

Identification and Molecular Characterization of an Efflux Pump Involved in *Pseudomonas putida* S12 Solvent Tolerance*

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Bacteria able to grow in aqueous:organic two-phase systems have evolved resistance mechanisms to the toxic effects of solvents. One such mechanism is the active efflux of solvents from the cell, preserving the integrity of the cell interior. *Pseudomonas putida* S12 is resistant to a wide variety of normally detrimental solvents due to the action of such an efflux pump. The genes for this solvent efflux pump were cloned from *P. putida* S12 and their nucleotide sequence determined. The deduced amino acid sequences encoded by the three genes involved show a striking resemblance to proteins known to be involved in proton-dependent multidrug efflux systems. Transfer of the genes for the solvent efflux pump to solvent-sensitive *P. putida* strains results in the acquisition of solvent resistance. This opens up the possibilities of using the solvent efflux system to construct bacterial strains capable of performing biocatalytic transformations of insoluble substrates in two-phase aqueous:organic medium.

The microbial transformation of hydrocarbons is important not only in environmental applications such as soil remediation and waste stream purification, but also in biocatalytic applications for the production of specialty chemicals. The metabolic pathways by which many of these compounds are degraded in various bacteria have been elucidated and in many cases the genes coding for the enzymes involved have been cloned and sequenced (1). A major problem in applying hydrocarbon degrading bacteria to industrial processes is their susceptibility to the toxic effects of the very substrate that the organism is utilizing as a carbon source. This is often due to accumulation of the hydrophobic compound in bacterial membranes which can cause devastating effects on membrane structure (2, 3). A second problem in the application of catabolic pathways in the synthesis of fine chemicals is that many of the desired substrates of enzymatic reactions are sparingly soluble in water and thus may not be fully bioavailable to microorganisms. The use of solvent tolerant bacteria allows the introduction of a

nonpolar phase to the medium, dissolving the desired substrate, and increasing the exposure of the cell to the substrate.

Many different mechanisms have been described that contribute to solvent resistance (Fig. 1) but despite these efforts no comprehensive overview is available to explain the physiological response of microorganisms to toxic organic solvents (for a recent review, see Weber and de Bont (4)). Our laboratories have been investigating the ability of *Pseudomonas putida* S12 to withstand toxic concentrations of toluene and other organic solvents (5). This organism has evolved at least two mechanisms to combat the accumulation of hydrophobic solvents in the membrane or the interior of the cell. One key observation was the detection of *trans*- rather than *cis*-unsaturated fatty acids in the membrane of the solvent-tolerant bacterium upon exposure to solvents (6, 7). The conversion of *cis*- to *trans*-unsaturated fatty acids by a direct isomerization alters the packing of the phospholipids in the bacterial membrane. This results in a change in membrane fluidity, making the membrane less likely to allow solvents to partition into it, decreasing the detrimental effects on the membrane due to solvent partitioning, and thus increasing the solvent resistance of the cell (8). Recently it has been shown that a second mechanism of solvent resistance is possessed by *P. putida* S12. This is an energy-dependent active efflux system for solvents such as toluene (9) that may function in a fashion similar to that for multidrug efflux pumps found in many antibiotic-resistant microorganisms. Thus, *P. putida* S12 employs at least two mechanisms for active defense against the detrimental effects of solvents: one functioning to keep solvents out of the interior of the cell and a second functioning to prevent solvents from partitioning into the cell membrane.

In the present report we describe the construction of transposon mutants of *P. putida* S12 that have lost the solvent tolerant phenotype. This allowed the cloning of the genes responsible for a solvent efflux pump that is involved in the ability of *P. putida* S12 to withstand toxic concentrations of organic solvents. The nucleotide sequence of the genes involved was determined and their relationship to other bacterial efflux systems is discussed.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Media, and Growth of Strains—*P. putida* S12 (10) is the wild-type strain capable of growth at supersaturated solvent concentrations (5) and is the object of the present investigation. *P. putida* JK1 is a solvent-sensitive mutant of *P. putida* S12 derived in the present work by transposon mutagenesis with TnMod-KmO. *P. putida* JJD1, also derived in the present work, contains a kanamycin gene/ColE1 origin cassette insertion in the genome of *P. putida* S12. The solvent-sensitive strain *P. putida* PPO200 is *P. putida* mt-2 cured of the TOL plasmid (11). The artificial transposable element TnMod-KmO¹ contains a kanamycin resistance gene and the ColE1 origin of replication between Tn5 inverted repeats. The Tn5 trans-

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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) AF029405.

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¹ J. J. Dennis and G. J. Zylstra, manuscript in preparation.

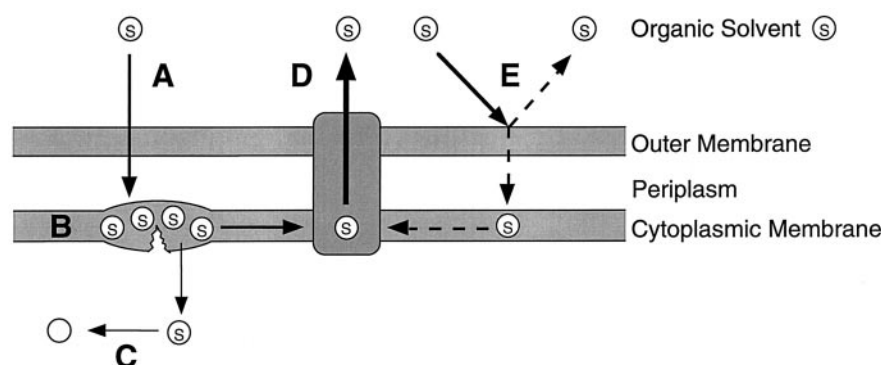


FIG. 1. Schematic representation of essential features of solvent-resistant bacteria. A, solvents diffuse to and preferentially partition to the cytoplasmic membrane where they cause disruptions in membrane functions by increasing membrane fluidity and affecting bilayer stability (3, 4). B, to compensate for these effects, solvent-resistant bacteria modify the composition of the membrane either by isomerizing *cis*- into *trans*-unsaturated fatty acids from the membrane lipids (9, 26) or by changing the headgroup composition (4, 37). This compensation will only be partial and thus in a dynamic process, solvents have to be removed continuously from the membrane. C, removal to a certain extent may be by degradation (32). D, very recently, on the basis of whole cell experiments, evidence was obtained that an unprecedented energy-dependent export system for hydrophobic solvents is in operation (9). E, in combination with a retarded influx of solvents due to modifications at the outer membrane (31, 44), the new efflux pump functions as the key factor in solvent tolerance.

posase gene and an origin of transfer for conjugation are present outside the inverted repeats. *Escherichia coli* JM109 (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)*) (F' *traD36 proAB lacI^qZΔM15*) (12)) was utilized as the host strain for all recombinant plasmids. The cloning vector pUC19 (12), the pGEM series of cloning vectors (Promega, Madison, WI), and the broad host range vector pUCP22 (13) were used for the construction of subclones.

L broth (14) was used as complete medium. Minimal medium was prepared as described by Stanier *et al.* (15). Solid media contained 2% agar. Ampicillin (100 μg/ml), kanamycin (50 μg/ml), or gentamicin (25 μg/ml) were added to the medium to maintain recombinant plasmids in *E. coli*. Gentamycin (25 μg/ml) was used to maintain the pUCP22 subclones in *P. putida* and kanamycin (250 μg/ml) was used in the selection of *P. putida* S12 transposon mutants. *E. coli* strains were routinely cultured at 37 °C and *P. putida* strains were grown at 30 °C. Growth of bacterial strains in the presence of various solvents (indicating solvent resistance) was determined essentially as described by Weber *et al.* (5). The experiments were conducted twice with duplicate samples each time.

Generation and Screening of TnMod-KmO Insertion Mutants—The conjugatable suicide transposon donor TnMod-KmO was introduced into *P. putida* S12 by triparental mating using pRK2013 as the mobilizing plasmid by established procedures (16, 17). Kanamycin-resistant colonies were tested for the ability to grow on L agar plates in the presence of saturating vapor amounts of toluene. This was accomplished by placing the agar plates in a sealed glass dessicator along with a small beaker containing toluene. Growth was scored after 12 h at 30 °C.

DNA Techniques—Total genomic DNA from *P. putida* strains was prepared by the CTAB procedure (18). Plasmid DNA was isolated by the alkaline-sodium dodecyl sulfate lysis method of Birnboim and Doly (19). DNA was digested with restriction enzymes and ligated with T4 ligase as recommended by the supplier (Life Technologies, Inc., Gaithersburg, MD). DNA restriction fragment and PCR² products were visualized by 0.7% or 1.0% agarose gel electrophoresis in 40 mM Tris, 20 mM acetate, 2 mM EDTA buffer. DNA from agarose gels was isolated using the method of Vogelstein and Gillespie (20). Plasmid DNA was introduced into either *E. coli* JM109 or *P. putida* JK1 cells by electroporation (21) using a Gene Pulser (Bio-Rad Laboratories).

All sequencing and PCR reactions were performed using a Gene Amp PCR System 9600 (Perkin-Elmer, Foster City, CA). Nucleotide sequencing reactions were performed with purified double strand plasmid DNA or PCR products using AmpliTaq FS DNA polymerase fluorescent dye terminator reactions (Perkin-Elmer) as recommended by the supplier. Sequencing products were detected using an Applied Biosystems 373A stretch automated DNA sequencer (Applied Biosystems Inc., Foster City, CA). Nucleotide sequence analysis was performed either with the Genetics Computer Group analysis package (22) or with the National Center for Biotechnology Information BLAST server (23). PCR reactions for amplifying the region of genomic *P. putida* S12 DNA contain-

TABLE I
Growth of *P. putida* S12 on L/acetate in the presence of various solvents

Strain JK1 is a transposon mutant of the genes for a solvent efflux pump. pJD105 and pJD106 are clones containing the three genes for the solvent efflux pump (*srpABC*) cloned into the vector pUCP22. pJD105 has the genes in the same orientation as the *lac* promoter on the plasmid while pJD106 has the genes in the reverse orientation. Different solvents (1% final concentration) were separately added to identical subcultures in L medium with 60 mM acetate during the early exponential growth phase. Growth of the culture was measured at various times after solvent addition with no continued growth indicating solvent sensitivity. A plus indicates growth with OD >0.7 after 24 h while a minus indicates no growth with OD <0.1 after 120 h. Log P_{ow} values were calculated according to the method of Rekker and de Kort (43) and is defined as the logarithm of the concentration of a solvent in octanol divided by the concentration of a solvent in water.

Solvent	Log P_{ow}	Growth on L/acetate and 1% solvent				
		<i>P. putida</i> S12	<i>P. putida</i> JK1	<i>P. putida</i> JK1 (pUCP22)	<i>P. putida</i> JK1 (pJD105)	<i>P. putida</i> JK1 (pJD106)
Hexadecane	8.8	+	+	+	+	+
Decane	5.6	+	+	+	+	+
<i>p</i> -Cymene	4.1	+	+	+	+	+
Propylbenzene	3.6	+	+	+	+	+
Hexane	3.5	+	—	—	+	+
Cyclohexane	3.2	+	—	—	+	+
Ethylbenzene	3.1	+	—	—	+	—
<i>p</i> -Xylene	3.0	+	—	—	+	—
Octanol	2.9	+	—	—	+	—
Toluene	2.5	+	—	—	+	—
Dimethylphthalate	2.3	+	—	—	+	—
Fluorobenzene	2.2	—	—	—	—	—
Benzene	2.0	—	—	—	—	—

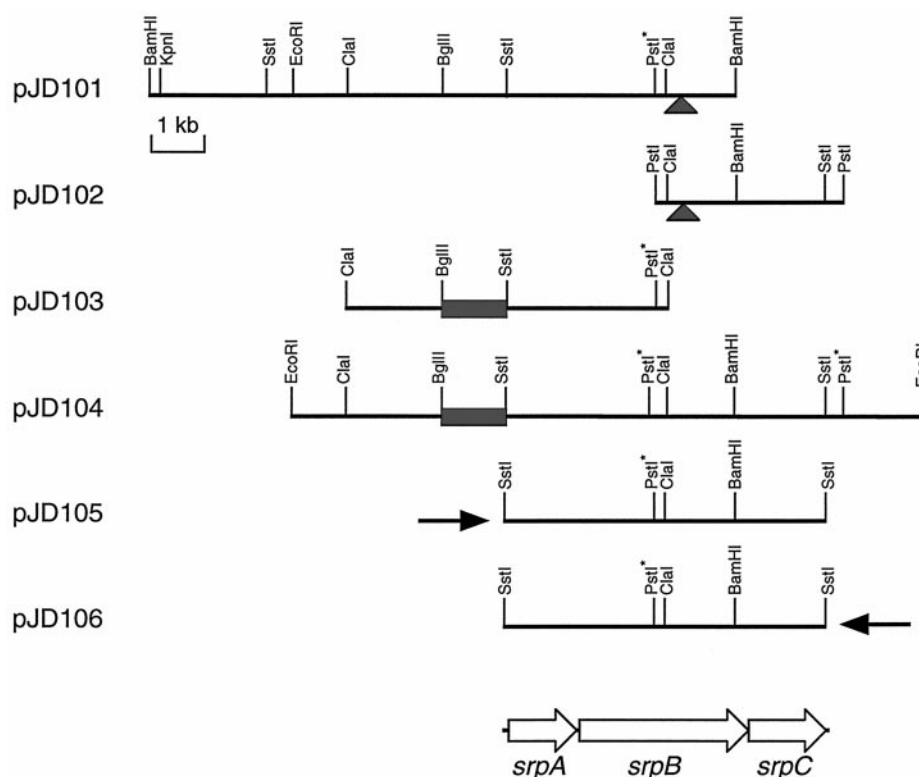
ing the insertion point for the transposon were performed using *Taq* DNA polymerase (Perkin-Elmer). The reaction mixture (100 μl) was treated for 1 min at 94 °C followed by 25 cycles of 1 min at 96 °C, 1 min at 55 °C, and 1 min at 72 °C before finishing for 10 min at 72 °C. Primers for this reaction were 5'-CGTTTGCAACCGGTGAG-3' and 5'-TATCGGACGCAAACG-3' corresponding to positions 3735 to 3752 and 4238 to 4253 of the nucleotide sequence, respectively.

RESULTS

Isolation of Solvent-sensitive Mutants—*P. putida* S12 was chosen for a molecular study of the basis of solvent resistance due to the extensive physiological studies that have been performed on the strain (4–6, 9, 24–26). The organism can grow in the presence of a wide variety of normally toxic solvents with log P_{ow} values ranging from 2.3 to 3.5 (Table I). Initially, several solvent-sensitive transposon mutants were constructed using TnMod-KmO. *P. putida* S12 mutants which are no longer

² The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase pair(s).

FIG. 2. Restriction maps of the clones derived from the transposon and insertional mutant strains. A cartoon of the *srpABC* nucleotide sequence is shown at the bottom, in proportion to the restriction maps and showing the positions of the genes relative to the restriction enzyme cutting sites. The triangles in the restriction maps of pJD101 and pJD102 and the boxes in the restriction maps of pJD103 and pJD104 indicate the location of the inserted kanamycin resistance gene and the ColE1 origin of replication in the cloned genomic DNA. The arrows next to pJD105 and pJD106 indicate the direction of transcription of the *lac* promoter from the vector pUCP22. The asterisk indicates that not all of the *Pst*I sites were mapped in these plasmids.



resistant to solvents were detected by the inability of kanamycin-resistant exconjugants to grow on L medium in the presence of supersaturated vapor concentrations of toluene as described under "Experimental Procedures." Several toluene-sensitive mutants were obtained and one of these, designated strain JK1, was chosen for further analysis. Besides toluene, JK1 is sensitive to a number of other solvents with log P_{OW} values less than or equal to 3.5 (Table I), indicating that a single genetic trait is responsible for resistance to all of the solvents tested.

Cloning and Analysis of the Genes for Solvent Resistance—To characterize the genes for solvent resistance in *P. putida* S12 in more detail, the region of the genome containing the transposon insertion in mutant JK1 was cloned. This was aided by the fact that the TnMod-KmO transposable element utilized in the construction of the mutants contains an origin of replication derived from plasmid ColE1. Total genomic DNA from JK1 was cleaved with *Bam*HI, ligated to form circular molecules, and electroporated into *E. coli* JM109 with selection for kanamycin resistance. Since *Bam*HI does not cleave the transposon, the resulting clone, pJD101 (Fig. 2), must contain DNA from both sides of the transposon insertion. Approximately 11 kilobases of genomic DNA was cloned along with the transposon which contains the origin of replication and the kanamycin resistance gene (2 kb). The point at which the transposon is inserted is only 1 kb away from one end of the *Bam*HI fragment cloned from strain JK1. This being the case, a second, overlapping 4-kb *Pst*I fragment was cloned from strain JK1 (designated pJD102, Fig. 2).

The cloned genomic DNA was initially analyzed by determining the nucleotide sequence to either side of the transposon insertion point using primers specific for either end of the transposon. Screening for similar nucleotide sequences in the GenBank data base revealed a significant match with genes coding for multidrug resistance export pumps, consistent with the hypothesis that a solvent efflux pump is a key solvent resistance mechanism of *P. putida* S12. The complete nucleotide sequence of an operon into which the transposon had

inserted was determined using a series of subclones and internal oligonucleotide primers. Both strands of a 6.5-kb *Sst*I fragment were sequenced in their entirety. To accurately determine the nucleotide sequence at the transposon insertion point, a 0.5-kb DNA fragment was amplified by PCR from the genome of the wild-type strain *P. putida* S12 using oligonucleotide primers flanking the transposon insertion point in strain JK1. A cartoon of the nucleotide sequence obtained is shown in Fig. 2 to show the relationship of the open reading frames with the two clones pJD101 and pJD102 and the complete nucleotide sequence is shown in Fig. 3. The three open reading frames show significant homology to the three proteins that assemble to form proton-dependent multidrug resistance efflux pumps (27, 28) and thus the genes were labeled *srp* for solvent resistance pump.

Complementation of *P. putida* JK1—To prove that the three open reading frames detected in the cloned fragment at the point of insertion of the TnMod-KmO transposon actually are responsible for solvent resistance, complementation experiments were performed. This required reconstruction of the operon since the clones obtained (pJD101 and pJD102) contain the transposon mutagenized DNA. A *Bgl*II-*Sst*I kanamycin resistance cassette also containing the ColE1 origin of replication from TnMod-KmO was inserted at the *Bgl*II and *Sst*I sites of a 6.7-kb *Cla*I fragment derived from pJD101. The resulting plasmid (pJD103, Fig. 2) was electroporated into *P. putida* S12 to construct a new mutation by site-specific reciprocal recombination. The resulting strain, JJD1, contains a kanamycin resistance gene adjacent to the *srpABC* genes in the genome. A 12-kb *Eco*RI genomic fragment containing the kanamycin/ColE1 cassette was cloned from JJD1. This plasmid, designated pJD104 (Fig. 2), contains the intact *srpABC* genes. A 6.5-kb *Sst*I fragment was cloned from pJD104 into the vector pUCP22 in both orientations with respect to the *lac* promoter. JK1 containing either of these two plasmids, designated pJD105 and pJD106 (Fig. 2), regained solvent resistance. JK1(pJD105), containing the *srpABC* genes in the same orientation as the *lac* promoter, regained resistance to all of the solvents that the

A

GAGCTCTCTGCTCCGCTAGCCAGCCACACATCGGAAACGCCCAATCGGTAAACCTCGACCAACGGCGCTCAGGCTTGC	120
TTAGCATTCAGCAATAGCCCCCGTCATCGGGGAGAGAGATGGAGCCAAACCGTGAGACAGATACGATCCCGCGTGCAT	240
TCAGGTAATCCCCCTTAACCTGCGCTGATGCTAATTTCCGGG	
M R Q I R S P R A L R V I P L T A L M L I S G	
ATGTGCTGAGAAGAAGCAGGTAGCTCTGCAACTCCACCACGGAGCTGGGCGTGTACACGCTGCGTGCACAAGCTTGACCC	360
CTGACCTGACTTGCCTCCGCGGACTTCGGCATTTCCG	
TGTAGCGGAAGTCCGCCCACAGGTTTCGGGGATTCTTCAGAAAGCGTTCGTTTGAAGGCGTGAAGTCAAGCTGGGT	480
CAGCAGCTTTATCAGATTGACCCACGAGCTACGAAGCGCA	
V A E V R P Q V S G I L Q K R S F V E G A E V K L G Q Q L Y Q I D P R T Y E A Q	
GTTGCGTCTGCAAGCGAATCGAACTAGCGCACAAACCTGGCTCGTCTATGAACCGCTGTTGAAGACCAAGGCAAGCAGCAGT	600
ACGACGATCGCCTTCCGCGATGGAA	
L R R A E A N R T S A Q N L A R R Y E T L L K T K A V S K Q Q Y D D A L A A W K	
ACAAGCAGAAGCGATACAGGTGGCTCGCATCGAGTTCAATACACAGGGTCTTCCCAATCTCCGGGCGCATTTGGTCTGCT	720
CGACGCTACCCGAGGGGCGCTCGTTACCAATGG	
Q A E A D Y Q V A R I D V Q Y T R V L S P I S G R I G R S T V T E G A L V T N G	
CCAAGCTCAGTCTGCTCGCCACCGTCACGAGCTGGACCCCATTTATGTCGACGCTCACTCAGCCCAATCACAAGCTTCT	840
TGGTCTGCAAAAGCGCTGGAGTCAGCCGCGCTTCAAAGAC	
Q A Q S L A T V T Q L D P I Y V D V T Q P I T K L L G L Q K A L E S G R L Q K T	
AGGCGAGAACCAGGCTGAAGTCAGCTGACCTGGATGACGGAAGTCTTATCCACTGCCAGGAACCTCTGAAGTTTCCGAGGT	960
GAGCGTGTATCCCAACAGGTTCTGTGACCTTCG	
G E N Q A E A V S L T L D D G S A Y P L P G T L K F S E V S V D P T T G S V T L R	
CGCCGAATTTCCCAATCTTAATCGAAGCTGCTACCAAGGCTGTTTGTGATGCTCTGCTGAAAGAGGCGCTGCAAAACCG	1080
CGCAATCTTGGTACCGCAACAGGCATCAGCCGCGATAC	
A E F P N P N R K L L P G M F V H A L L K E G V Q N A A I L V P Q Q A I S R D T	
GAGAGGTGTGCCAAGCGTCTGGTCTGAAGGCTGACAACCGTCAAGTCCCGTGAAGTTCAGACCTCCGCAACCTGGGCAAT	1200
GCTGGTGTATCAGCAACCGCTGACCGGAGGAGA	
R G V P S V W V V K A D N T V E S R E I Q T L R T V G N A W L I S N G V T E G E	
GCGGATCATTACCAAGGTGTTCAACGTGTTTCGACGCGCATCGCTGTCAACGCGGTGAGGCGCAAGATGTGAATTTGGT	1320
TAGACGCGCTTCGCGCTACCAAGAGCTTCGGCTAACTG	
R I I T E G Y Q R V R S G I A V N A V E A K N V L V D G F A A T E A S A N *	
AGGGGCGCAGATTATCTCTGTTCTTTATCGACAGGCGGATTTTTCGCTGGGTCTCGCTATCGCTGGCCATCTTGGCGT	1440
CGCTGTCTTGGCGAAATGCCCATATGCAATATC	
M S R F F I D R P I F A W V L A I V A M L L A G A L S L A K M P I S Q Y	
CAACATTCGCGGCCCTGCCGTTTCGATCCAAGTCAGTACCCAGGTGCTTCTGCACAAACCGTGACGACACAGTGGTTCAGGT	1560
CATTGACGAGCAGCTCAGTGGGCTGGATGGGTTCC	
P N I A A P A V S I Q V S Y P G A S A Q T V Q D T V V Q V I E Q Q L S G L D G F	
GATACATGTCGCGAGAGCGCTCTGACGCGCAGCATGACCATCATCTGACCTTTGAACAGGCAACCGATCCGGATATCGCACAG	1680
GTTCAGGTGCGAACAAGCTCGACGTGGCAACAC	
R Y M S A E S A I R E Q N V T F E G G T D P D I A Q V Q V Q N K L Q A T C	
CTCGTTTCGCGAAGAGGTTCAGCGTCAGGCTCTTCGTGTAGTGAAGTACAGATGAACCTTCTTCGTGATGAGCTTGGTGGAT	1800
CGTTTCGCGAAGCTCGATTAACCTTCGACCTGGGCA	
P R L P E E V Q R G L R V Q G L R V Q M N F L V M S L V D R S G K L D N F D L G	
ACCTGATCGCGTCACAGCTCCAGGACCCCATTCGCGCATCCAGGCGTCGCGGATTTCAGCTGTTTGGCTCGCCTTATGCCAT	1920
CGCTATCTGGCTGGACCCAGGCAAGCTCAATAGCT	
N L I A S Q L Q D P I S R I P G V G D F Q L F G S P Y A M R I W L D P G K L N S	
ATCAGCTGACGCCCATCGCTCGCAGCGCTATCCGAGAGCAGAAGTTCAGGTGTCCTCCGCGAGTTGGTGGATTACCAACT	2040
CGCTCAGGCGTCCAGCTGAATGCGACCGTTCCTGG	
Y'Q L T P T D V A S A I R E Q N V T F E G G T D P D I A Q V Q V Q N K L Q A T C	
GCAAGACGCGCATGACCAAGCTAGCCAGTTCGATGAGATTCTGGTCAAGGTAACCCCGGATGGCTCGCAGGTACGAGTGAAG	2160
GATGGTGGTTCGCGAAGCTTGGGCTGACAGCTTCG	
G K T R M T T P S Q F D E I L V K V N P D G S Q V R V K D V G R A E L G A D S F	
CCATTTCGCGCATACAGGACTCGCAACCGCAAGCTGCGCTGTGCCACCGCGGCAATCTGCTGGAACCGTAGACGCGGT	2280
CAAAAAGCTGATGGAGCAGCAAAAGGCCT	
A I S A Q Y K D S P T A S L A L R L S T G G N L L E T V D A V K K L M E Q Q K A	
ACTTCCAGAGCGCTGGAGTCATTTCCTACGATACGACACCGGTTGTTAGCGCTCGATTGAGTACACACGATCTTTAGGCGGT	2400
GGTACTGGTCTTCTGGTCAATGT	
Y L P D G V E V I Y P Y D T T P V V E A S I E S V V H T I F E A V V L F L V M	
ATCTGTTCTCGAGAGCTTCGCGGCACACTGATCCCCACCTGGCAGTTCAGTGGTGTCTGCTCGCCACTTTCGCCCTTTT	2520
CGCGTACTTCGGGCTGAATCAACGTGCTCACCATGT	
Y L F L Q S I L P T L A V P L L A V P L L A T F A L L P Y F G L N I N V L T M	
ATGCCATGGTGTGGCTATCGGCTTGTGTGTGAGATGCAATCTGCTGGTTCGAGAACGTTGAGCGGCTCATGATGACGAGG	2640
CTTGTGCGCTCTTGGGCTACCCGCAAGTCGATGG	
Y A M V L A I G L L V D D A I V V E N V E R L M H D E G L S P L E A T R K S M	
ATCAGATTTACGGGCTTGGTGGGATCGGATGGTGTGCTGCTGCTGATTCGCTTACCCATGCGCTTCTTGGTGGCTCCGCT	2760
GGCATCATCTATCAACAATTTGGCATCACCATGTAG	
D Q I S G A L V L S A V F V P M A F F G G S A G I Y Q Q F A I T I V	
TCGTGATGGGCTTTCGATCTTGGTCTGCTTGTGTTCAACCCAGCACTTTGCTGACCATCTCAAAGCTTCGAGGGTAATTC	2880
CACACCAGAGAAAGGGCTTCTGCGCTGGTTTA	
V C M G L S I L A L V F T L P A L C V T I L K A P E G N S H H E R K G G F F G W F	
ACCGCATCTTCGACCGCGCACGCGCGGTTTCGAGCGAGGCGTCGCGCAATGCTGAAGGGCGCGGTAGGTACCTGCTTGTGCT	3000
CATCACCGGAGGAACCGGCTATCTGTTC	
N R I F D R G T R R F E R G V G A M L K G R G R Y L L A F L L I T G G T G Y L F	
CCAGATCCCTAAGCATTCTGCCAACAGGATCAGGGCTTGTGATGATGATGAGTGCCTACACCGCAACGCTTCCGCTGAACG	3120
TACCGAGGTGCTTCAAGAGGTGCGAGACT	
T Q I P K A F L P N E D Q G L M M I E V R T P A N A S A E R T E G V L Q E V R D	
ACTTGGCCCAATGATGAAGGTGATGCTTGTGAGCACTTCATGACGGTGAATGGCTTCAACTTGTGCTGCTGCTGAGAACT	3240
CGGGCTGGTGTGATCACTTCAAGGACTGGAAGGAGC	
Y L A N D E G A L V E H F M T V N G F N F A G R G Q N S G L V L I T F K D W K E	

Fig. 3. Nucleotide sequence of an *Sst*I fragment containing the *srpABC* genes. The deduced amino acid sequences of the encoded proteins are shown below the nucleotide sequence. Termination codons are indicated with an asterisk, putative ribosome-binding sites are underlined, a putative RNA polymerase-binding site is underlined, and the TnMod-KmO insertion point is marked with an arrow. The GenBank accession number for the *srpABC* sequence is AF029405.

original strain, *P. putida* S12, was resistant to (Table I). However, JK1(pJD106), containing the *srpABC* genes in the opposite orientation to the *lac* promoter, regained resistance to only two solvents, hexane and cyclohexane, with log P_{OW} values near the border of the resistance phenotype. These results are consistent with the solvent resistance phenotype being dependent on the level of expression of the *srpABC* genes.

Transfer of the Solvent Resistance Phenotype—*P. putida* S12 displays multiple physiological responses to organic solvents (see Introduction). Intuitively, a solvent efflux pump would be the most important mechanism of solvent resistance since it would be involved in actively removing solvents from the cell. Experiments were therefore performed to determine whether the solvent efflux pump by itself is capable of imparting the solvent resistance phenotype on other *P. putida* strains. The two plasmids, pJD105 and pJD106, were electroporated into the normally solvent sensitive *P. putida* PPO200. The resulting recombinant strains are able to grow on rich medium in a toluene-saturated atmosphere, whereas the parent strain PPO200 with the vector pUCP22 could not (Fig. 4). PPO200-

(pJD106) grew slightly slower than PPO200(pJD105), probably due to the fact that the *srpABC* genes are expressed from the *lac* promoter in pJD105. In liquid culture, PPO200(pUCP22) could withstand concentrations of toluene up to 2.8 mM while both PPO200(pJD105) and PPO200(pJD106) showed resistance to the toxic effects of toluene up to a concentration of 4.9 mM (Table II). Neither of the recombinant strains were resistant to a second phase of toluene (5.6 mM). These experiments indicate that the solvent resistance phenotype can be transferred to other bacterial strains and that the resistance can be enhanced by higher levels of gene expression.

DISCUSSION

In the past several years solvent-resistant microorganisms have been isolated directly from the environment (5, 29–33) or through the process of mutation of a solvent-sensitive strain (34–36). It is evident that these organisms must therefore have specific adaptation mechanisms that impart the solvent resistance. Physiological studies on microorganisms isolated from the environment that are naturally resistant to high levels of

B

GTCACGGTGTGGGCAAGCGTATTCAGCATCGCGCAACGGGCTAATCAGCATTTTGCCAAAGATCAAAGATGCCAGCGTCATGGCGTTCGTCCTCCAGCCATCTTGGAATGGGCAACG	3360
R H G A G Q D V F S I A Q R A N Q H F A K I K D A S V M A F V P P A I L E M G N	
CGATGGGCTTCAACTTGTATCTGCAGGACAACCTCGGCTAGGCCACGAAGCATGTATGGCAGCTCGAAACCAATTCCTGCAACTGGCAAGCCAGAACCCTAAGCTCCAGGCAGTTCGTC	3480
A M G F N L Y L Q D N L G L G H E A L M A A R N Q F L Q L A S Q N P K L Q A V R	
CAAAAGCGCAAGGACGACGAGCGCAATTCAGGATCGATGACGAAAAGCGCGCTGCAAGTAAAGCATCGCGTCCATCAACGAACAGTGTCCGCGAGCTGGGGCTCGATGT	3600
P N G K D D E P Q F Q V N I D D E K A R A L Q V S I A S I N E T M S A A W G S M	
ATGTCAACGACTTCATCGACGCGCGCGGCTCAACCGGCTGATCGTCAGGCGAAGACATCTCGGAAATCTCCCTGAGGAGCTTCGATAAATGGTACGTGCGCAATAGCTGGGCCAGA	3720
Y V N D F I D R G R V Y V Q G E D I S R I S P E D F D K W Y V R N S L G Q	
TGGTTCGGTTCGCGGTTTGCAACCGGTGAGTGGGTGAACGGCTCACCGAAGCTGGAGCGCTACGGTGGCATTTTCGTCCTGAACATCTCCGCGAGCCAGCCCTGGATACAGCACGG	3840
M V P F S A F A T G E W V N G S P K L E R Y G G I S S L N I L G E P A P G Y S T	
GTGACGCGATGATAGCTATCGTGAATCATGCAACAACCTGCCTGCCGCGATGGCTGAGCTACACCGGCTCTCATATGAAGAAATCCAACTGGCGACCGGACCATTTGCTCTATG	3960
G D A M I A I A E I M Q Q L P A G I G L S Y T G L S Y E E I Q T G D G A P L L Y	
CGTTGACTGTGCTGATCGTTTCTGTGCTGGCGCTGTATGAGAGCTGGTGGTACCGGCTCAGTATCATGGTGTCTCTGGTATTTTGGGCGCGGTGCTGGCAACGCTTT	4080
A L T V L I V F L C L A A L Y E S W S V P V S V I M V P L G I L G A V L A G T	
GGCGCGACCTGACGCGAGATGTACTTCCAGGTGGGCTTGATGACCAAGGTTGGCTTGTCCGCCAAGAACCCATCTGATCGTAGAGTTCGCGAAAGAGCTGTATGAGAAGAGGGTT	4200
W R D L T A D V Y F Q V G L M T T V G L S A K N A I L I V E F A K E L Y E K E G	
ATCCGATCGTCAAAGCTGCGATAGAGGCCGCAAGCTGCGTTTTCGTCGCGATTTGATGACCTCGTGGCATTCACCTTCGGTGTGCTGCCGATGGCCATAGCATCCGCGCAGGCGCTG	4320
Y P I V K A I A E A K L R L R P I L M T S L L A F T F G V L P M A I A S G A G A	
GCAGCCAGCATTCATGCTACCGGCTGCTGCGAGGCGATGACACAGCAACGGTGTGCTGGCTGCTCTTCTTGTTCGCGCTTCTACGTAGTAGTGTGCAAGCTTTTCGAGGGGCTCATGA	4440
G S Q H S I A T G V V G G M I T A T V L A V F V P L F Y V V V K L F E G L M	
AAAGAAAGCCAAACGCGGTCAAGGAGTGACTCATGAAGTTTAAGTCACTCCCATGTTTGGCTTGTGCTGATGCTCGGCGGATGCAGCTTGATCCCGACTATCAGCAGCCAGCGCACCG	4560
K R K P N A V K E V T H E V *	
M K F K S L P M F A L L M L G G C S L I P D Y Q Q P A A P	
ATGCAGGCACATGGCCAAAGCGCCAGGCTACGGCGGCGAGGAGCAACGCGATTCGCGAGCGCTCTCCCAAGGCAAGAGTTTTAAAGATCCAGCGCTGGTCCGCTCCTC	4680
M Q A Q G W P T G Q A Y G G Q G D Q R S I A T A L P K A K E F F K D P A L V R L L	
GATGCAGCATAGAGCAACAGAGATCTTCGAATCGCTGCAAAATGTTGAATCTACAGAGCGCTGACCGGATCCAAAGAGCAGAGCGGTTTCCACACTTGATGGCCAGGCTCT	4800
D A A L E N N R D L R I A A K N V E S Y R A L Y R I Q R A E R F P T L D G Q A S	
GGAAATCGAACCCGCTTGCTGACGATTTATCCCCACCGCGATTTCTCGGATCGATAGCCAGTACCAAGTAGGCTTGGTACTGCTACGAGTTGGACTTATTTGGCCGAATCCGAAGC	4920
G N R T R L P D D L S P T G D S R I D S Q Y Q V G L V T A Y E L D L F G R I R S	
TTGTGCAACCAAGCACTTGAAAATACCTCGTACTGAGGAGGCGCAGCGCTTCAGATCGCACTCATAGGCGATGTGGCAACCACTATTTTCTGTGCGCAACCGACCAAGCGCTC	5040
L S N Q A L E K Y L A T E E A Q R S V Q I A L I G D V A T T Y F L W R T D Q A L	
TTGGAGCTGACCGAAGCGACCTGACGAGCTACGTTGAAAGCTTGGCAATGATCGAGTGCAGTGTGGGCGAGGAACCTCGTGGAGCTAGATTTTCTGCGCCAGGACTTTGGTGAAC	5160
L E L T E A T L T S Y V E S L A M I E S S A W A G T S S E L D V R Q A R T L V N	
CAAGCCAGGCGCAGCAGCGCTTGATACCCGCTCGAATAGCGCAGGATGTGAATGCATGGAATTTGCTGGGCGCAAGATTCCAACGGATTTCGCAAAAACCTCGCCAGTGGCCATG	5280
Q R A Q Q R I A L Q D V N A L E L L S K I P T D L P K N S P L A M	
AGCGCCCTAGGGAAGTTCCAGCTGGGCTACGAGTGTCTGCTGAACAGACCGGACATCCGTTCTGCTGAACATCAACTTATGGCCGCAAGCAAAATATCGGCGAGCTGTGCG	5400
S A L G K V P A G L P A D L L N R P D I R S A E H Q L M A N A N I G A A R A	
GCATTTTTCACGCACTTACGCTGACAGCTTCAGCGGCTCAGCGAGCGATCTTGATGAGCTTTCAACAGCGGATCTGACAGCTGGAGCTTCGCTCCACAAATCAGCGTGGCGATT	5520
A F F P R I S L T A S A G S A S S D L D G L F N S G S D S W S F A P Q I S V P I	
TTTAACGAGAAAGCTGCGAGCAATCTCGATTACGCTGAGCTTCAGAAGGATGTGGCGGTAGCGACCTACGAGAAGAGATCCAAACCGCTTTCCGAGAGGTAGCCGAGCGGATCGCT	5640
F N A G K L R A N L D Y A E L Q K D V G V A T Y E K S I Q T A F R E V A D G L A	
GCGAGGGAAGTACGGCAACAGCTCAGTGTCTCAAAGCGAGCTGGTTGATAATTATAAGCCTATTTACGTTTGGCCAGCAGCGCTATGACGAGGCGCTCGATAGCTATTTGACAGTG	5760
A R G T Y G K Q L S A Q S E L V D N Y K A Y F S L A Q Q R Y D Q G V D S Y L T V	
CTGGATGCTCAACGCGAAGCTTTTCTCTCAACAGAAAGCTTTGAATGATCAGCTCGATCAATAGTGAAGTCCAGCTTTACAAAGCCCTCGGGGCGGCTGGAGCGTGGAGTCAA	5880
L D A Q R E L F S S Q Q K L L N D Q L D Q I N S E V Q L Y K A L G G G W S V S Q	
AACTAAGATCCTGGGCGCGCTAGCAGTAGCGCTGCCTAGATTTCAAATATTGAAATATTTTTCGAAGTGAAGTGGCGAATTATCGTCGATTTTAAATCGGGAGTATTAGCAATGGGG	6000
N *	
TTGATATCAGCAAGCGGCTTAGAGGTTTCGTTTCGAAGACCGGCTGACCATGATGACTTTTACCGGTAGACGACCTGGTTAAGCTGCTTCGCACTGCGCGGGGTGTATGAGCTATACG	6120
ATAATCGCGCTGAACAAGTACTTCAGTTTCGATGATATGCGGCTTCGAGGACAATGACAAAGATATGATAGATCATTTTTCAGCTCAAGTTTCGCGACATTCAGTATGCTTGAC	6240
TCCATTTCCGCAAGCATCTCTTTTAGAGCATTTGTTTATGGATAATGAAATGCGGGGCAACATGCAGATCTGAAAGCGCTACTGGATGCAGATTTACACAGCTGATTACTTTCTT	6360
GGTGGTATATCAAGAAAGGGAGTGACTCGGGCTTCTGAGCGCTTAGGTGTAAACAAACAGCATATCAGCAATACCTAGTAAAGCTTCGCAACAGATTTCGTGATGAGCTC	6472

FIG. 3—continued

organic solvents have revealed that many different factors may play a role (4, 7). Naturally solvent-resistant bacteria have been shown to alter the composition of the cell membrane by increasing the ratio of *trans*- to *cis*-unsaturated fatty acids (26) or by changing the headgroup composition (37). This change in the cell membrane produces a physical barrier, preventing solvents from entering the cell by decreasing membrane fluidity. This would not entirely prevent solvents from entering the cell, only slow down their diffusion into the cell and increase the time needed to reach equilibrium with the external environment. An intuitively better method of solvent resistance would be to physically remove the solvent from the cell. One way of doing this would be to degrade the solvent but this would only be effective against low concentrations of solvents. Evidence was recently obtained that an energy-dependent export system for hydrophobic solvents functions to remove solvents from the interior of whole cells (9). This solvent efflux pump should be a key element in solvent resistance by naturally solvent-resistant bacteria. The genes for such a solvent efflux pump in *P. putida* S12 were identified via transposon mutagenesis to construct a solvent-sensitive strain. The genes were cloned and sequenced and their role in solvent resistance verified through complementation of the transposon mutation. The three genes involved were labeled *srpABC* for solvent resistance pump.

The deduced amino acid sequences of the proteins encoded by the *srpABC* genes have extensive homology with those for proton-dependent multidrug efflux systems of the resistance/nodulation/cell division family (27, 28). This "RND" family of efflux pumps is composed of three protein components that together span the inner and outer membranes of Gram-negative bacteria: an inner membrane transporter (SrpB analogues), an outer membrane channel (SrpC analogues), and a periplasmic linker protein (SrpA analogues). Members of this family have been shown to be involved in export of antibiotics, metals, and oligosaccharides involved in nodulation signaling. Based on the work presented here, this family can be broadened to include a new class of efflux pump, involved in export of solvents. Dendrograms showing the phylogenetic relationship of SrpA, SrpB, and SrpC to other proteins involved in multidrug resistance are shown in Fig. 5. The *srpABC*-encoded proteins show the most homology with those for the *mexAB/oprM*-encoded multidrug resistance pump found in *Pseudomonas aeruginosa* (38, 39). SrpA, SrpB, and SrpC are 57.8, 64.4, and 58.5% identical to MexA, MexB, and OprM, respectively. The three dendrograms show that the Srp and the Mex proteins fall into a distinct class, separate from but still closely related to the other members of the RND family of efflux pumps. The evolutionary relationship of the solvent resistance pump to multidrug resistance pumps is not surprising since they both

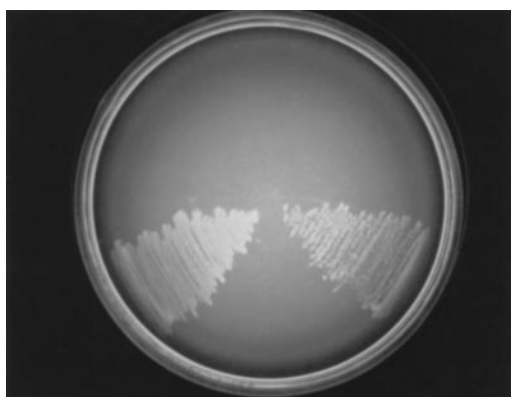


FIG. 4. Photograph of the solvent-sensitive *P. putida* PPO200 plated on solid L medium in the presence of saturating vapor concentrations of toluene with and without the *srpABC* genes. Upper: *P. putida* PPO200(pUCP22), no growth. Lower left: *P. putida* PPO200(pJD105), good growth. Lower right: *P. putida* PPO200(pJD106), slow growth.

TABLE II
Growth of *P. putida* PPO200 containing the cloned *srp* genes on L medium in the presence of toluene

Toluene was added at different concentrations to identical subcultures in L medium during the early exponential growth phase. A plus indicates growth with OD >0.7 and a minus indicates no growth with OD <0.1 after 24 h.

Toluene Concentration	Growth on L medium with toluene		
	<i>P. putida</i> PPO200 (pUCP22)	<i>P. putida</i> PPO200 (pJD105)	<i>P. putida</i> PPO200 (pJD106)
mM			
0.0	+	+	+
0.7	+	+	+
1.4	+	+	+
2.1	+	+	+
2.8	+	+	+
3.5	—	+	+
4.2	—	+	+
4.9	—	+	+
5.6	—	—	—

function to export hydrophobic molecules from the cell. It is logical that a solvent efflux pump would have evolved since it would enable microorganisms to survive in close proximity to oil or coal deposits (a rich source of carbon and energy) in the environment.

There have been several attempts to clone genes that are involved in solvent resistance leading in many cases to the identification of a protein that is somehow involved in making the cell resistant to a single solvent or to a group of related solvents. Most of these studies took place using *E. coli* that was forced under selective pressure to become solvent resistant. Mutations in a number of different genes can result in an increased tolerance to a particular chosen solvent, any one of which can allow the cell to grow in the presence of the solvent. Genes implicated in increased organic solvent tolerance of *E. coli* include the uncharacterized *ostA* (for organic solvent tolerance) (40), *ahpC* encoding alkylhydroperoxide reductase (35), *robA* encoding a global regulatory protein (41), and *soxS* encoding regulatory proteins controlling the superoxide response regulon (42). These genes enhance the survivability of the organism in the presence of a particular solvent but are not responsible for solvent resistance *per se* since they do not aid in understanding the true mechanism(s) of solvent resistance found naturally in environmental isolates. This article, however, represents the first example of cloning and characterization of genes for a major solvent resistance mechanism: a

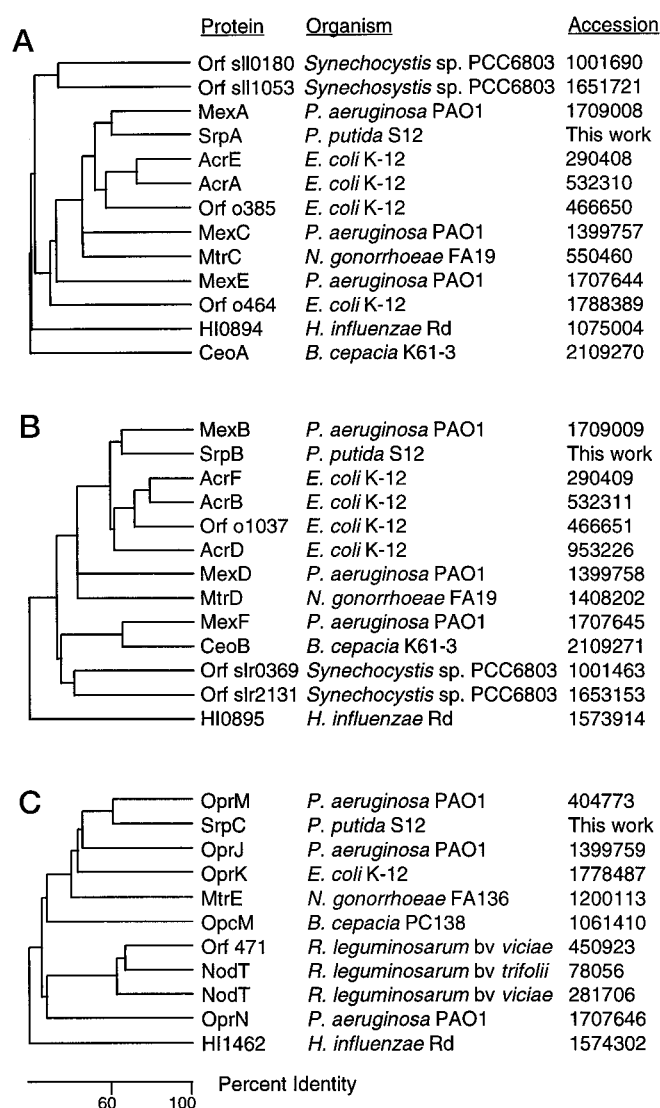


FIG. 5. Dendrograms showing the levels of homology between the amino acid sequences of different proteins involved in proton-dependent efflux systems. A, dendrogram of periplasmic linker proteins. B, dendrogram of inner membrane transporter proteins. C, dendrogram of outer membrane channel proteins. The accession column refers to the GenPep data base accession number.

proton-dependent solvent efflux pump.

We have shown that the cloned genes can be transferred to another *P. putida* strain with the concomitant gain of solvent resistance by that organism. This has far reaching implications for industrial applications in the fine chemistry area. Existing and potential biocatalytic processes for compounds such as catechols, phenols, medium chain alcohols, and enantiopure epoxides are suboptimal because the products formed are very toxic to normal microorganisms. Product accumulation to a concentration which allows economic downstream processing is inherently prevented by the physical characteristics of these compounds. The ability, as demonstrated here, to take a normally solvent-sensitive strain and make it solvent resistant will greatly enhance the ability of a given strain to perform a desired biocatalytic reaction resulting in otherwise toxic products.

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