	5 ± 1.5
S12TS(pBBR1MCS-2)	5 ± 2.6
S12TS(pBBR1-2-*srpR*)	5 ± 2.3
S12TS(pBBR1-2-*srpS*)	0.0003 ± 0.0002
S12TS(pBBR1-2-*srpRS*)	0.0002 ± 0.0001

[a] All assays were repeated three times with three separate cultures. The given values are the means ± standard deviations of triplicate measurements.
[b] The names of the introduced plasmids are in parentheses.

The authors of a previous study examining the survival frequencies of *P. putida* S12 and an *srpS* insertion mutant in response to toluene shock concluded that the increased survival rate of the mutant drops to wild-type levels only if both *srpR* and *srpS* are complemented (24). We obtained different results by performing a similar complementation experiment. DNA fragments containing the *srpR*, *srpS*, and *srpRS* genes were amplified using primers 12 (GGGAATTCTATGGCCA GAAAGAC) and 13 (CCGGATCCTCACTCGAAG), 14 (C CGAATTCTATGAACCAATCAG) and 15 (TTGGATCCTC TAGGGAGCTTTC), and 13 and 14, respectively. All three amplified fragments were ligated into pBBR1MCS-2 (11) by using incorporated BamHI and EcoRI sites. The corresponding constructs were designated pBBR1-2-*srpR*, pBBR1-2-*srpS*, and pBBR1-2-*srpRS*, respectively, and each was introduced into the S12TS mutant by electroporation using standard methods (3). Kanamycin was added to a final concentration of 250 μg/ml to maintain the plasmids when necessary. The survival frequencies were determined by measuring the numbers of CFU on LB plates after 20 h of incubation in LB medium with 20% (vol/vol) toluene or 20% (vol/vol) distilled water as a control. The survival frequency assay results are listed in Table 3. They show that the *srpS*::IS*Ppu21* mutant S12TS had a 17,000-fold-greater survival frequency in the presence of a 20% (vol/vol) toluene shock than wild-type S12, which was not influenced by the introduction of the empty vector pBBR1MCS-2. Interestingly, both S12TS(pBBR1-2-*srpS*) and S12TS(pBBR1-2-*srpRS*) showed survival frequencies close to parental levels, suggesting that the survival frequency of *P. putida* S12TS returns to the wild-type level when *srpS* is complemented. Complementation with *srpR* did not affect survival frequency, as the S12TS(pBBR1-2-*srpR*) value remained similar to that for S12TS (~5%). Like SrpABC, the TtgGHI efflux pump of *P. putida* DOT-T1E has two regulatory genes, *ttgV* and *ttgW*, located upstream from the structural genes *ttgGHI*. These two regulatory genes are polycistronic, are transcribed divergently from the *ttgGHI* operon (18), and are 95% identical to *srpRS* at the DNA sequence level. A mutant deficient in *ttgV*, but not in *ttgW*, has much higher expression activity from the *ttgGHI* and *ttgVW* promoters than the wild type, suggesting that *ttgV* encodes a repressor for the expression of *ttgGHI* and itself. A similar result for a *ttgVW* double mutant has also been observed. However, the mutant lacking *ttgW* but carrying intact *ttgV* does not show any significant changes in the expression levels of *ttgGHI* and *ttgVW*. Thus, it was proposed that *ttgW* plays no major role in this regulatory system (18). Further investigation of the 0.0003% of wild-type cells surviving 20% (vol/vol) toluene for 30 min was performed. Total DNA was isolated from nine individual colonies and analyzed by PCR with primers 8 and 9. The results showed that four of nine samples had IS*Ppu21* within *srpS*, while the other five had intact *srpS* (data not shown). This finding again suggests that more than half of the surviving S12 cells do not need *srpS* inactivation in order to tolerate toluene shock.

Insertion sequences have been shown to be important for the genomic plasticity of certain bacteria. The literature contains many examples of insertion sequences causing the inactivation of genes by insertion, the activation of neighboring genes by the induction of outwardly directed promoters, or genomic rearrangements by homologous recombination. However, there are few published examples in which insertion sequence transposition is shown to be directly responsible for a change in cellular phenotype (21). The inactivation of *srpS* by IS*Ppu21* is a clear example of an insertion sequence inactivating a gene encoding a repressor directly responsible for controlling efflux pump expression, and therefore preadapting the bacterial cell for normally lethal organic solvent stress. Our detection of a second *P. putida* S12 insertion sequence element capable of inactivating *srpS* indicates that IS*S12* is not a specific mutator element, as suggested previously by Wery et al. (24). It is also possible that other transposable elements besides IS*S12* and IS*Ppu21* can insert themselves into *srpS* in order to generate constitutively expressed SrpABC efflux pump variants.

Based on our discovery of IS*Ppu21* in two different target sequences within the *srpS* ORF in different isolates of solvent-shocked S12, the target site recognition of the IS*Ppu21* transposase is not highly specific. At insertion site 1, the putative IS*Ppu21* target sequence in *srpS* is CCTTA; at insertion site 2, the putative target sequence is CTAG. In the *P. aeruginosa* putative O antigen biosynthesis cluster, the IS*Ppu21* target site appears to be TTT or TAG, although further experimental tests are required to confirm this. As shown in Fig. 2, determination of the IS*Ppu21* target sequence is complicated by the random addition of 4 to 6 bp at each end of the insertion that do not correspond to either target DNA (e.g., *srpS*) or IS*Ppu21*. It is unknown whether other examples of IS*5* family insertion elements behave similarly. Figure 3 illustrates the addition of one IS*Ppu21* copy into the genome after IS replication to give four copies, suggesting that IS*Ppu21* transposes by a replicative mechanism. It is expected that insertion sequence inactivation of *srpS* occurs prior to toluene shock. It has been demonstrated previously that toxic toluene accumulation in the *P. putida* S12 membranes occurs within 10 min of toluene introduction (8), whereas toluene induction of the *srpABC* promoter reaches half maximal transcription activation only after ~60 min (10). The inability of 99.99% of cells to survive solvent shock relates to the inability of cells to express and assemble the SrpABC efflux pump in sufficient time to prevent membrane disruption and cell death. Interestingly, we found that only approximately 33% of toluene-tolerant *P. putida* S12 isolates contained IS insertions in the *srpS* gene, the majority of these insertions being IS*Ppu21* rather than IS*S12*. Sequence analysis of the other toluene-tolerant S12 isolates not containing an insertion sequence did not show mutations in *srpS* or in other, nearby DNA regions. This result is puzzling in light of

the fact that immediate SrpABC expression appears to be a requirement for S12 solvent tolerance, and suggests that other mechanisms may be involved in controlling SrpABC expression. We are currently working to elucidate the role of SrpR in SrpABC expression in *P. putida* S12 under solvent stress conditions, especially with respect to S12 strains that have no insertion sequence interruption of *srpS*.

Nucleotide sequence accession number. The DNA sequence of IS*Ppu21* has been deposited in GenBank under accession number FJ905320 and in the Insertion Sequence Database (http://www-is.biotoul.fr/).

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