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An Antirepressor, SrpR, Is Involved in Transcriptional Regulation of the SrpABC Solvent Tolerance Efflux Pump of *Pseudomonas putida* S12▽

Xu Sun,† Zileena Zahir,† Karlene H. Lynch, and Jonathan J. Dennis*

Department of Biological Sciences, M354 Biological Sciences Building, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

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Organic compounds exhibit various levels of toxicity toward living organisms based upon their ability to insert into biological membranes and disrupt normal membrane function. The primary mechanism responsible for organic solvent tolerance in many bacteria is energy-dependent extrusion via efflux pumps. One such bacterial strain, *Pseudomonas putida* S12, is known for its high tolerance to organic solvents as provided through the SrpABC resistance-nodulation-cell division (RND) family efflux pump. To determine how two putative regulatory proteins (SrpR and SrpS, encoded directly upstream of the SrpABC structural genes) influence SrpABC efflux pump expression, we conducted transcriptional analysis, β-galactosidase fusion experiments, electrophoretic mobility shift assays, and pulldown analysis. Together, the results of these experiments suggest that expression of the *srpABC* operon can be derepressed by two distinct but complementary mechanisms: direct inhibition of the SrpS repressor by organic solvents and binding of SrpS by its antirepressor SrpR.

Several mechanisms have been proposed to account for organic solvent tolerance in Gram-negative bacteria. These include the formation of membranous vesicles (21), organic solvent metabolism (5, 28), and increasing membrane rigidity (16, 17, 29, 40). Although a permeability barrier in the form of a cellular membrane is indispensable for bacteria to resist the toxic effects of organic solvents, it is a relatively passive mechanism, and many solvents can diffuse across this barrier over time (7, 29, 30). Consequently, extrusion of toxic solvents by membrane-spanning protein complexes, termed efflux pumps, is a crucial active mechanism to remove these compounds from the bacterial cell.

Efflux pumps are divided into five families: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, and the resistance-nodulation-cell division (RND) family (25). To be effective, bacterial efflux pumps are necessarily located in the cell membrane of Gram-positive bacteria or the cell envelope of Gram-negative bacteria, and their function is energy dependent (23, 25). The efflux of antimicrobial compounds as a resistance mechanism was first reported for tetracycline in *Escherichia coli* (27) and was later discovered in many bacteria resistant to antimicrobials. During the past decade, several efflux systems have also been discovered to be involved in bacterial tolerance to organic solvents (2), including the AcrAB-TolC efflux pump in *E. coli* (25), the MexAB-OprM, MexCD-OprJ, and MexEF-OprN efflux pumps in *Pseudomonas aeruginosa* (24), the SrpABC efflux pump in *Pseudomonas putida* S12 (19, 20), and the TtgABC, TtgDEF, and TtgGHI efflux pumps in *P. putida* DOT-T1E (30, 32). The characterization of these efflux pump systems has helped to elucidate bacterial mechanisms of tolerance to extremely high concentrations of toxic organic solvents.

The expression of most RND-type multidrug and solvent efflux systems is controlled by both complex global regulatory networks and a local repressor (10, 13, 25); the gene for the latter is typically situated upstream and is transcribed divergently from the efflux pump genes (22, 39). For example, the TtgGHI efflux pump of *P. putida* DOT-T1E (the system most closely related to the SrpABC pump in *P. putida* S12) is locally regulated by the repressor TtgV (33). The genes *ttgV* and *ttgW* (a pseudogene that does not regulate pump expression) are located upstream from the structural genes *ttgGHI* and are transcribed divergently from this operon (33). A mutant deficient in *ttgV*, but not in *ttgW*, was shown to possess much higher expression from the *ttgGHI* and *ttgWV* promoters, suggesting that *ttgV* encodes a repressor for the expression of both *ttgGHI* and itself (32, 33). Further studies revealed that TtgV is an IclR family protein that represses the transcription of these genes by binding to the *ttgV-ttgG* intergenic region, thereby preventing the binding of RNA polymerase to promoter sequences. Substrates of the TtgGHI pump such as 1-hexanol can release TtgV from its DNA-binding site and induce the expression of the efflux pump (11). More recently, Guazzaroni et al. (12) identified additional compounds that play a role similar to that of 1-hexanol in inducing TtgGHI expression and established a clear relationship between a compound's affinity for TtgV and its efficiency at inducing *ttgGHI* expression.

The genes encoding the SrpABC efflux pump in *P. putida* S12 possess two putative regulatory genes upstream and divergently transcribed from *srpABC*: *srpS* (putatively encoding an IclR family regulator) and *srpR* (putatively encoding a TetR family repressor). Wery et al. (41) discovered a 2.6-kb insertion sequence, IS*S12*, that can insert into the repressor gene *srpS* and block its expression, suggesting that an insertion sequence may also be involved in the regulation of this RND-type efflux

* Corresponding author. Mailing address: M354 Biological Sciences Building, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9. Phone: (780) 492-2529. Fax: (780) 492-9234. E-mail: jon.dennis@ualberta.ca.

† X.S. and Z.Z. contributed equally to this work.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Invitrogen
E. coli BL21	F^- *ompT hsdS* (r_B^- m_B^-) *gal*	Amersham
E. coli M15	Nal^s Sm^s Rif^s Thi^- Lac^- Ara^+ Gal^+ Mtl^- F^- $RecA^+$ Uvr^+ Lon^+	Qiagen
P. putida S12	Wild-type; *srpABC*$^+$ *srpR*$^+$ *srpS*$^+$	14
P. putida JK1	*srpB*::Tn*Mod*-KmO	19
P. putida S12 *lacZ*	*srpABC*$^+$ *srpS*$^+$ *srpR*$^+$ *lacZ*, in a single-copy transcriptional fusion behind P*srpA*	This study
P. putida SrpS$^-$ *lacZ*	*srpABC*$^+$ *srpS* mutant *srpR*$^+$ *lacZ*, in a single-copy transcriptional fusion behind P*srpA*	This study
P. putida SrpR$^-$ *lacZ*	*srpABC*$^+$ *srpS*$^+$ *srpR* mutant *lacZ*, in a single-copy transcriptional fusion behind P*srpA*	This study
Plasmids		
pCR2.1-TOPO	TOPO TA cloning vector for direct insertion of PCR products, blue/white screening; Ap^r Km^r	Invitrogen
pJD101	Derived from a BamHI digestion of *P. putida* JK1 chromosome; contains *srpR* through a partial open reading frame of Tn*Mod*-KmO plasposon-mutated *srpB*	19
pJD102	Derived from a PstI digestion of *P. putida* JK1 chromosome; contains a partial open reading frame of Tn*Mod*-KmO plasposon-mutated *srpB* through *srpC*	19
pJD203	Constructed by digestion (EcoRV and BamHI) and ligation of pJD101 and pJD102; contains a partial open reading frame of Tn*Mod*-KmO plasposon-mutated *srpB* through *srpC*	This study
pJD500	Ap^r Km^r; promoterless *trp-lacZ* fusion downstream of the *srpA* promoter, for the construction of single-copy chromosomal *lacZ* reporter fusions	This study
pGEX-4T-1	Ap^r; cloning vector for overexpression of N-terminal GST fusion proteins	Amersham
pGEX-*srpR*	Entire coding region of *srpR* cloned into pGEX-4T-1 (BamHI-EcoRI)	This study
pQE31	Ap^r; cloning vector for overexpression of N-terminal His_6 fusion proteins	Qiagen
pQE31-*srpS*	Entire coding region of *srpS* cloned into pQE31 (BamHI-KpnI)	This study
pQE31-*arpR*	Entire coding region of *arpR* cloned into pQE31 (SstI-PstI)	This study
pREP4	Km^r; introduced into *E. coli* M15; constitutively expresses LacI	Qiagen

pump. The discovery of a second insertion sequence, IS*Ppu21*, that inserts into *srpS* and derepresses efflux pump expression (37) confirms that SrpS is the *srpABC* efflux pump repressor but eliminates the hypothesis that IS*S12* is a specific *srpS* mutator element (41). No insertion sequences were detected inserted in *srpS* when cells were treated with 1% (vol/vol) toluene shocks, whereas approximately one-third of the cells that survived a 20% (vol/vol) toluene shock carried either IS*Ppu21* (37) or IS*S12* (41) within *srpS*. Mutant S12TS, carrying a copy of IS*PpuS12* within *srpS*, was shown to have a 17,000-fold increased survival frequency to toluene shock in comparison with wild-type S12. The increased survival frequency in S12TS dropped to wild-type levels when the strain was complemented with *srpS* but not with *srpR*. These results are consistent with the hypothesis that SrpS, but not SrpR, is the repressor of SrpABC. Importantly, this study indicated that two-thirds of the cells that survived extreme toluene levels do not require *srpS* gene inactivation.

The objective of this study was to determine how the putative regulators SrpS and SrpR are involved in controlling expression of the *srpABC* genes. Since the *srpABC-srpSR* gene cluster and the *ttgGHI-ttgVW* gene cluster are highly homologous, it was expected that SrpSR would perform in the same manner as TtgVW (SrpR having little to no importance). However, the results presented here suggest that SrpS and SrpR function together to control the production of SrpABC and that this activity is affected by the presence of organic solvents in the cell.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The *E. coli* and *P. putida* strains and the plasmids used in this study are listed in Table 1. Bacterial strains were routinely grown in Luria-Bertani (LB) medium (34). Solid medium contained 1.5% (wt/vol) agar. When required for selection, the culture medium was supplemented with different antibiotics including ampicillin (100 μg/ml), kanamycin (50 μg/ml), gentamicin (25 μg/ml), and streptomycin (150 μg/ml). *E. coli* and *P. putida* cultures were incubated at 37°C and 30°C, respectively. Liquid cultures were shaken on a horizontal shaker at 200 rpm. When *P. putida* culture plates were supplied with toluene via the gas phase, a sealed glass chamber was used, and saturated toluene vapor was achieved by adding toluene to the bottom of the chamber. When *P. putida* liquid cultures were supplied with certain concentrations of toluene, the culture flasks were sealed with foil-covered stoppers.

DNA techniques. Total genomic DNA was isolated from *P. putida* strains by the hexadecyl trimethyl ammonium bromide (CTAB) procedure (3). Plasmid isolations were performed with a QIAprep Spin Miniprep Kit (Qiagen Inc., Mississauga, ON). Digestions with restriction enzymes were performed according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). Ligations were performed with T4 DNA ligase (Promega, Madison, WI). Chemically competent *E. coli* DH5α cells (Invitrogen) were transformed according to the manufacturer's instructions. Electroporation of *P. putida* and *E. coli* strains was performed as described previously (8) using a MicroPulser (Bio-Rad Laboratories, Hercules, CA). Genetic mutations in *srpS* and *srpR* were constructed in the S12 chromosome using a standard technique (34): the double crossover of a nonreplicating plasmid carrying an amplified S12 gene fragment with an internal antibiotic resistance marker insertion (9). Based on previous reports (41) and unpublished data, it was determined that due to polar effects, the *srpS* knockout is, in fact, an *srpS srpR* double mutant. DNA fragments were purified from agarose gels using a GeneClean II kit (Q. BIOgene, Carlsbad, CA). DNA fragments amplified by PCR for use in sequencing reactions and restriction enzyme digestion were purified using a QIAquick PCR Purification Kit (Qiagen). Samples for sequencing were prepared with an Amersham DYEnamic ET kit (Amersham Biosciences Corp., Piscataway, NJ) and provided to the University of Alberta Molecular Biology Services Unit (MBSU) for automated sequencing using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Nucleotide and amino acid sequence analysis was carried out with the BLAST program on the National Center for Biotechnology Information (NCBI) server (1).

RNA preparation and RT-PCR. Total RNA from *P. putida* S12 was isolated with an RNeasy Mini Kit and RNAprotect Bacteria Reagent (Qiagen) according to the manufacturer's instructions. The RNase-Free DNase Set (Qiagen) was used to eliminate DNA contamination. Reverse transcriptase PCR (RT-PCR) was performed with 1 μg of RNA/ml in a 20-μl reaction volume using the

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)[a]	Use
S1	TTGGAGGTGAATACTGG	With primer S2 to PCR amplify a 205-bp fragment within *srpS*; used in RT-PCR to amplify the first-strand cDNA of *srpS*
S2	TCGGTCTGCCTGGCTTCT	With primer S1 or with R0 in RT-PCR to amplify a 940-bp fragment overlapping *srpS* and *srpR*
R0	CGCCGATTGAGGTTTGAAG	RT-PCR to amplify the first-strand cDNA of *srpR* or *srpSR*; used with primer R2 to amplify a 450-bp fragment within *srpR*
R2	AGGCGGAGGAGACAAGA	With primer R0 to PCR amplify the cDNA of *srpR*
SP1	AACCTGTTCTTTCTCACCAC	With AP2, PCR amplification of a 490-bp fragment in the *srpS-srpA* intergenic region used in EMSAs
AP2	TTCTTCCAGAGCGTTGATGA	With SP1, PCR amplification of a 490-bp fragment in the *srpS-srpA* intergenic region used in EMSAs
SF-01	ATGTCGACTACAGTGGCGGC	With SR-02, PCR amplification of an 831-bp fragment encompassing the coding sequence of *srpS* to clone into pMAL-c2X
SR-02	TTAAGCTTCTAGGGAGCTTTCTTC	With SF-01, PCR amplification of an 831-bp fragment encompassing the coding sequence of *srpS* to clone into pMAL-c2X
RF-01	TAGTCGACATGGCTAGAAAGACG	With RR-02, PCR amplifies a 642-bp fragment encompassing the coding sequence of *srpR* to clone into pMAL-c2X
RR-02	ATAAGCTTTACTCGAAGGATTTGACTT	With RF-01, amplifies a 642-bp fragment encompassing the coding sequence of *srpR* to clone into pMAL-c2X
SR	ACCACTCTGCCTCACTTCG	RT-PCR to amplify the first-strand cDNA of *srpS*
SF0	TGCTGAATCGTAATGCGGT	With primer SR to determine the transcription start site of *srpS*
SF1	CCGTTGGTCGAGGTTTACC	With primer SR to determine the transcription start site of *srpS*
SF2	CCAGAGCAGCCTCGATCA	With primer SR to determine the transcription start site of *srpS*
AR	CGTGGGTCAATCTGATAAAG	RT-PCR to amplify the first-strand cDNA of *srpA*
AF0	ATCGCATAATGGTAGACTCT	With primer AR to determine the transcription start site of *srpA*
AF1	AGACTCTACCGCATTACGAT	With primer AR to determine the transcription start site of *srpA*
AF2	ATTACGATTCAGCAATAGCC	With primer AR to determine the transcription start site of *srpA*
AR5Sst	AAGAGCTCGATGGTCCGTC	PCR amplification of a 657-bp fragment encompassing the entire coding sequence of *arpR* to clone into pQE31
AR3Pst	GGCTGCAGCAAAGTGTCAT	
S5Bam	AAGGATCCTATGAACCAATCA	PCR amplification of a 794-bp fragment encompassing the entire coding sequence of *srpS* to clone into pQE31
S3Kpn	CTTATCTAGGGTACCTTCTTCGAC	
R5Bam	AAGGATCCATGGCCAGAAAGAC	PCR amplification of a 649-bp fragment encompassing the entire coding sequence of *srpR* to clone into pGEX-4T-1
R3Eco	GGGAATTCGGATTTGACTTGC	

[a] Engineered restriction sites are underlined.

SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen), and PCR amplification of the cDNA was performed with Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen). The first-strand cDNA synthesis step was conducted at 55°C for 50 min, and the cycling conditions for PCR amplification were as follows: a 2-min denaturation period at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min per kb of DNA template at 68°C; and a final 2-min extension period at 68°C. Positive and negative controls were performed in all assays. The primers and their uses in this study are listed in Table 2. The concentration of toluene used in all experiments is above its solubility limit in an aqueous solution (0.47 g/liter). To ensure that SrpS came into contact with toluene in the *in vitro* RT-PCR assays, we used an excess of toluene at 6 M (20%, vol/vol). In order to ensure cell viability throughout our reporter fusion experiments (see below), the toluene concentration was reduced to 6 mM, a concentration just over the solubility limit of toluene (0.552 g/liter).

β-Galactosidase assays. The plasmid pJD500, based on the kanamycin- and ampicillin-resistant narrow-host range vector pGEM-5Zf(+) (Promega Corp., Madison, WI), was constructed containing a promoterless *trp-lacZ* gene transcriptionally fused to the *srpA* gene downstream of the *srpA* promoter. To create the *srpA-lacZ* reporter gene fusion strains, *P. putida* S12 *lacZ*, an SrpS$^-$ *lacZ* strain, and an SrpR$^-$ *lacZ* strain, pJD500 was transformed by electroporation using standard techniques (8) into wild-type *P. putida* S12 and the S12 SrpS$^-$ and S12 SrpR$^-$ mutants. Transformants were selected using kanamycin antibiotic selection. Chromosomal PCR analysis was performed to verify the occurrence of a single chromosomal homologous recombination event, leaving a functional *srpABC* efflux pump operon. β-Galactosidase assays, including the calculation of Miller units, were performed according to the method of Slauch and Silhavy (36). Briefly, formation of *o*-nitrophenol (ONP) was monitored by measuring A_{420}. Readings were taken for a 60-min period, and the β-galactosidase activity was determined by calculating the ratio of A_{420} to the optical density at 600 nm (OD_{600}). The cultures used in these assays were supplemented with or without 6 mM toluene. All assays were averages of at least three independent trials.

Overexpression and purification of MBP-SrpS and MBP-SrpR. The putative regulators SrpS and SrpR were overexpressed in *E. coli* DH5α as N-terminal maltose binding protein (MBP) fusion proteins for use in the *in vitro* protein-DNA binding studies. The *srpS* gene was PCR amplified from the *P. putida* S12 chromosome using the primers SF-01 and SR-02, which contain engineered SalI and HindIII sites, respectively. The amplified fragment was digested accordingly and cloned in frame downstream of the *malE* gene in the plasmid pMAL-c2X (New England BioLabs, Mississauga, ON). Likewise, the *srpR* gene was cloned into pMAL-c2X using the primers RF-01 and RR-02, which also contain engineered SalI and HindIII sites, respectively. To overexpress the fusion proteins, *E. coli* DH5α cells transformed with the respective plasmids were grown in 1 liter of LB medium with 100 μg/ml ampicillin at 37°C to an OD_{600} of 0.5 to 0.6 and induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested after 6 h of induction at 37°C, resuspended in 30 ml of column buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA) containing 0.01 mM phenylmethylsulfonyl fluoride (PMSF), and stored at −20°C overnight. Subsequently, the cells were thawed on ice and lysed by sonication in four pulses of 15 s using a Branson Sonifier 450. Following centrifugation at 9,000 × *g* for 30 min at 4°C, the supernatant containing the soluble protein fraction was diluted 1:2 with column buffer. The column buffer used throughout the protein purification steps was supplemented with 0.01 mM PMSF. Purification of the fusion protein from the crude extract was performed in a 20-ml syringe containing 15 ml of amylase resin (New England BioLabs, Mississauga, ON). The resin was initially washed with 8 column volumes of column buffer. Subsequently, the diluted crude extract was added, and the resin was washed with 12 column volumes of column buffer. The fusion protein bound to the amylase resin was eluted in 3-ml fractions using column buffer containing 10 mM maltose. Samples were analyzed by measuring the A_{280}, and the purified protein fractions were pooled and quantified using a Bio-Rad protein assay kit. Quantified fusion proteins were divided into aliquots and stored at −80°C.

Electrophoretic mobility shift assays (EMSAs). A 490-bp DNA fragment within the *srpS-srpA* intergenic region, containing the promoters of both *srpS* and *srpA* and the regulatory region containing potential operators, was PCR amplified from the plasmid pJD101 using the primers SP1 and AP2. The amplified fragment was purified and end labeled with [γ-^{32}P]dATP. Binding reactions were carried out in 20-μl volumes and consisted of increasing amounts of purified MBP-SrpS or MBP-SrpR, 1× binding buffer [65 mM Tris-HCl (pH 7.8), 0.2 M KCl, 25 mM $MgCl_2 \cdot 6H_2O$, 5 mM dithiothreitol (DTT), 0.25% NP-40, 12.5% glycerol, 1 μg of poly(dI-dC)], labeled target DNA (2×10^6 to 4×10^6 cpm), and sterile Milli-Q H_2O to adjust the volume. Reaction mixtures were incubated at 30°C for 30 min, followed by the addition of a one-fifth volume of 5× loading buffer. The samples were then separated by electrophoresis in a 4% nondenaturing polyacrylamide–1× TAE (Tris-acetate EDTA) gel before exposure to X-ray film and visualization by autoradiography. Competition assays were performed under the same conditions with the addition of competitive or noncompetitive DNA. When toluene was included in the assays, it was added to the reaction mixtures either prior to or following the binding reaction.

Overexpression and purification of GST-SrpR, His-SrpS, and His-ArpR. The *srpR* gene was PCR amplified from pJD101 using the primers R5Bam and R3Eco, which contain engineered BamHI and EcoRI sites, respectively. The amplified fragment was digested accordingly and cloned in frame downstream of the glutathione *S*-transferase (GST) gene in the plasmid pGEX-4T-1 (Amersham), resulting in the N-terminal GST-tagged construct pGEX-*srpR*. The *srpS* gene was PCR amplified from pJD101 using the primers S5Bam and S3Kpn, which contain engineered BamHI and KpnI sites, respectively. The amplified fragment was digested accordingly and cloned in frame downstream of the 6× histidine gene in the plasmid pQE31 (Qiagen), resulting in the N-terminal His_6-tagged construct pQE31-*srpS*. Likewise, *arpR* was cloned into pQE31 with the primers AR5Sst and AR3Pst and used as a negative control for the pulldown assay. *E. coli* BL21 was used to express GST and GST fusion proteins, and *E. coli* M15(pREP4) was used to express His_6 fusion proteins. To induce expression of the fusion proteins, *E. coli* cells transformed with the respective plasmids were grown at 37°C in 2× YT broth (1.6% [wt/vol] tryptone, 1.0% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl) until the OD_{600} reached 0.7, and IPTG was added to a final concentration of 0.05 mM. Incubation was continued for one additional hour at 30°C before the cells were harvested by centrifugation at 7,000 × *g* for 10 min and resuspended in ice-cold phosphate-buffered saline (PBS) buffer. The cells were then lysed by sonication, and the sonicates were pelleted by centrifugation at 10,000 × *g* for 15 min to remove insoluble material. The supernatants were saved as the crude cell lysate samples. These total protein samples were quantified using a Bio-Rad protein assay kit and stored at −80°C before being used in the pulldown assay.

GST pulldown assays. A test group (GST-SrpR and His-SrpS) and three negative-control groups (GST-SrpR and His-ArpR, GST and His-SrpS, and PBS buffer and His-SrpS) were assayed simultaneously in the pulldown experiments. Preliminary tests were performed to determine the concentrations of cell lysate samples to be used, such that the test group and control groups would contain the same amount of GST-SrpR and GST, as well as the same amount of His-SrpS and His-ArpR. Formal pulldown assays were then performed accordingly. One milliliter of 0.83 mg/ml cell lysate containing GST-SrpR, 1 ml of 0.14 mg/ml cell lysate containing GST, or 1 ml of PBS buffer was incubated with 20 μl of 50% glutathione-Sepharose beads (Amersham) at 4°C for 1 h. The beads were then washed twice with 1 ml of PBST (PBS and 0.05% Tween 20) and once with 1 ml of PBS. Following the wash steps, 1 ml of 0.22 mg/ml cell lysate containing His-SrpS or His-ArpR was added to the beads, and the samples were incubated with gentle agitation at 4°C for 2 h. Subsequently, the beads were washed twice with PBST and twice with PBS and heated in SDS-PAGE loading buffer at 95°C for 5 min. Finally, the beads were briefly pelleted by centrifugation, and the supernatant was separated on an SDS-PAGE gel and analyzed by Western blotting. The His_6 fusion proteins were detected by Penta-His Antibody (Qiagen) and peroxidase-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The pulldown assay results were the product of three independent trials.

RESULTS

Transcriptional analysis of the *srpSR-srpABC* gene cluster. It is unclear whether *srpR* and *srpS* are coexpressed in the presence or absence of organic solvent. To analyze *srpSR* expression, total RNA was isolated from cells grown in the presence or absence of 20% (vol/vol) toluene, and RT-PCR assays were performed with primer pairs within *srpR* (primers R0 and R2), *srpS* (primers S1 and S2), and across *srpSR* (primers R0 and S2). *srpR* and *srpS* were transcribed as a single unit in both the presence and absence of toluene (Fig. 1), thus confirming that *srpR* and *srpS* are encoded on a polycistronic transcript.

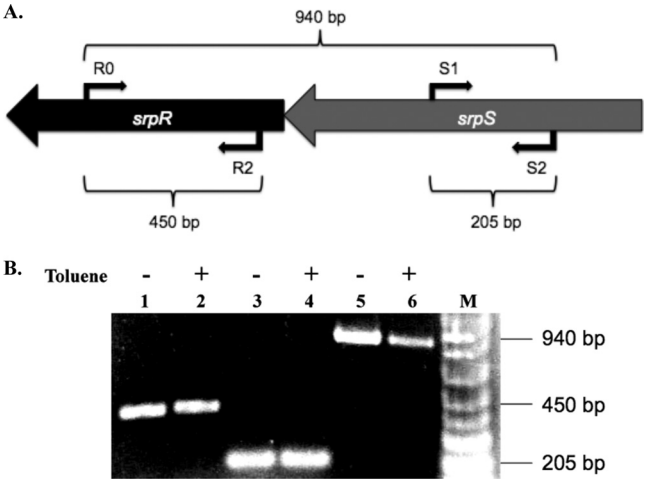

FIG. 1. RT-PCR analysis of *srpSR* in *P. putida* S12. (A) RT-PCR primers and amplified region. Primers R0 and R2 were used to amplify *srpR*, primers S1 and S2 were used to amplify *srpS*, and primers R0 and S2 were used to amplify *srpSR*. The expected fragment sizes are shown. (B) RT-PCR results. Lanes 1 and 2, *srpR* amplification; lanes 3 and 4, *srpS* amplification; lanes 5 and 6, *srpR-srpS* amplification; lane M, molecular size markers (1 Kb Plus DNA Ladder; Invitrogen) (0.1 kb to 1.0 kb shown). RNA templates in lanes 1, 3, and 5 were isolated from cells grown without toluene; RNA templates in lanes 2, 4, and 6 were isolated from cells grown with 20% (vol/vol) toluene. Negative controls without reverse transcriptase added were included with all of the reaction mixtures (data not shown).

P. putida DOT-T1E is another solvent-tolerant *P. putida* strain. The TtgGHI efflux pump in this strain is highly homologous (98% nucleotide sequence identity) to the SrpABC efflux pump in *P. putida* S12 (32). The structural genes *ttgGHI* correspond to *srpABC*, and *ttgV*, encoding the repressor, and *ttgW* correspond to *srpS* and *srpR*, respectively. In an alignment of the *srpS* sequences with the *ttgV* sequence features identified by Rojas et al. (33), the transcription start site of *ttgV* corresponds to the highlighted G residue in Fig. 2A ($+1P_S$), and the translation start codon of *ttgV* corresponds to the arrow labeled *srpS* in Fig. 2A. To determine if these sites are similarly important in the *srpSR* operon, RT-PCR assays were performed with primers upstream (primer SF0) and downstream (primers SF1 and SF2) of the position $+1P_S$. Total RNA samples isolated from cells grown with or without 20% (vol/vol) toluene were used as templates, and *srpS* first-strand cDNA was amplified with primer SR. PCR amplification was then performed by adding primer SF0, SF1, or SF2. Amplification was achieved only when primers downstream of $+1P_S$ were used (SF1 and SF2), and no band was obtained with the primer upstream of $+1P_S$ (SF0) (Fig. 2B). This result confirms that the transcription start site of the *srpSR* operon is between SF1 and SF0 both in the presence and absence of toluene. Together with the *ttgV* bioinformatics comparisons, these RT-PCR results are consistent with the prediction that the G residue indicated by $+1P_S$ in Fig. 2A is the transcription start site of the *srpSR* operon.

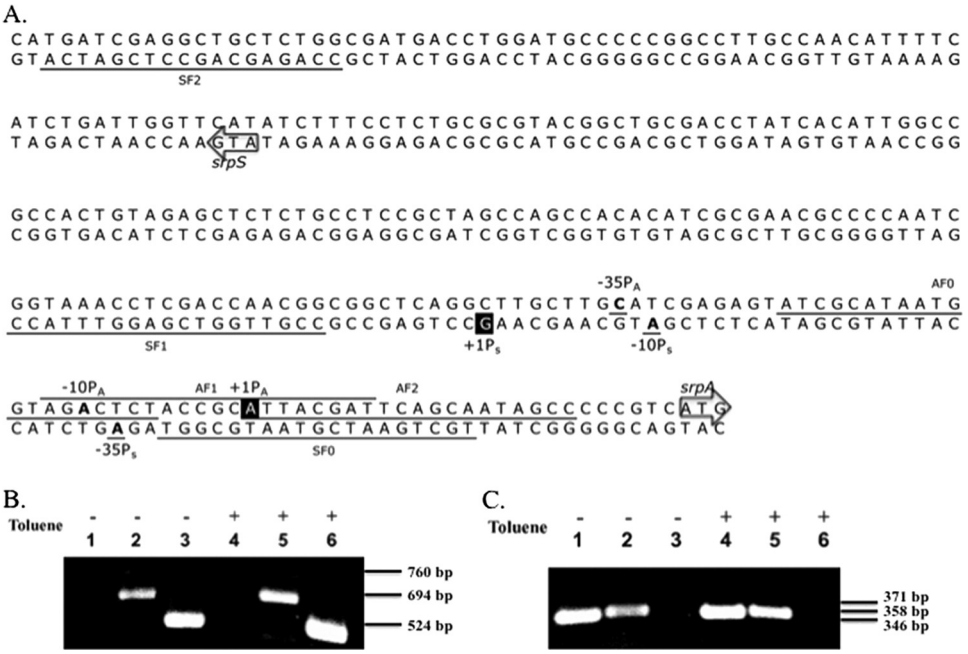

FIG. 2. Determination of the transcription start regions of the *srpS* and *srpA* genes in *P. putida* S12. (A) Overlap region of the *srpS* and *srpA* promoters. The translation start codons of *srpS* and *srpA* are indicated by arrows. The predicted transcription start sites for *srpS* and *srpA* (based on bioinformatics and RT-PCR analysis) are highlighted and designated $+1P_S$ and $+1P_A$, respectively. The base pairs at the putative −10 and −35 positions of each promoter are bolded and underlined. The primers used in RT-PCR assays are underlined (or overlined for AF1), with names indicated in small font above or below the sequence. (B) RT-PCR results for determination of the transcription start region of *srpS*. Lanes 1 and 4, amplification with primer SF0 (expected size, 760 bp); lanes 2 and 5, amplification with primer SF1 (expected size, 694 bp); lanes 3 and 6, amplification with primer SF2 (expected size, 524 bp). (C) RT-PCR results for determination of the transcription start region of *srpA*. Lanes 1 and 4, amplification with primer AF2 (expected size, 346 bp); lanes 2 and 5, amplification with primer AF1 (expected size, 358 bp); lanes 3 and 6, amplification with primer AF0 (expected size, 371 bp). RNA templates in lanes 1, 2, and 3 of panels B and C were isolated from cells grown without toluene; RNA templates in lanes 4, 5, and 6 were isolated from cells grown with 20% (vol/vol) toluene.

Similarly, the transcription start region of the *srpABC* operon was determined. According to prior *srpABC* reverse transcriptase experiments in our lab and predictions based on the *ttgGHI* experiments by Guazzaroni et al. (11), the putative transcription start site of *srpA* in *P. putida* S12 is the A nucleotide indicated by $+1P_A$ in Fig. 2A. To confirm that the start site is in this region, RT-PCR assays were performed with primers upstream of $+1P_A$ (primer AF0), covering $+1P_A$ (primer AF1), and downstream of $+1P_A$ (primer AF2). Amplification was achieved only using primers AF1 or AF2 (Fig. 2C). Together with the bioinformatics comparisons, these RT-PCR results suggest that the A indicated by $+1P_A$ in Fig. 2A is the transcription start site of the *srpABC* operon.

SrpS is a repressor and SrpR is an antirepressor of the *srpA* promoter. Based on the results of β-galactosidase assays, Rojas et al. (33) concluded that *ttgV* encodes a repressor that prevents expression of the *ttgGHI* operon, whereas *ttgW* does not play a significant role in regulation. The *srpR* and *ttgW* genes have 96% identity at the nucleotide level over the length of *ttgW*, but *srpR* encodes a 213-amino-acid protein, whereas *ttgW* encodes a significantly shorter 134-amino-acid protein. We performed β-galactosidase assays to determine the functions of SrpS and SrpR. The single-copy chromosomal *srpA-lacZ* reporter *P. putida* S12 *lacZ*, SrpS$^-$ *lacZ*, and SrpR$^-$ *lacZ* gene fusion strains were constructed as described in Materials and Methods. Table 3 shows the averages for at least three independent assays performed for each strain in the presence or absence of 6 mM toluene. In the wild-type background, expression from the *srpA* promoter was observed even in the absence of toluene (892 ± 13 Miller units). However, expression was increased approximately 6-fold with the addition of toluene (5,023 ± 363 Miller units). In the absence of toluene, expression from the *srpA* promoter increased approximately 5.5-fold in the *srpS* deletion background compared to the wild-type background. In the presence of toluene, expression from the *srpA* promoter failed to increase in the *srpS* deletion background, suggesting that toluene directly contributes to the inhibition of SrpS repressor activity in wild-type cells. Deletion of *srpR* caused expression from the *srpA* promoter to decrease almost 6-fold compared to the wild-type background in the

TABLE 3. Transcription from the *srpA* promoter in the presence or absence of toluene in *srpS* or *srpR* deletion backgrounds compared to the wild-type background

P. putida strain	β-Galactosidase activity (Miller units)[a]	
	Without toluene	With toluene
S12 *lacZ* strain	892 ± 13	5,023 ± 363
SrpS$^-$ *lacZ* strain	4,500 ± 223	4,528 ± 102
SrpR$^-$ *lacZ* strain	153 ± 7	1,555 ± 95

[a] β-Galactosidase assays were performed in the *srpA-lacZ* reporter SrpS$^-$ *lacZ*, SrpR$^-$ *lacZ*, and S12 *lacZ* gene fusion strains in the presence or absence of 6 mM toluene. The values in the table are the averages of at least three independent assays followed by the standard deviations.

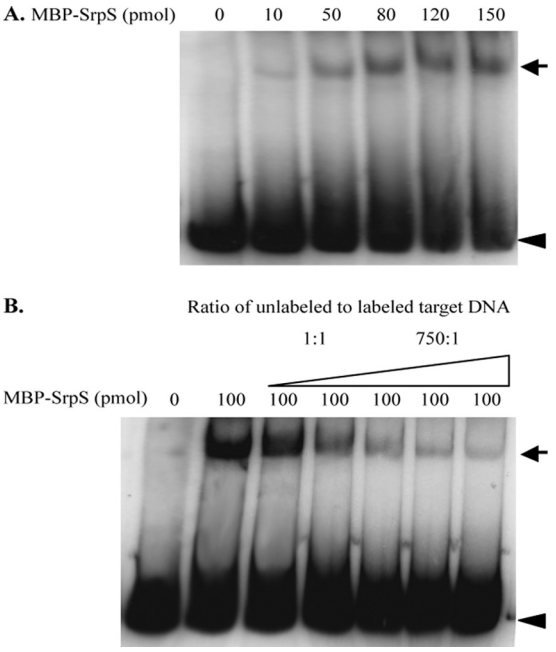

FIG. 3. EMSAs used to assess the interaction of SrpS within the *srpS-srpA* intergenic region. (A) The 490-bp DNA fragment within the *srpS-srpA* intergenic region was end labeled with [γ-^{32}P]dATP. Purified MBP-SrpS at the indicated amounts was allowed to bind to the labeled DNA fragment (2×10^6 to 4×10^6 cpm) in 1× binding buffer at 30°C for 30 min. (B) The 490-bp DNA fragment within the *srpS-srpA* intergenic region in both the unlabeled and labeled forms (with the ratio of unlabeled to labeled DNA ranging from 1:1 to 750:1) was mixed with 100-pmol samples of MBP-SrpS. The reaction mixtures were analyzed on a 4% nondenaturing polyacrylamide–1× TAE gel. The black arrows indicate the shifted band caused by the binding of SrpS to the target DNA. The arrowheads indicate the unbound DNA.

absence of toluene (153 ± 7 versus 892 ± 13 Miller units) and approximately 3-fold in the presence of toluene (1,555 ± 95 versus 5,023 ± 363 Miller units). These results indicate that SrpR positively influences *srpABC* efflux pump gene expression in both the presence and absence of toluene.

***In vitro* assay of the protein-DNA interactions between SrpS and SrpR and the *srpS-srpA* intergenic region.** To further investigate the regulatory mechanisms of SrpS and SrpR, electrophoretic mobility shift assays (EMSAs) were performed to assess the interactions of the SrpS and SrpR proteins with the *srpS-srpA* intergenic region. A 490-bp DNA fragment within the *srpS-srpA* intergenic region was PCR amplified and end labeled with [γ-^{32}P]dATP, and increasing amounts of purified MBP-SrpS or MBP-SrpR were allowed to bind to this fragment. When the labeled DNA was incubated with increasing amounts of MBP-SrpS, a single shifted band was observed (Fig. 3A). The shifted band appeared following addition of as little as 10 pmol of MBP-SrpS, and the intensity of the shifted band increased with the addition of increasing amounts of MBP-SrpS, suggesting that SrpS binds with high affinity to the target DNA within the *srpS-srpA* intergenic region. Competition assays were performed under the same conditions and a fixed amount of MBP-SrpS to determine if the binding was specific. When the 490-bp unlabeled DNA fragment was added to the reaction mixtures as a competitive inhibitor in ratios ranging from 1:1 to 750:1, a gradual decrease in intensity of the shifted band was observed (Fig. 3B). However, in a similar experiment where noncompetitive DNA [poly(dI-dC)] was added to the reaction mixtures, the intensity of the shifted band remained the same (data not shown). These results confirm that SrpS functions as a specific repressor that binds to an operator in the *srpS-srpA* intergenic region, repressing expression of the *srpABC* operon.

Several attempts were made to determine if SrpR binds to the same 490-bp DNA fragment as SrpS. EMSAs were initially performed using the same buffer and binding conditions as for SrpS, and subsequently several variations of buffer compositions, temperatures, toluene additions, and increased DNA fragment sizes were also tested. None of these experiments showed a band shift with MBP-SrpR. Because *srpA-lacZ* reporter results suggested that SrpR positively influences *srpABC* expression 2, but SrpR does not bind to the *srpS-srpA* intergenic region, we hypothesized that SrpR may act as an antirepressor that binds to SrpS, inhibiting SrpS binding to promoter DNA and derepressing transcription from the *srpA* promoter. To test this hypothesis, EMSAs were performed to determine if SrpR, with or without toluene, affected the binding of SrpS to the *srpS-srpA* intergenic region. A fixed amount of MBP-SrpS was allowed to bind to the labeled target DNA, and shifted bands were visualized following the addition of increasing amounts of MBP-SrpR (with the ratio of MBP-SrpS to MBP-SrpR ranging from 1:1 to 1:14). The samples were incubated at 30°C for 30 min to allow SrpR to interact with SrpS (Fig. 4). Another set of EMSAs was performed such that both 1 mM toluene and MBP-SrpR (with the ratio of MBP-SrpS to MBP-SrpR ranging from 1:1 to 1:14) were added simultaneously to the prebound MBP-SrpS–DNA reaction mixtures. The samples were incubated at 30°C for 30 min to allow SrpR to interact with SrpS (Fig. 4B). Interestingly, regardless of whether MBP-SrpR was added to the reaction mixtures after MBP-SrpS was allowed to bind to the DNA (Fig. 4) or MBP-SrpR and MBP-SrpS were added simultaneously (data not shown), the intensity of the DNA-SrpS band gradually decreased as the amount of MBP-SrpR added increased. Also, as shown in Fig. 4B, toluene exhibits a direct inhibitory effect on SrpS DNA binding, independent of the presence of SrpR (complete SrpS dissociation occurring at above 280 pmol of SrpR both in the presence (Fig. 4B), and absence (Fig. 4A) of toluene). Overall, these results indicate (i) that SrpR prevents the binding of SrpS to the *srpS-srpA* intergenic region, (ii) that SrpR is capable of dissociating SrpS prebound to this region, and (iii) that, consistent with the β-galactosidase assay results (Table 3), both toluene and SrpR have direct inhibitory effects on the binding of SrpS to its target DNA.

***In vitro* analysis of SrpR-SrpS protein-protein interactions.** The EMSA results described above suggest that SrpR may act as a regulator that counteracts the repressor activity of SrpS. To test this hypothesis, an *in vitro* glutathione *S*-transferase (GST) pulldown assay was used to determine if these two regulatory proteins interact directly with one other. *srpR* and *srpS* were cloned into the GST gene fusion vector pGEX-4T-1 and the His_6 tag gene fusion vector pQE31, respectively, as described in Materials and Methods. *arpA*, a regulatory gene that encodes the ArpABC efflux pump repressor in *P. putida* S12 (18), was cloned into pQE31 and included in the pulldown

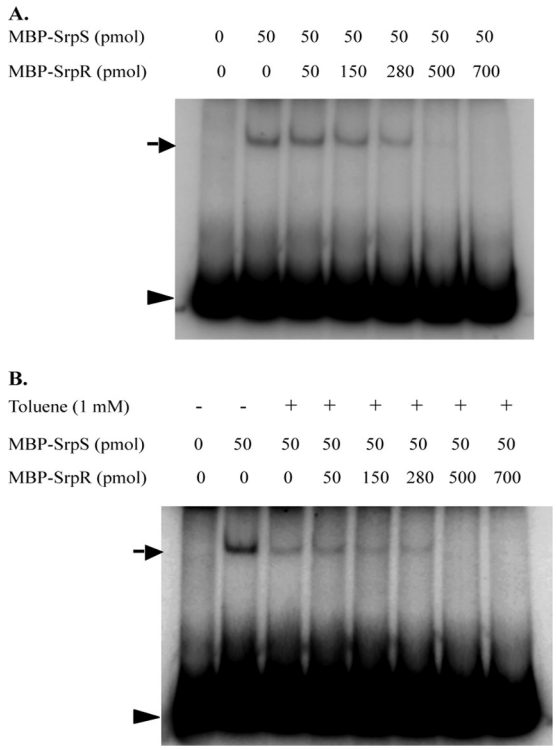

FIG. 4. EMSAs used to assess if SrpR, in the presence or absence of toluene, affects the binding of SrpS within the *srpS-srpA* intergenic region. The 490-bp DNA fragment within the *srpS-srpA* region was end labeled with [γ-^{32}P]dATP. Samples of MBP-SrpS at a fixed amount of 50 pmol were allowed to bind to the labeled DNA fragment (2×10^6 to 4×10^6 cpm) in 1× binding buffer at 30°C for 30 min. (A) Following the binding reaction, purified MBP-SrpR was added to the reaction mixtures in the indicated amounts and incubated at 30°C for 30 min. (B) Purified MBP-SrpR in the indicated amounts and toluene (final concentration of 1 mM) were added simultaneously to the reaction mixtures following the binding reaction and incubated at 30°C for 30 min. The reaction mixtures were analyzed on a 4% nondenaturing polyacrylamide–1× TAE gel. The black arrows indicate the shifted band caused by the binding of SrpS to the target DNA. The arrowheads indicate the unbound DNA.

assay as a negative control for SrpS. In the GST pulldown assays, GST-SrpR was used as the bait protein, and His-SrpS was used as the prey protein. Three negative-control groups were tested simultaneously: GST-SrpR and His-ArpR, GST and His-SrpS, and PBS buffer and His-SrpS. The results of the pulldown assays were visualized using Western blotting, and Penta-His antibody was used to detect the His_6 fusion proteins (Fig. 5). A strong band was present for the test group (lane 1), whereas no band (lane 2) or very faint bands (lanes 3 and 4) were present for the control groups, indicating that GST-SrpR and His-SrpS specifically interact with each other. This result supports our hypothesis that SrpR directly interacts with SrpS and acts as an antirepressor in the transcriptional regulatory system of the SrpABC efflux pump.

DISCUSSION

The SrpABC organic solvent efflux pump in *P. putida* S12 is a unique RND-type efflux system that has an unusual regulatory system consisting of two regulatory genes, *srpS* and *srpR*,

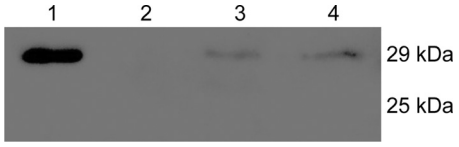

FIG. 5. Results of the GST-pulldown assays visualized by Western blotting. The His_6 fusion proteins were detected by Penta-His antibody and peroxidase-conjugated goat anti-mouse secondary antibody. Lane 1, GST-SrpR and His-SrpS (test group); lane 2, GST-SrpR and His-ArpR (negative control); lane 3, GST and His-SrpS (negative control); lane 4, PBS buffer and His-SrpS (negative control). The expected size of His-SrpS is 29 kDa, and the expected size of His-ArpR is 25 kDa. These assays were repeated three times; this figure, resulting from one of the three experiments, is representative of all three trials.

located upstream of and transcribed divergently from the structural genes. Although the DNA sequence for the RND-type efflux system TtgGHI in *P. putida* DOT-T1E is highly similar to that encoding SrpABC, the pseudogene *ttgW* encodes a protein of only 134 amino acids compared to the 213-amino-acid SrpR protein. Therefore, while the truncated TtgW protein does not appear to be involved in regulation of the TtgGHI efflux pump (33), we show that SrpR is able to derepress SrpABC expression by inactivating SrpS activity in *P. putida* S12.

According to the transcription start site predictions based on alignment with the homologous *ttgV* and *ttgG* genes characterized previously by Rojas et al. (33), there is a 42-bp intergenic region between *srpS* and *srpA*, covering both the −10 and −35 regions of the promoters of these genes. We characterized *srpA* promoter activity in SrpS-deficient and SrpR-deficient backgrounds by constructing *srpA-lacZ* reporter gene fusion strains and performing β-galactosidase assays (Table 3). Expression from the *srpA* promoter increased 5.5-fold in the SrpS-deficient mutant in the absence of toluene but decreased almost 6-fold in the SrpR-deficient mutant, indicating that SrpS represses and SrpR derepresses *srpABC* expression. The *srpS* mutant (*srpR* polar mutation) showed complete derepression in the presence or absence of toluene. The data in Table 3 indicate that SrpR is responsible for approximately 83% of the *srpABC* derepression in the absence of toluene through its effect on SrpS and for 69% of the *srpABC* derepression in the presence of toluene. Furthermore, when SrpR is absent (SrpR$^-$ *lacZ* strain), toluene increases expression of the *srpABC* operon 10-fold (153 ± 7 versus 1,555 ± 95 Miller units), but no effect is observed when SrpS is absent (4,500 ± 223 versus 4,528 ± 102 Miller units), suggesting that toluene must interact directly with SrpS. However, it is interesting that full derepression of *srpABC* in the presence of toluene does not occur in the absence of SrpR (1,555 ± 95 versus 5,023 ± 363 Miller units), suggesting that either toluene potentiates SrpR to remove SrpS or else that toluene and SrpR act together to remove SrpS from the DNA. Given that SrpS is directly affected by toluene (Fig. 4), we predict that both mechanisms play an independent role in causing SrpS removal from an operator region upstream of *srpABC*: for toluene, activity is calculated as 5,023 ± 363 minus 892 ± 13 Miller units, or 4131 of 5,023 Miller units (or ~82%); and for SrpR, activity is calculated as 892 ± 13 minus 153 ± 7 Miller units or 739 of 892

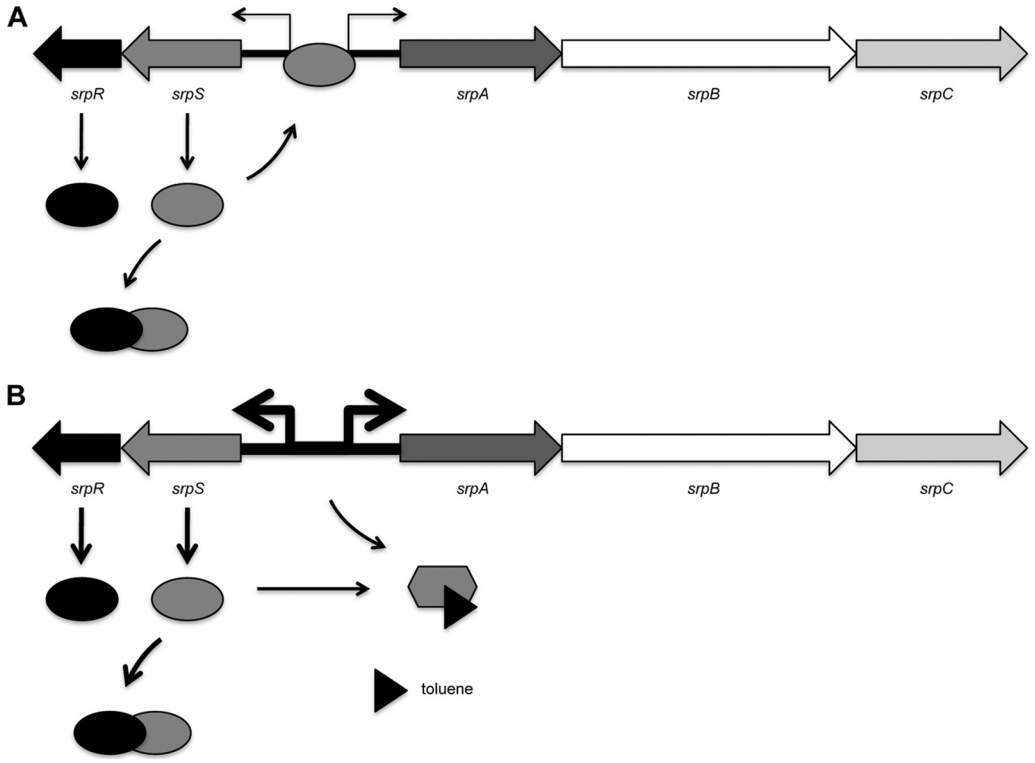

FIG. 6. Proposed mechanism of transcriptional regulation of the *srpABC* operon in *P. putida* S12. (A) In the absence of toluene, SrpR (antirepressor) binding to SrpS (repressor) reduces the ability of SrpS to bind the operator site, permitting low-level expression from the *srpS* and *srpA* promoters. (B) In the presence of toluene, toluene binds SrpS, limiting its ability to bind the operator site. This derepression (in addition to the derepression caused by SrpR) strongly increases the transcription level from the *srpS* and *srpA* promoters. The opposing black arrows represent the promoters of *srpS* and *srpA*, with the thickness of the arrows indicating the level of transcription. The black triangle represents toluene.

Miller units (or ~83%). Together, toluene and SrpR act to dissociate SrpS from its operator upstream of *srpABC*.

Electrophoretic mobility shift assays show that SrpS binds to the *srpS-srpA* intergenic region, thus repressing the transcription of *srpA* (Fig. 3 and 4). Although SrpR does not bind to this DNA region, it nevertheless inhibits the binding of SrpS to its operator (Fig. 4A), indicating that the derepressing effect of SrpR is achieved through the direct interaction of SrpR with SrpS. In Fig. 4, SrpS dissociates from target DNA at the same concentration of SrpR, regardless of whether toluene is present, suggesting that there is a threshold level of SrpR required to complex SrpS. However, in contrast to the *in vivo lacZ* promoter fusion data that suggest that SrpR is responsible for 69% of the SrpS derepression observed in the presence of toluene, a large proportion of the SrpS derepression *in vitro* appears to be due to the direct activity of toluene on SrpS (compare the 50-pmol MBP-SrpS and 50-pmol MBP-SrpR bands in Fig. 4A with the 50-pmol MBP-SrpS and 50-pmol MBP-SrpR plus toluene bands in Fig. 4B). The much greater SrpS dissociation shown in Fig. 4B can be at least partially explained by the fact that the comparison being made is between the effects of toluene directly on an *in vitro* system versus the indirect penetration of toluene into a live cell. It is reasonable to assume that the S12 cells were able to reduce the internal concentration of toluene to a level that not only permitted cell survival but also allowed SrpR to play a greater role in coregulating SrpS relative to the amount of toluene added. With higher cytoplasmic concentrations of toluene (e.g., as viable cells are nearing maximum toluene tolerance), it is possible that high levels of cytoplasmic toluene would lead to increased toluene inactivation of SrpS.

To further investigate the direct interaction of SrpR and SrpS, we conducted GST pulldown assays to identify protein-protein interactions between these two regulators. The bait protein GST-SrpR specifically binds to the prey protein His-SrpS but not to the negative control His-ArpR (the repressor protein for the *P. putida* S12 paralogous ArpABC efflux pump) (Fig. 5). This *in vitro* evidence further supports the contention that SrpR directly binds to SrpS and acts as an antirepressor in the transcriptional regulation of the SrpABC efflux pump. A model of the proposed interactions between SrpS and SrpR in regulating *srpABC* expression in both the presence and absence of toluene (organic solvent) is shown in Fig. 6.

Several mechanisms for antirepression have been previously described. Many mobile genetic elements have regulatory systems similar to an integrative and conjugative transposon named ICE*Bs1* found in the *Bacillus subtilis* genome (4). The SOS response and cell-cell signaling activate the conjugative transfer of ICE*Bs1* following inactivation of the ImmR repressor. Although the ImmR protein sequence is similar to that of many characterized phage repressors, its inactivation is not through RecA-dependent coproteolysis but, rather, results from direct proteolysis by the ICE*Bs1*-encoded antirepressor ImmA. This antirepressor mechanism of action appears to be

quite common, and ImmA homologs are widely conserved in many mobile genetic elements. A second functional class of antirepressors, especially those encoded within phage genomes, exhibits inhibitory activity through direct interaction and binding to their cognate repressors. For example, satellite phage RS1 produces antirepressor RstC that forms complexes with the RstR repressor of the phage CTX (6). Similarly, antirepressors Tum, Coi, E, and Ant from phages 186, P1, P4, and P22, respectively, are all thought to complex with their respective repressors and inhibit the ability of these repressors to bind DNA (15, 26, 35, 38). Even though no sequence or structural similarity is apparent between any of these antirepressors and SrpR, it is the second functional category of antirepressors to which SrpR belongs. Interestingly, SrpR is also predicted to belong to the TetR family of transcriptional regulators. Other members of this family are transcriptional repressors with a high degree of amino acid similarity at the helix-turn-helix (HTH) DNA-binding domain (31). Although SrpR contains this signature HTH domain in its N terminus, the results from this study indicate that SrpR is functionally neither a DNA-binding protein nor a transcriptional repressor.

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