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Bacterial organic solvent tolerance mechanisms and their regulation

by

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Abstract

The efflux system SrpABC in *Pseudomonas putida* S12 is involved exclusively in organic solvent efflux. Unlike most efflux pump operons, *srpABC* has two putative upstream regulatory genes, designated *srpS* and *srpR*. Primer extension analysis revealed that *srpS* is transcribed from a single promoter. Analyses of the effects of *srpS* or *srpR* deletion mutations on *srpA* promoter activity indicate that SrpS represses, and SrpR activates the transcription of *srpABC*. Electrophoretic mobility shift assays revealed that SrpS binds to the *srpS – srpA* intergenic region. SrpR was observed to reduce the operator binding ability of SrpS, suggesting that SrpR functions as an anti-repressor.

Enrichment procedures proved effective in isolating several new organic solvent tolerant bacteria. The strains were identified by comparison of 16S rDNA sequences and carbon source utilization profiles. The Gram positive isolates exhibited tolerance to more polar highly toxic solvents and showed higher tolerance to toluene shock as compared to the Gram negative isolates. Examination of the ultrastructure of the *Staphylococcus* sp. strain ZZ1 revealed that the cells display polysaccharide capsules when grown in the presence of toluene.

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List of Abbreviations

A	Adenine
APS	Ammonium persulfate
ATP	Adenosine triphosphate
bp	Base pair
C	Cytosine
CCCP	Carbonyl cyanide *m*-chlorophenyl benzene
cDNA	complementary DNA
cpm	Counts per minute
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ds	Double stranded
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
G	Guanine
IPTG	Isopropyl β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
LB	Luria Bertani medium
MBP	Maltose-binding protein
MBSU	Molecular biology services unit

mRNA	messenger RNA
MW	Molecular weight
NaOAc	Sodium acetate
NEB	New England Biolabs
OD	Optical Density
ONP	*o*-nitrophenol
ONPG	*o*-nitrophenyl β-D-galactopyranoside
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PIA	*Pseudomonas* Isolation Agar
PMSF	Phenylmethylsulfonyl fluoride
PNK	Polynucleotide kinase
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
sp	species
SSC	Standard saline citrate
STE	Sodium-Tris-EDTA buffer
T	Thymine
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
TEMED	Tetramethyl ethylene diamine
Tm	Melting temperature
tol	Toluene
U	Unit (measure of enzyme activity)
UV	Ultraviolet
v/v	Volume per volume

w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Chapter 1:

Introduction

1.0 Introduction

Microorganisms are constantly exposed to a variety of noxious chemicals. Besides those chemicals which exist naturally in the environment as a result of natural biosynthesis like natural antibiotics and trace amounts of organic solvents, and bile salts encountered in the guts of higher organisms, human activities have led to a tremendous increase in the release of harmful chemicals into the environment. Large quantities of chemical wastes are produced and released into the environment routinely. Herbicides and pesticides are introduced into the environment for weed and pest control. The accumulation of toxic organic solvents in the biosphere is increasing as a result of their wide-spread use in industrial processes, fuels, and everyday items like paints. Other chemicals like semisynthetic antibiotics or biocides are synthesized and released into the environment to specifically target microbes. Continuous exposure to noxious chemicals have led some microbes to evolve mechanisms to allow high levels of tolerance to toxic substances (Fernandes *et al.*, 2003; Juttner and Henatsch, 1986; Segura *et al.*, 1999). Recently, bacteria that exhibit tolerance to high concentrations of organic solvents have been identified. The high potential of these bacteria in environmental applications like soil remediation and wastewater treatment for the removal of toxic chemicals, as well as in biocatalytic applications for the production of fine chemicals in two-phase systems, has resulted in enormous efforts to explore and uncover the mechanisms behind their solvent tolerance.

A manuscript based on some of the results described in this thesis is currently in press. Zahir, Z., Seed, K.D., and Dennis, J.J (2005) Isolation and characterization of novel organic solvent tolerant bacteria. *Extremophiles.*

1.1 Toxic effects of organic solvents

Organic solvents include aromatic, cyclic, aliphatic hydrocarbons and alcohols. In general, organic solvents even in small quantities are extremely toxic to microorganisms. The effects of organic solvents like toluene, *n*-hexane and cyclohexane on the structure of *Escherichia coli* cells have been observed by electron microscopy. Solvent accumulation was observed in the cytoplasmic membrane causing the displacement and the partial disintegration of the cytoplasmic membrane from the outer membrane. Remnants of the cytoplasmic membrane were observed as diffuse bands in the cell cytoplasm, while the outer membrane remained relatively unperturbed, demonstrating that the main target of toxic effect of organic solvents is the cytoplasmic membrane (Aono *et al.*, 1994; de Smet *et al.*, 1978; Jackson and DeMoss, 1965; Woldringh, 1973). The preferential accumulation of organic solvents in the cytoplasmic membrane results in the swelling of the membrane, disturbing the interaction between the acyl chains of the membrane phospholipids. The alteration of membrane structure causes the disruption of bilayer stability and a concomitant increase in membrane fluidity (Antunes-Madeira and Madeira, 1989; Sikkema *et al.*, 1994). This results in the disruption of the normal functioning of the membrane as a permeability barrier. Leakage of macromolecules such as RNA, phospholipids and proteins have been observed in *E. coli* after exposure to toluene (Jackson and DeMoss, 1965). Treatment of *E. coli* cells with phenol has been shown to result in the leakage of potassium ions and cellular metabolites such as ATP as well other nucleotides (Heipieper *et al.*, 1991). Additionally, a variety of cyclic hydrocarbons has been shown to cause the leakage of protons and other ions across artificial and bacterial membranes, dissipating the pH gradient and the electrical

potential, disrupting energy production and transduction via protein motive force (Sikkema *et al.*, 1994). Moreover, even minor changes in membrane fluidity caused by solvent accumulation have also been shown to affect the normal functioning of various membrane associated proteins (Yuli *et al.*, 1981). For instance, the total disruption of the galactose permease system has been observed in *E. coli* cells when exposed to toluene (Jackson and DeMoss, 1965). Thus, organic solvents affect bacterial cells by disorganizing the membrane structure and impairing the vital membrane functions, ultimately leading to cell death.

1.2 Toxicity of organic solvents based on log P_{OW} values

Since the toxic effects of organic solvents occur mainly in the cytoplasmic membrane, an estimation of the solvent's ability to accumulate in the membrane in a membrane-buffer two-phase system is important to determine the toxicity of the solvent. Studies have been performed with *E. coli* phospholipid liposomes and ^{14}C labeled organic solvents to determine the correlation between the hydrophobicity of a solvent and the solvent's ability to partition into the membrane from the aqueous phase. The hydrophobicity of a solvent is typically expressed as a measure of the logarithm of the partition coefficient of a solvent in a standard octanol-water two-phase system, which is termed as the log P_{OW} value of the solvent. Partition coefficients in a membrane-buffer two-phase system (log P_{MB}) were calculated for ten different organic solvents by testing increasing amounts of each ^{14}C labeled solvent on a fixed amount of a liposome/buffer solution and plotting the aqueous concentrations of the solvent against the solvent to liposome ratios. The calculated log P_{MB} values when plotted against their corresponding

log P_{OW} values showed that the partitioning of solvents in octanol-water two-phase systems parallels that in membrane-buffer two-phase systems despite the structural differences in the solvents tested. Hence, the log P_{OW} value of a solvent has been taken as a measure of the partitioning of the solvent into the membrane from the aqueous phase (Sikkema *et al.*, 1994).

Solvent dissolution in the aqueous phase needs to precede the subsequent partitioning into the membrane. Thus, the level of solvent accumulation in the membrane depends both on the solubility of the solvent in the aqueous phase and the partitioning of the solvent into the membrane from the aqueous phase (Sikkema *et al.*, 1994). Accordingly, based on the aqueous concentration of a solvent and its log P_{OW} value, an estimation of the toxicity of the solvent can be determined. Table 1.1 shows the log P_{OW} values of several organic solvents. Generally, organic solvents with log P_{OW} values < 1 are observed to be not toxic to microbes as they have a low affinity for the cell membrane. Solvents with log P_{OW} values > 4 are again observed to be generally not toxic; they have a high affinity to accumulate in the membrane, but are only slightly soluble in the aqueous phase and so are unable to reach a high membrane concentration. However, solvents with log P_{OW} values between 1-4 are observed to be extremely toxic because they are more soluble in the aqueous phase and concurrently their degree of partitioning from the aqueous phase to the bacterial membrane is relatively high, therefore, they reach a high membrane concentration (Inoue and Horikoshi, 1991; Osborne *et al.*, 1990; Sikkema *et al.*, 1994; Vermue *et al.*, 1993). Nevertheless, the partitioning of a solvent in a membrane-aqueous phase system is not as simple as the partitioning of a solvent in the octanol-water two-phase system. It has been shown that the particular composition of

Table 1.1. Organic solvents and their corresponding log P_{OW} values*

Solvent	log P_{OW} value
Decalin	4.8
Diphenyl ether	4.3
Cyclo octane	4.2
Propyl benzene	3.8
Tetralin	3.8
Methyl cyclohexane	3.7
Hexane	3.5
Cyclohexane	3.2
Ethyl benzene	3.1
p-Xylene	3.0
Styrene	3.0
Octanol	2.9
Carbon tetrachloride	2.7
Toluene	2.5
Heptanol	2.4
Dimethylphthalate	2.3
Fluorobenzene	2.2
Benzene	2.0
Chloroform	2.0
Cyclohexanol	1.5
Phenol	1.5
n-Butanol	0.8

*Adapted from Heipieper *et al.* (1994) and Sardessai and Bhosle (2002a)

lipids in a membrane lipid bilayer significantly influences the partitioning of a solvent into a membrane. Partitioning of the insecticides parathion, lindane, DDT and malathion has been studied in membranes with varying lipid compositions and these compounds were observed to partition preferentially in membranes composed of short-aliphatic-chain lipids (Antunes-Madeira and Madeira, 1984, 1985, 1986, 1987). Additionally, an increase in membrane surface density, either by varying temperature, phospholipid chain lengths or cholesterol incorporation, has been shown to result in a decrease in partitioning ability of the solvents benzene and hexane, independent of the method used to change the membrane surface density, suggesting that the structural organization of the lipid bilayer influences solvent partitioning (De Young and Dill, 1988, 1990). Thus, quantitative estimations of the toxicity of a solvent to an organism cannot be based solely on the solubility and the log P_{OW} value of a solvent.

1.3 Organic solvent tolerant bacterial strains

Despite the extreme toxicity of organic solvents, a number of organic solvent tolerant bacterial strains have been isolated. The first organic solvent tolerant strain identified was the *Pseudomonas putida* strain IH-2000. This strain was isolated from oil contaminated soil and was observed to survive in the presence of high concentrations of toluene (Inoue and Horikoshi, 1989). Since then, a number of other *P. putida* strains capable of surviving in the presence of high concentrations of a second phase of toluene as well as other aromatic and aliphatic organic solvents and alcohols have been isolated from normal soil environments (Cruden *et al.*, 1992; Kim *et al.*, 1998; Weber *et al.*, 1993). The solvent tolerant *P. putida* strain DOT-T1E was isolated from water samples

obtained from a wastewater treatment plant (Ramos *et al.*, 1995). Strains of other *Pseudomonas* species like *P. aeruginosa*, *P. fluorescens* and *P. mendocina* that show high levels of solvent tolerance have also been isolated from various soil environments (Aono *et al.*, 1992; Ikura *et al.*, 1997; Nakajima *et al.*, 1995a; Ogino *et al.*, 1994). A strain of the *Flavobacterium* species which survives in the presence of high concentrations of solvents like *p*-xylene, toluene and benzene was isolated from a more extreme location, the deep sea (Moriya and Horikoshi, 1993).

Recently, several organic solvent tolerant Gram positive bacteria have also been isolated from normal as well as more extreme environments. A *Bacillus cereus* strain R1 isolated from a normal soil habitat was found to tolerate high concentrations of toluene and several other organic solvents but not aliphatic alcohols (Matsumoto *et al.*, 2002). A *Rhodococcus* species isolated from a chemical contaminated site in Australia has been shown to have exceptional tolerance to high levels of liquid benzene (Paje *et al.*, 1997). A *Bacillus* sp. strain SB-1 which can survive high concentrations of various organic solvents such as toluene, benzene and *n*-butanol was isolated from mangrove sediments (Sardessai and Bhosle, 2002b) and a *Bacillus* sp. strain BC1 isolated from coastal sediments was observed to have high levels of tolerance to chloroform (Sardessai and Bhosle, 2003). Since the majority of the organic solvent tolerant strains identified to date are Gram negative, a large amount of research has focused on elucidating the solvent tolerance mechanisms in Gram negative bacteria.

1.4 Organic solvent tolerance mechanisms in Gram negative bacteria

1.4.1 Cytoplasmic membrane adaptations in response to organic solvents

Since the initial effect of solvent accumulation in the cytoplasmic membrane is the swelling of the membrane and an increase in membrane fluidity (Antunes-Madeira and Madeira, 1989; Sikkema *et al.*, 1994), several cytoplasmic membrane modification mechanisms appear to be utilized by bacteria in order to readjust the membrane fluidity and thus restore the normal membrane functioning.

1.4.1.1 *cis*- to- *trans* isomerization of unsaturated fatty acids

The *cis* configuration of an unsaturated fatty acid has a 30° bend in the acyl chain, causing steric hindrance, which reduces dense packing and gives high membrane fluidity. However, the *trans* configuration lacks this bend and has a long extended steric conformation which allows highly ordered packing of the membrane and gives a lower membrane fluidity (MacDonald *et al.*, 1985). Isomerization of *cis* configuration of unsaturated fatty acids to *trans* configuration has been observed in response to exposure to organic solvents in various *Pseudomonas putida* strains. In the *P. putida* strain P8, increasing concentrations of phenol were observed to increase the content of the *trans* isomers and decrease the content of the respective *cis* isomers in a concentration-dependent manner even at phenol concentrations that inhibited cell growth. Additionally, analysis of cells under bacteriostatic conditions showed isomerization of *cis* to *trans* unsaturated fatty acids after exposure to 4-chlorophenol. Incorporation experiments performed with ^{14}C labeled acetate showed that *de novo* synthesis of lipids was absent in these cells when compared to actively growing cells, suggesting that the increase in *trans*

fatty acid content is not the result of fatty acid biosynthesis (Heipieper *et al.*, 1992). This was further demonstrated as the exposure to 4-chlorophenol led to the observation of the *trans* configuration of ^{14}C labeled oleic acid in the membranes of growing *P. putida* P8 cells incorporated with ^{14}C labeled oleic acid in the *cis* configuration. Because oleic acid cannot be synthesized by these cells it was proven that the increase in content of the *trans* unsaturated fatty acids when exposed to organic solvents is a result of the direct isomerization of its corresponding *cis* isomers (Diefenbach and Keweloh, 1994). Time course experiments showed an increase in *trans* to *cis* ratio of unsaturated fatty acids immediately after exposure of *P. putida* S12 cells to toluene or ethanol and the final *trans* to *cis* ratio was observed within 30 minutes (Heipieper and de Bont, 1994).

The conversion of *cis* to *trans* unsaturated fatty acids has been shown to be driven by *cis-trans* isomerases (Cti). The gene for Cti has been identified in several *Pseudomonas* strains (Junker and Ramos, 1999). Cti was purified from the periplasmic fractions of *P. oleovorans* GPo12 and *Pseudomonas* sp. strain E-3, suggesting that this enzyme is located in the periplasmic space (Okuyama *et al.*, 1998; Pedrotta and Witholt, 1999). When the *cti* gene sequences from P. *putida* DOT-T1E, *P. putida* P8 and *P. oleovorans* GPo12 were compared to the corresponding N-terminal sequences from the mature Cti proteins, it was revealed that the Cti is derived from a precursor with a signal peptide which is cleaved after the enzyme is targeted to the periplasmic space (Holtwick *et al.*, 1997; Junker and Ramos, 1999; Pedrotta and Witholt, 1999). A *cti* gene deletion mutant of *P. putida* DOT-T1E lacked the ability to isomerize *cis* to *trans* unsaturated fatty acids. The survival rate of the mutant strain was observed to be lower than the wild-type strain when shocked with toluene and also showed a longer lag phase when grown in

the presence of toluene, suggesting that the Cti enzyme and *cis-trans* unsaturated fatty acid isomerization functions to counteract the toxic effects of organic solvents (Junker and Ramos, 1999). Primer extension analysis showed that the Cti enzyme is constitutively expressed in *P. putida* DOT-T1E. However, the purified isomerase from *P. oleovorans* GPo12 allowed *cis-trans* isomerization of unsaturated fatty acids in crude membranes isolated from *Pseudomonas* and *E. coli* only in the presence of organic solvents and not in the absence, suggesting that the presence of solvents in the membrane triggers certain factors which allow the constitutively expressed periplasmic isomerase to act on the fatty acids. Penetration of the enzyme into the membrane due to increased fluidity has been postulated, but the molecular basis behind the regulation of the enzyme activity is not known (Junker and Ramos, 1999; Pedrotta and Witholt, 1999). Toluene adapted *P. putida* S12 cells possessing a higher membrane content of the *trans* unsaturated fatty acids were observed to have a higher transition temperature when compared to non-adapted cells, suggesting that the cells exhibit a denser packed membrane with a lowered membrane fluidity to compensate for the increase in fluidity caused by organic solvent accumulation (Weber *et al.*, 1994). However, the gene for the Cti enzyme has been identified and *cis* to *trans* isomerization of unsaturated fatty acids has been observed after solvent exposure in strains that are sensitive to solvents, suggesting that other essential solvent tolerance mechanisms function in solvent tolerant bacteria (Junker and Ramos, 1999; Pinkart *et al.*, 1996). Thus, *cis* to *trans* isomerization of unsaturated fatty acids has been proposed as an immediate short term response to overcome initial membrane damage, allowing cells to survive and trigger long term responses. *Cis* to *trans* isomerization of unsaturated fatty acids has also been observed in

response to exposure to antibiotics, heavy metals or high temperature (Heipieper, 1996; Isken *et al.*, 1997), suggesting that this response is part of a general stress response and not a specific response to organic solvents.

1.4.1.2 Increase in the saturated fatty acid content

Several *Pseudomonas* and *E. coli* strains have been observed to shift the saturated to unsaturated fatty acid ratio when exposed to organic solvents. An increase in the saturated fatty acid content has been observed in *E. coli* after exposure to non-toxic concentrations of a variety of aromatic hydrocarbons and long chain alcohols (Ingram, 1977; Keweloh *et al.*, 1991). A similar increase in the saturated fatty acid content has been observed in the membranes of *P. putida* S12 when exposed to non-toxic concentrations of toluene but not at concentrations that inhibit cell growth, suggesting that the increase in saturated fatty acid content is the result of fatty acid biosynthesis (Heipieper and de Bont, 1994). Time course experiments of the fatty acid saturation in *P. putida* strain Idaho showed that the initial increase in saturated fatty acid content occurred 15 minutes after exposure to *o*-xylene and the final saturated to unsaturated fatty acid ratio was observed after 2h (Pinkart and White, 1997). Saturated fatty acids have a long extended steric conformation, much like *trans* unsaturated fatty acids, which enables highly ordered packing of the membrane. Accordingly, an increase in the saturated fatty acid content results in a membrane with lower fluidity (Segura *et al.*, 1999). Thus, this mechanism has been proposed as a long term response requiring time-consuming *de novo* biosynthesis of saturated fatty acids to compensate for the increase in fluidity caused by solvent accumulation. However, an unexpected increase in the unsaturated fatty acid

content has been observed when *E. coli* and *P. putida* S12 cells were exposed to relatively polar solvents like ethanol and acetone (Heipieper and de Bont, 1994; Ingram, 1977). Increase in unsaturated fatty acids in the membrane would have a propensity to increase membrane fluidity as has been observed in *E. coli* phospholipid liposomes exposed to ethanol (Dombek and Ingram, 1984). It was postulated that the increase in unsaturated fatty acid content is the result of the polar solvent induced inhibition of the enzymes required for saturated fatty acid synthesis. As such, an increased synthesis of unsaturated fatty acids would be required for the non-selective increase in the phospholipid content in the membrane (Buttke and Ingram, 1978; Heipieper and de Bont, 1994).

1.4.1.3 Alteration in the composition of the phospholipid head groups

Changes in phospholipid head group composition have been observed in several *P. putida* strains when exposed to organic solvents. The major phospholipids identified in *P. putida* strains are phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and diphosphatidylglycerol or cardiolipin (CL). *P. putida* S12 cells when grown in a chemostat culture in the presence of toluene showed a decrease in the membrane content of PE and an increase in content of CL (Weber and de Bont, 1996). Incorporation experiments with ^{32}P labeled orthophosphate in *P. putida* DOT-T1E showed that in the presence of toluene more than 90% of the labeled phosphate was directed towards CL synthesis. Consequently, toluene exposure resulted in an increase in the membrane content of CL and a decrease in PE, while there was no significant change in the level of PG (Ramos *et al.*, 1997). Similar effects have been observed in solvent tolerant mutants

of *E. coli* (Clark and Beard, 1979). The transition temperature of CL is about 10°C higher than PE, thus increasing the membrane content of CL was proposed to stabilize the membrane and compensate for the increase in fluidity caused by solvent accumulation. Also, CL has a larger phospholipid headgroup area and it was suggested that the increase in membrane CL content will counteract the increase in volume caused by solvent accumulation and so has a stabilizing effect (Weber and de Bont, 1996). However, ^{32}P incorporation experiments in *P. putida* strain Idaho showed an increase in membrane content of PE following exposure to *o*-xylene. The membrane content of PG was observed to be less but close to PE while the level of CL decreased. This effect is uncommon but PE has a higher transition temperature than PG and so increasing membrane content of PE over PG was proposed to stabilize the membrane and decrease membrane fluidity (Pinkart and White, 1997).

1.4.1.4 Increase in the rate of phospholipid biosynthesis

The highly damaging effects of solvents on the membrane require not only a shift in the composition of the membrane phospholipids to counteract the damaging effects, but the damaged membrane also needs to be rapidly repaired for survival in the presence of organic solvents. Exposure of *P. putida* Idaho to *o*-xylene has been observed to result in a significant increase in the rate of phospholipid biosynthesis when compared to the absence of *o*-xylene. The total phospholipid content in these cells was observed to double in the presence of *o*-xylene. However, the solvent sensitive strain *P. putida* MW1200 showed only a slight increase in the rate of phospholipid biosynthesis and a 70% decrease in the total phospholipid content after exposure to *o*-xylene. Thus, the increase in the rate

of phospholipid biosynthesis was proposed as a solvent-induced response in the *P. putida* strain Idaho to allow rapid repair of the damaged membrane, while the limited ability to synthesize phospholipids was suggested to contribute to the increased solvent sensitivity of the *P. putida* strain MW1200 (Pinkart and White, 1997). Incorporation experiments performed with ^{13}C labeled acetic acid to determine the rate of phospholipid biosynthesis in a toluene sensitive mutant of *P. putida* DOT-T1E showed that this strain synthesized high levels of phospholipids in the absence of toluene while it was unable to synthesize phospholipids in the presence of toluene. In contrast, the wild-type *P. putida* DOT-T1E strain showed a high rate of phospholipid biosynthesis both in the presence and absence of toluene. The sensitivity of the mutant strain to toluene was mostly attributed to its inability to synthesize phospholipids. Accordingly, membrane phospholipid biosynthesis has been proposed as an important mechanism to allow the repair of the damaged membrane and to maintain membrane integrity in the presence of organic solvents (Segura *et al.*, 2004).

1.4.1.5 Increase in membrane protein content

Besides the changes in the membrane phospholipid composition and the increase in the dynamics of phospholipid biosynthesis, changes in the membrane protein content have been observed in response to exposure to organic solvents. As mentioned previously, it has been observed that *E. coli* phospholipid liposomes when exposed to ethanol showed an increase in fluidity caused by an increase in the unsaturated fatty acid content. However, examination of intact membranes of *E. coli* cells after exposure to ethanol showed a lower fluidity when compared to unexposed cells, suggesting the

involvement of a non-lipid component to compensate for the ethanol-induced increase in fluidity. Analysis of the membrane composition showed an increase in the protein-to-lipid ratio in cells after exposure to ethanol (Dombek and Ingram, 1984). A similar increase in protein-to-lipid content has also been observed in *E. coli* cells when exposed to phenol (Keweloh *et al.*, 1990). Proteins, which exist as bulky rigid domains, would hinder the motion of the lipids in the membrane and so an increase in protein content would increase the lipid ordering and membrane stability (Weber and de Bont, 1996).

1.5 Outer membrane adaptations in response to organic solvents

The outer membrane of Gram negative bacteria consists of an inner monolayer of phospholipids and an outer monolayer mainly composed of highly hydrophilic strongly negatively charged lipopolysaccharide (LPS) molecules. The electrostatic charge repulsion between adjacent LPS molecules are stabilized by non-covalent bridges formed by the divalent cations Ca^{2+} and Mg^{2+}. This arrangement results in a stable, densely packed structure with a low permeability for hydrophobic compounds (Nikaido and Nakae, 1979; Vaara *et al.*, 1990; Yoshimura and Nikaido, 1982). Enhanced toluene tolerance has been observed in several *Pseudomonas* strains when the growth medium was supplemented with Ca^{2+} and Mg^{2+}, indicating the importance of a stable outer membrane to allow solvent tolerance (Ramos *et al.*, 1995; Weber and de Bont, 1996). Several adaptive changes in the outer membrane have been observed in response to exposure to organic solvents. Toluene adapted *P. putida* S12 cells have been observed to have a lower cell surface hydrophobicity as measured by the water contact angle of the cells and these cells were observed to be more tolerant to toluene than non-adapted cells

(Weber and de Bont, 1996). A similar decrease in cell surface hydrophobicity was observed in solvent tolerant mutants of *E. coli* K12 when compared to the solvent sensitive parent strain. Microscopic analysis showed a lower cell surface adherence of the mutant strains to *n*-octanol droplets when compared to the parent strain. Analysis of the cell envelope components showed an increased content of LPS in the mutant strains with no significant change in the chemical composition of the LPS molecules (Aono and Kobayashi, 1997). However, a change in LPS composition has been observed in the *P. putida* strain Idaho after exposure to *o*-xylene, probably making the cell surface less hydrophobic (Pinkart *et al.*, 1996). These results suggest that the outer membrane LPS molecules are involved in protecting the cells against organic solvents, although more detailed studies are required.

1.5.1 Alteration of porin proteins

Also embedded in the Gram negative outer membranes are porin proteins, which are water-filled channels that have been shown to function either as general channels or as substrate specific channels for the passage of small hydrophilic molecules (Hancock, 1984). A toluene tolerant mutant of *P. aeruginosa* has been observed to be deficient in the major outer membrane porin protein OprF, suggesting that the loss of OprF decreased the diffusion of toluene across the outer membrane (Li *et al.*, 1995a). Similarly, solvent tolerant mutants of *E. coli* K12 were shown to be deficient in the porin protein OmpF in their outer membranes, suggesting that organic solvents are allowed to diffuse through porin proteins and their deficiency increases solvent tolerance (Aono and Kobayashi, 1997). Porin deficient mutants of *E. coli* K12 and *P. aeruginosa* have also been shown to

have an increased outer membrane content of LPS and phospholipids. The loss of porin proteins is possibly compensated for by an increase in these outer membrane components and the solvent tolerance observed in these strains is the result of a decrease in solvent diffusion due to loss of porins and a concomitant increase in LPS (Aono and Kobayashi, 1997; Gotoh *et al.*, 1989).

Porin proteins have also been shown to be involved in the maintenance of the cell shape and the integrity of the outer membrane. Electron microscopy revealed altered cell shapes and formation of blebs in the outer membranes of mutant *P. aeruginosa* strains deficient in the porin protein OprF. Leakage of a considerable amount of periplasmic proteins was also observed, suggesting the importance of OprF in maintaining the integrity of the outer membrane (Gotoh *et al.*, 1989). Similarly, a mutant strain of *P. putida* KT2440 lacking the porin protein OprN was shown to have a deformed wavy appearance of the outer membrane when analyzed by electron microscopy. This strain was also observed to be more sensitive to detergents and EDTA than the parent strain (Rodriguez-Herva and Ramos, 1996). In addition, an OprN deficient mutant of *P. putida* DOT-T1E has been shown to be hypersensitive to toluene, suggesting the importance of the outer membrane integrity for the effective exclusion of organic solvents (Ramos *et al.*, 1997)

1.5.2 Formation of membrane vesicles

The toluene tolerant strain *P. putida* IH-2000 has been shown to produce membrane vesicles from its outer membrane when exposed to toluene. Analysis by electron microscopy showed that these cells when exposed to toluene, initially formed

swellings on the outer membrane which are then released into the medium as vesicles, leaving the outer membrane intact. It was determined that these membrane vesicles consisted of phospholipids, LPS, a small amount of proteins and contained toluene molecules adhering to them. A toluene sensitive mutant of this strain with altered LPS and outer membrane proteins was unable to form vesicles when exposed to toluene. Thus, vesicle formation in *P. putida* IH-2000 has been proposed as a mechanism to eliminate organic solvents from the cell surface (Kobayashi *et al.*, 2000). Similar swellings have also been observed on the outer membrane of the *P. putida* strain DOT-T1E when exposed to high concentrations of toluene, suggesting the existence of toluene induced vesicle formation. However, it has not been demonstrated if vesicles function to eliminate solvents in this strain (Ramos *et al.*, 1995).

1.6 Solvent metabolism or transformation

Many microbes are able to metabolize or transform toxic organic solvents and several of these hydrocarbon-degrading strains have also been shown to survive in high concentrations of the solvent. For instance, a recently isolated benzene degrading *Rhodococcus* strain has been shown to have exceptional tolerance to high levels of liquid benzene (Paje *et al.*, 1997). Metabolism or modification of organic solvents can help reduce their toxicity and may confer some level of tolerance to these solvents. A solvent tolerant mutant of *E. coli* has been shown to have increased expression of the enzyme alkylhydroperoxide reductase which is responsible for the reduction of organic hydroperoxides to alcohols. This strain was observed to have a higher level of tolerance to several organic solvents including tetralin and cyclohexane and showed an increased

rate of reduction of tetralin hydroperoxide as compared to the parent strain. It was proposed that these solvents are transformed in the cell into toxic hydroperoxides by a yet unknown mechanism and that the mutant strain is able to more effectively reduce the hydroperoxides to non-toxic alcohols and thus increase tolerance to these solvents (Ferrante *et al.*, 1995).

However, many solvent tolerant strains are able to survive in the presence of a second phase of a variety of organic solvents that cannot be metabolized or transformed by these strains. For instance, the toluene tolerant strains *P. putida* IH2000 and *P. putida* S12 are unable to metabolize or transform toluene (Inoue and Horikoshi, 1989; Isken and de Bont, 1996) and *E. coli* K12 mutants tolerant to *n*-hexane do not metabolize this compound (Aono *et al.*, 1991). Furthermore, a mutant strain of toluene degrading *P. putida* DOT-T1E, defective in its ability to metabolize toluene, was observed to be as tolerant to toluene as the parent strain (Mosqueda *et al.*, 1999). Thus, solvent degradation or transformation may not be important solvent tolerance mechanisms.

1.7 Active efflux as a mechanism of solvent tolerance

An effective mechanism for solvent tolerance would be a dynamic mechanism that actively transports solvents from the membrane to the external medium, reducing the level of accumulation of solvents in the membrane. Indeed, active efflux of organic solvents has been observed to work in conjunction with membrane modification mechanisms in a number of solvent tolerant bacterial strains. To date, five major families of efflux transporter proteins have been identified in bacteria, all able to accommodate and expel a wide range of structurally unrelated antimicrobial compounds. These include

the ATP-binding cassette (ABC) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) family, and the resistance-nodulation-division (RND) family. Members of the ABC family utilize ATP hydrolysis as the energy source for efflux, while the rest of the transporter families utilize energy derived from proton motive force or drug-Na^+ antiport systems (Saier and Paulsen, 2001).

Thus far, all efflux systems identified for organic solvent extrusion belong to the RND family. Efflux systems of this type typically traverse both the cytoplasmic membrane and the outer membrane by using three protein components. A proton motive force-driven RND transporter protein located in the cytoplasmic membrane is connected via a periplasmic membrane fusion protein (MFP) to an outer membrane channel-forming protein to allow extrusion of toxic substances from the cytoplasm and the periplasm across both the membranes directly to the external medium in a single energy-coupled step (Murakami *et al.*, 2002; Nikaido, 1996; Touze *et al.*, 2004). However, variations of this arrangement have been observed in some efflux systems. The RND transporter protein AcrD, which is involved in the efflux of aminoglycosides in *E. coli,* has been shown to function as a single component system without the requirement for a periplasmic or an outer membrane protein component. It has been proposed that this arrangement is still effective for aminoglycoside extrusion. Aminoglycosides are highly hydrophilic polycationic compounds that should not pose the problem of spontaneous re-entry across the cytoplasmic membrane once transported to the periplasm (Rosenberg *et al.*, 2000). Additionally, the RND transporter protein MexK in *P. aeruginosa* has been shown to associate only with the periplasmic membrane fusion protein MexJ during the

efflux of the amphiphilic biocide triclosan, suggesting that two-component RND systems may be adequate to effectively extrude specific amphiphilic compounds (Chuanchuen *et al.*, 2002).

1.7.1 Involvement of RND-type efflux systems in organic solvent tolerance

Involvement of an active efflux mechanism in solvent tolerance was first observed in the *P. putida* strain S12. Studies with ^{14}C labeled toluene showed that toluene-adapted *P. putida* S12 cells accumulated 50% less toluene compared to non-adapted cells. But the addition of the energy inhibitors potassium cyanide and carbonyl cyanide *m*-chlorophenyl benzene (CCCP) significantly increased accumulation of toluene in the adapted cells while no change in accumulation was observed in the non-adapted cells. Since *P. putida* S12 does not metabolize or transform toluene, it was proposed that a toluene-induced energy dependent efflux system functions in the toluene-adapted cells to reduce the level of toluene accumulation (Isken and de Bont, 1996). Genetic analysis of a solvent sensitive mutant of *P. putida* S12 obtained by transposon mutagenesis led to the identification of three genes (*srpABC*) arranged as an operon. These genes encode proteins whose amino acid sequences show high homology to those of the proteins comprising the three-component RND efflux systems, indicating the involvement of an RND-type efflux system in organic solvent tolerance in this strain (Kieboom *et al.*, 1998a). A variety of aromatic and aliphatic hydrocarbons and alcohols has been shown to induce the expression of the *srpABC* genes, however, these genes were not induced by antibiotics or heavy metals (Kieboom *et al.*, 1998b). It has been observed that inactivation of the *srpABC* genes did not make the strain more susceptible to antibiotics.

In addition, accumulation assays with ^{14}C labeled toluene have shown that antibiotics do not compete with toluene for efflux via the SrpABC system, indicating that antibiotics are not substrates of this efflux system (Isken and De Bont, 2000). This is not typical for RND-type efflux systems that have been shown to be involved in the efflux of a variety of antibiotics and that are described as the major contributors to multiple antibiotic resistance in Gram negative bacteria.

Involvement of a common mechanism for antibiotic resistance and organic solvent tolerance was first proposed when cyclohexane tolerant mutants of *E. coli* K12 were also observed to have an increased resistance to several antibiotics including ampicillin, chloramphenicol, and tetracycline (Aono *et al.*, 1995; Aono *et al.*, 1991). Further characterization showed that these mutants expressed increased levels of TolC and AcrA, the outer membrane and the periplasmic protein components respectively, of the multidrug resistance RND-type efflux system AcrAB-TolC. Thus, it was proposed that the AcrAB-TolC system which has been shown to confer multiple antibiotic resistance in *E. coli*, also confers organic solvent tolerance (Aono *et al.*, 1998; Okusu *et al.*, 1996). This system has also been shown to play a major role in the resistance of *E. coli* to biocides such as triclosan (McMurry *et al.*, 1998). In addition, toxic short chain fatty acids and bile salts, which are likely encountered by *E. coli* in the gut, have also been observed as substrates of this efflux system (Ma *et al.*, 1995).

Inactivation of the *acrB* gene has been shown to result in *E. coli* strains hypersensitive to organic solvents due to a non-functional AcrAB-TolC system. Solvent tolerant mutants of this hypersensitive strain were observed to produce increased levels of the proteins AcrE and AcrF whose amino acid sequences show high homology to AcrA

and AcrB, respectively. Genetic analysis revealed an insertion sequence upstream of the *acrEF* operon that was shown to cause an increase in expression of the *acrEF* genes in these solvent tolerant mutants. Complementation experiments showed that solvent tolerance was restored in the *acrB* mutant when *acrEF* is introduced only with the upstream insertion sequence and provided that the *tolC* gene was present. Therefore, it was proposed that an alternative efflux system comprised of AcrEF-TolC functions in *E. coli* to confer organic solvent tolerance (Kobayashi *et al.*, 2001). However, the AcrEF-TolC system has been observed to be only weakly expressed in the wild-type strain (Ma *et al.*, 1994) and inactivation of this system did not make the wild-type strain hypersensitive to solvents, suggesting that the AcrEF-TolC system is not essential for organic solvent tolerance. Instead, AcrEF-TolC may be a system that takes over in the absence of AcrAB-TolC (Kobayashi *et al.*, 2001). Similarly, overexpression of *acrEF* as a result of the upstream insertion element, has been shown to increase multiple antibiotic resistance in *acrAB* deletion mutants (Jellen-Ritter and Kern, 2001). Additionally, the genes *yhiUV* which encode proteins homologous to AcrAB have been shown to confer some level of tolerance to less toxic solvents in *acrAB* deletion mutants (Tsukagoshi and Aono, 2000). Thus, it is evident that the AcrAB-TolC system is the major contributor to organic solvent tolerance in *E. coli*, although a number of other systems may be able to contribute to solvent tolerance in its absence.

Several RND-type systems involved in the efflux of multiple antibiotics have been characterized in *P. aeruginosa*. The constitutively expressed MexAB-OprM system has been shown to be the major efflux system contributing to the high intrinsic antibiotic resistance of this strain (Li *et al.*, 1995b). Tolerance to *n*-hexane and *p*-xylene has also

been observed in wild-type strains expressing the MexAB-OprM system. Hyperexpression of this system was shown to result in an increase in tolerance to these organic solvents, while mutational inactivation of this system was shown to render the cells hypersensitive to these solvents. Thus, it was proposed that the MexAB-OprM system plays a significant role in the solvent tolerance of this strain and that the level of tolerance parallels the level of expression of the system (Li *et al.*, 1998).

The two systems, MexCD-OprJ and MexEF-OprN are not expressed constitutively in the wild-type *P. aeruginosa* strain, but these systems have been shown to confer multiple antibiotic resistance when overexpressed in laboratory mutants and in clinical isolates (Fukuda *et al.*, 1995; Poole *et al.*, 1996a). To check the involvement of these systems in organic solvent tolerance, each of these systems was overexpressed in mutants lacking the MexAB-OprM system. It was observed that these systems when overexpressed contributed to the tolerance to *n*-hexane and *p*-xylene, albeit at very low levels compared to the MexAB-OprM system (Li *et al.*, 1998).

The genes for the RND-type efflux system TtgABC was identified in *P. putida* strain DOT-T1E by genetic analysis of a toluene sensitive mutant obtained by transposon mutagenesis (Ramos *et al.*, 1998). Subsequent screenings for homologous genes in the chromosome of *P. putida* DOT-T1E led to the identification of two additional RND-type efflux systems, TtgDEF and TtgGHI, that contribute to the solvent tolerance of this strain (Mosqueda and Ramos, 2000; Rojas *et al.*, 2001). The TtgABC and TtgGHI systems have been shown to be expressed constitutively, although the *ttgGHI* expression is slightly increased when induced by organic solvents. These two systems have been shown to accommodate and exclude several organic solvents including toluene, styrene,

ethylbenzene, propylbenzene and *m*-xylene, as well as several antibiotics. In contrast, the TtgDEF system has been shown to be expressed only when induced by organic solvents and appears to be more substrate-specific as it is involved only in the efflux of the organic solvents toluene and styrene and not antibiotics (Mosqueda and Ramos, 2000; Ramos *et al.*, 1998; Rojas *et al.*, 2001). Triple efflux pump deletions were shown to result in mutants hypersensitive to organic solvents. However, double mutations did not compromise the solvent tolerance unless the mutations were in both TtgABC and TtgGHI systems. These mutants were shown to be hypersensitive to ethylbenzene, propylbenzene and *m*-xylene, as these are not substrates of the remaining TtgDEF system. Therefore, it was suggested that these efflux systems with overlapping functions have an additive effect on the organic solvent tolerance of *P. putida* DOT-T1E (Rojas *et al.*, 2001).

Furthermore, the organic solvent tolerance observed in a variant of the solvent sensitive strain *P. putida* KT2442 was attributed to an RND-type efflux system designated MepABC, which was also shown to be involved in the efflux of certain antibiotics (Fukumori *et al.*, 1998). Recently, an RND-type efflux system has also been identified in the polycyclic aromatic hydrocarbon degrader *P. fluorescens* cLP6a. The EmhABC system was observed to be involved in the efflux of the polycyclic aromatic compounds phenanthrene, anthracene and fluoranthene, and specific antibiotics (Hearn *et al.*, 2003). Thus, active efflux via RND-type efflux systems appears to play a crucial role in the solvent tolerance of a number of bacteria. The physiological role of these efflux systems is not clear since the expression of most of these systems is not induced by the wide range of antimicrobial substrates they can accommodate and extrude.

1.7.2 Regulation of expression of RND-type efflux systems involved in organic solvent efflux

Most RND-type efflux systems have a single repressor gene situated upstream and transcribed divergently from the efflux pump structural genes. In *E. coli*, the *acrR* gene divergently transcribed from the *acrAB* operon encodes a protein belonging to the TetR family of transcriptional regulators. AcrR has been shown to bind to the *acrR-acrA* intergenic region and repress both *acrAB* and its own transcription (Ma *et al.*, 1996). However, involvement of global regulatory proteins in the regulation of expression of the AcrAB-TolC system was indicated by the observation of mutations in the *marR* gene in an organic solvent tolerant mutant of *E. coli* (Asako *et al.*, 1997). MarR is the transcriptional repressor of the *marRAB* operon and MarA is a protein belonging to the AraC family of transcriptional activators. Inactivation of MarR derepresses the *marRAB* operon increasing the level of MarA, which in turn activates the transcription of a large number of genes that comprise the *mar* regulon, including the *marRAB* operon (Alekshun and Levy, 1997). Overexpression of MarA or its homologues SoxS or RobA, has been shown to increase the organic solvent tolerance in *E. coli* (Asako *et al.*, 1997; Nakajima *et al.*, 1995b; Nakajima *et al.*, 1995c). Simultaneously, overexpression of MarA, SoxS or RobA has been shown to increase the expression of AcrA and TolC. As well, a putative *mar-rob-sox* box has been identified upstream of the *tolC* gene, suggesting the involvement of these global regulatory proteins in the regulation of AcrAB-TolC expression (Aono *et al.*, 1998). Under general stress conditions the expression of *acrR* was also observed to increase. Therefore, it has been proposed that the function of the

local repressor AcrR is to fine tune and modulate the expression of AcrAB-TolC system under stress conditions to ensure that the system is not overexpressed (Ma *et al.*, 1996).

In *P. aeruginosa*, MexR has been identified as a repressor of its own transcription and that of the *mexAB-oprM* operon. This protein is encoded by a gene situated upstream and transcribed divergently from the *mexAB-oprM* genes and shows close homology to MarR, the repressor of the *marRAB* operon in *E. coli* (Poole *et al.*, 1996b). The *mexR* and *mexA* genes have been observed to have single overlapping promoters that also overlap with the MexR binding site (Evans *et al.*, 2001; Sanchez *et al.*, 2002). Mutations that increase the transcription from one promoter have been shown to decrease the transcription from the other, suggesting promoter competition for RNA polymerase binding (Sanchez *et al.*, 2002). In addition, expression of the *mexAB-oprM* genes has been observed to be growth phase-dependent with minimal expression at lag phase and the highest expression observed at the onset of stationary phase, perhaps suggesting the involvement of a quorum sensing autoinducer. Indeed, supplementing the culture medium with one of the two types of quorum sensing autoinducers produced by this strain, N-butyryl-L-homoserine lactone (C4-HSL) increased the expression of the *mexAB-oprM* genes. Moreover, observation of a similar pattern of C4-HSL-mediated increase in expression of the *mexAB-oprM* genes in *mexR* deletion mutants suggested that another regulator besides MexR functions to regulate the growth phase-dependent expression of this system (Evans and Poole, 1999; Maseda *et al.*, 2004; Sawada *et al.*, 2004). Consistent with this, mutations resulting in overexpression of the MexAB-OprM system have been mapped to two additional genes *nalC* and *nalD* encoding proteins belonging to

the TetR family of transcriptional repressors, emphasizing the involvement of a complex regulatory network. (Cao *et al.*, 2004; Sobel *et al.*, 2005a)

Transcription of the *mexCD-oprJ* operon in *P. aeruginosa* has been shown to be regulated by the repressor protein NfxB, the gene for which is situated upstream and transcribed divergently from the efflux pump genes. A point mutation in the *nfxB* gene has been shown to derepress its own expression and that of the *mexCD-oprJ* genes (Poole *et al.*, 1996a). Unlike most RND-type efflux systems which are negatively regulated, expression of the *mexEF-oprN* operon in *P. aeruginosa* has been observed to be positively regulated by an activator protein MexT, which is encoded by an upstream gene transcribed in the same direction as the efflux pump genes and shows high homology to the LysR family of transcriptional activators (Kohler *et al.*, 1999). Transcription of *mexEF-oprN* has been shown to occur as a result of mutations in *mexT* that transforms inactive MexT to its active form (Maseda *et al.*, 2000). MexT has also been shown to activate the transcription of the *mexS* gene situated upstream of *mexT*. MexS is a homologue of oxidoreductase/dehydrogenases and mutational inactivation of *mexS* has been shown to result in increased expression of the *mexEF-oprN* genes, suggesting its function as a negative regulator, but how this is achieved has yet to be determined (Kohler *et al.*, 1999; Sobel *et al.*, 2005b). Intriguingly, MexT has recently been observed to act as a repressor of the growth phase-dependent increase in expression of the *mexAB-oprM* genes (Maseda *et al.*, 2004), suggesting regulator cross-talk and further accentuating the complexity of RND efflux pump gene regulation in *P. aeruginosa*.

In the *P. putida* strain DOT-T1E, the TtgR protein that belongs to the TetR family of transcriptional repressors, is encoded by an upstream gene divergently transcribed

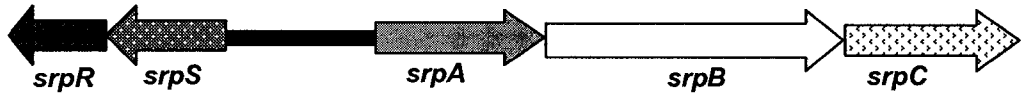
from the *ttgABC* operon. It has been observed that TtgR negatively regulates the expression of the *ttgABC* operon as well as its own expression (Duque *et al.*, 2001). Primer extension analysis and DNase I footprinting experiments have revealed that the *ttgR* and *ttgA* genes have single overlapping promoters that also overlap with the TtgR binding site (Duque *et al.*, 2001; Teran *et al.*, 2003). Transcription from these overlapping promoters has been observed to increase in the presence of the antibiotics chloramphenicol and tetracycline, which are both substrates of the TtgABC efflux system. Gel mobility shift assays and DNase I protection experiments confirmed that these antibiotics directly inhibited the binding of TtgR to the operator DNA. However, no such effect was observed with any other antibiotics, suggesting that chloramphenicol and tetracycline may resemble some natural substrates of this efflux system (Teran *et al.*, 2003). Furthermore, even in the presence of these inducers transcription from both *ttgA* and *ttgR* promoters never reached as high a level as in the *ttgR* deletion mutant. Therefore, it was proposed that simultaneous derepression of the overlapping *ttgA* and *ttgR* promoters ensures that the level of TtgR in the cell is always kept high and the TtgABC system is not overexpressed (Duque *et al.*, 2001; Teran *et al.*, 2003). Additionally, mutations in a gene homologous to the global regulator Lrp (leucine-responsive regulatory protein) have been shown to increase *ttgR* expression with a concomitant decrease in the *ttgABC* expression, suggesting that global regulators may be involved in the regulation of expression of the *ttgABC* operon (Duque *et al.*, 2001).

Unlike most RND-type efflux systems, the *ttgGHI* operon in *P. putida* DOT-T1E is reported to have two upstream divergently transcribed polycistronic genes *ttgV* and *ttgW*, encoding regulators belonging to the IclR and TetR family, respectively (Rojas *et*

al., 2003). Primer extension analysis followed by *in vitro* transcription assays showed that the *ttgVW* and *ttgGHI* operons are transcribed from single overlapping promoters (Guazzaroni *et al.*, 2004; Rojas *et al.*, 2003). Deletion of *ttgV*, with *ttgW* expressed in *trans*, has been shown to decrease the expression from both *ttgG* and *ttgV* promoters and similar decreases were observed in a *ttgVW* double mutant. However, deletion of *ttgW* in a strain with intact *ttgV* showed the same level of activity as the wild-type strain. Therefore, it was proposed that TtgV acts as a repressor of *ttgVW* and *ttgGHI* expression while TtgW plays no significant role in the regulation of these genes. Moreover, gel mobility shift assays and DNase I footprinting experiments confirmed that TtgV binds to a region overlapping the *ttgV* and *ttgG* promoters (Rojas *et al.*, 2003). Organic solvents, which are substrates of this system, have been shown to directly inhibit the binding of TtgV to the operator DNA, increasing the transcription from the *ttgG* promoter as observed by *in vitro* transcription assays. However, antibiotics which are also substrates of this efflux system did not have similar effects (Guazzaroni *et al.*, 2004), raising the possibility that organic solvents are the natural substrates of this system. While these studies did not allow the identification of a role of TtgW, it is expected that TtgW is somehow involved in the regulation of the *ttgGHI* expression since the *ttgW* gene is located at such close proximity with the efflux pump genes and is arranged as an operon with *ttgV*.

Much like the *ttgGHI* genes, the *srpABC* genes in *P. putida* S12 have two upstream divergently transcribed genes (Dennis and Zylstra, 1999; Wery *et al.*, 2001) (Figure 1.1). The *srpS* and *srpR* genes encode regulatory proteins that show the most homology to TtgV and TtgW, respectively (Dennis and Zylstra, 1999). Inactivation of

(A)



(B)

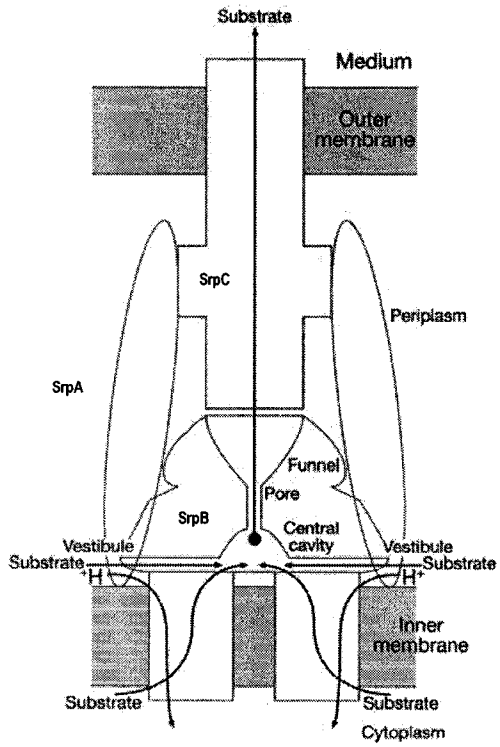


Figure 1.1. The RND-type efflux system SrpABC in *P. putida* S12. (A) Schematic representation of the *srpABC-srpSR* cluster. (B) Structure of the SrpABC system. The inner membrane transporter protein SrpB is connected via the periplasmic membrane fusion protein SrpA to the outer membrane channel-forming protein SrpC. Adapted from Murakami *et al.* (2002)

srpS by an insertion sequence has been shown to result in a tremendous increase in solvent tolerance of this strain. But complementation with both *srpS* and *srpR* were required to restore wild-type levels of solvent tolerance, suggesting that both the regulators are involved in the regulation of solvent tolerance in this strain (Wery *et al.*, 2001). How these regulators control the expression of the *srpABC* genes was not determined. It is apparent that regulation of expression of RND-type efflux systems is a complex process, involving multiple regulatory factors that vary between the different systems.

1.8 Organic solvent tolerance mechanisms in Gram positive bacteria

Gram positive bacteria do not have an outer membrane but have a thick cell wall mainly consisting of peptidoglycan. Covalently linked to the peptigoglycan are the linear anionic polymers teichoic acids and lipoteichoic acids, which give the cell wall a net negative charge. This structure facilitates penetration of molecules with molecular weights ranging between 30,000 and 57,000 kDa (Scherrer and Gerhardt, 1971), therefore most antimicrobials are allowed easy access into the cell membrane and the target sites. Nonetheless, a number of Gram positive bacteria have been observed to tolerate a variety of antimicrobials. Several mechanisms involved in antibiotic and biocide resistance have been identified. Active efflux of antibiotics and biocides via transporter proteins belonging to the ATP-binding cassette (ABC) family, the major facilitator superfamily (MFS), and the small multidrug resistance (SMR) family have been observed in a number of Gram positive bacteria (Poole, 2005). However, the rapid substrate influx through the cell wall will require these single component systems located

in the cytoplasmic membrane to work in conjunction with other mechanisms. It has been observed that vancomycin resistant *Staphylococcus aureus* strains have thickened cell walls and altered peptidoglycan structures that function to reduce the penetration of vancomycin (Cui *et al.,* 2000). Similar mechanisms may be involved in organic solvent tolerance. Recently, it was observed that the solvent tolerant *Bacillus cereus* strain R1 accumulated higher levels of toluene in the presence of energy inhibitors, suggesting that an active efflux mechanism may be involved in the solvent tolerance of this strain (Matsumoto *et al.*, 2002). Endospore formation and solvent metabolism or modification have also been suggested as mechanisms that may contribute to solvent tolerance in Gram positive bacteria (Sardessai and Bhosle, 2002a), but the exact mechanisms involved have yet to be determined.

1.9 Objectives

The SrpABC system in *P. putida* S12 is unique in that it is involved only in organic solvent efflux (Isken and De Bont, 2000) and the genes encoding this system have two upstream regulatory genes, unlike most RND-type efflux systems in Gram negative bacteria (Dennis and Zylstra, 1999; Wery *et al.,* 2001). Moreover, organic solvents induce the expression of this system (Kieboom *et al.*, 1998b), suggesting that it may have evolved specifically to confer organic solvent tolerance. One objective of this research was to determine how the two regulators SrpS and SrpR are involved in the expression of the *srpABC* genes. Northern hybridization analyses helped to determine the transcription levels of both the regulators in the presence and absence of toluene. Attempts were made to map the transcription start sites of *srpS* and *srpA* to determine if

the expression of *srpS* and the *srpABC* operon is coordinately regulated. Creation of strains containing *srpA-lacZ* reporter gene fusions was helpful in determining the *srpA* promoter activity in *srpS* deletion and *srpR* deletion mutants when compared to the wild-type strain during growth in the presence and absence of toluene. Finally, electrophoretic mobility shift assays helped determine the protein-DNA interactions of the SrpS and SrpR proteins within the *srpS-srpA* intergenic region and were useful in elucidating the roles that these proteins play in SrpABC efflux pump gene expression.

The second objective of this research was to use enrichment procedures to isolate novel organic solvent tolerant bacteria from both hydrocarbon-contaminated and normal soil environments. Isolated strains were characterized by determining the range of solvents they were able to tolerate as well as their survival under solvent shock conditions. Comparison of 16S rRNA gene sequences and carbon source utilization profiles with other bacteria helped to determine the taxonomic classification of the isolated strains. Finally, the ultrastructure of an isolated Gram positive strain was examined after growth in the presence and absence of toluene in an attempt to identify a mechanism that contributes to organic solvent tolerance in Gram positive bacteria.

Chapter 2:

Materials and Methods

2. Materials and Methods

2.1 Characterization of SrpS and SrpR, the regulators of the SrpABC solvent efflux pump in *Pseudomonas putida* S12

2.1.1 Bacterial strains, plasmids and growth conditions

2.1.1.1 Bacterial strains

The *Escherichia coli* and *Pseudomonas putida* S12 strains used in this study are listed in Table 2.1.1

2.1.1.2 Plasmids

Recombinant plasmids previously constructed in the lab and their uses in this study are listed in Table 2.1.2. The cloning vectors used in this study are listed in Table 2.1.3. The recombinant plasmids constructed in this study, together with their uses are listed in Table 2.1.4.

2.1.1.3 Media and Growth conditions

Luria-Bertani (LB) medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.5% [w/v] NaCl) (Sambrook *et al.*, 1989) was routinely used as complete medium for growth. Solid medium contained 1.5% (w/v) agar. Where indicated, M9 minimal medium (47.8 mM $Na_2HPO_4.7H_2O$, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl,

Table 2.1.1. Bacterial strains used in this study

Strain	Relevant characteristics	Reference or Source
E. coli		
DH5α	F^-ϕ80*lacZ*ΔM15Δ(*lacZY* A-*argF*)U169 *deoR recA1 endA1 hsdR17*(r^-_k,m^+_k) *phoA supE44 thi-1 gyrA96 relA1*λ	Invitrogen Corp., Carlsbad, CA
***P. putida* S12**		
Wild-type	*srpABC*$^+$, *srpR*$^+$,*srpS*$^+$	(Hartmans *et al.*, 1990)
SrpS$^-$	Wild-type derivative with the *srpS* gene disrupted by a Gm^R cassette	Collaborative efforts by the members of the Dennis lab (2001-2003)
SrpR$^-$	Wild-type derivative with the *srpR* gene disrupted by a Sm^R cassette	Collaborative efforts by the members of the Dennis lab (2001-2003)
S12/*lacZ*	Wild-type derivative containing an *srpA-lacZ* reporter gene fusion	This study
SrpS$^-$/*lacZ*	SrpS$^-$ derivative containing an *srpA-lacZ* reporter gene fusion	This study
SrpR$^-$/*lacZ*	SrpR$^-$ derivative containing an *srpA-lacZ* reporter gene fusion	This study

Table 2.1.2. Recombinant plasmids constructed previously and used in this study

Plasmid	Parent plasmid	Antibiotic resistance marker	Relevant characteristics	Use
pJD500	pGEM5Z	Ampicillin Kanamycin	Plasmid containing a transcriptional fusion of *srpA-lacZ*. Contains a promoterless *trp-lacZ* gene fused into *srpA* downstream of *srpA* promoter. (Previously known an psrptrplacZKm; constructed by J. Dennis, personal communication)	Creation of the S12/*lacZ*, SrpS$^-$/*lacZ* and SrpR$^-$/*lacZ* strains with the *srpA-lacZ* reporter gene fusions
p1B1/pJD101		Kanamycin	Derived from a *Bam*HI digestion of *P. putida* S12 chromosome containing plasposon TnMod-KmO. Contains *srpR* through partial open reading frame for *srpB* (Kieboom *et al.*, 1998a)	Template for the manual sequencing of the *srpS-srpA* intergenic region. Also template for the amplification of the *srpS-srpA* intergenic region to be used as the probe for electrophoretic mobility shift assays.

Table 2.1.3. Cloning vectors used in this study

Plasmid	Antibiotic resistance marker	Relevant characteristics	Reference or source
pMAL-c2X	Ampicillin	High copy number plasmid for overexpression of maltose-binding protein fusion proteins	New England Biolabs (NEB), Ipswich, MA

Table 2.1.4. Recombinant plasmids constructed and used in this study

Plasmid	Parent plasmid	Antibiotic resistance marker	Insert size	Relevant characteristics	Use
pMAL-c2X-srpS	pMAL-c2X	Ampicillin	831 bp	Contains the entire coding sequence of *srpS* cloned downstream of the *E. coli malE* gene using engineered *Sal*I and *Hind*III sites	Overexpression of MBP-SrpS in *E. coli*
pMAL-c2X-srpR	pMAL-c2X	Ampicillin	642 bp	Contains the entire coding sequence of *srpR* cloned downstream of the *E. coli malE* gene using engineered *Sal*I and *Hind*III sites	Overexpression of MBP-SrpR in *E. coli*

2 mM $MgSO_4.7H_2O$, 0.1 mM $CaCl_2$) (Sambrook *et al.*, 1989) supplemented with 0.2% glucose as the carbon source was used as defined medium. When required for selection, the culture medium was supplemented with different antibiotics including gentamicin (25 μg/ml), streptomycin (150 μg/ml), kanamycin (50 μg/ml) and ampicillin (100 μg/ml). Where indicated, cultures were supplemented with toluene to a final concentration of 6.0 mM and the flasks were sealed with foil-covered stoppers. Liquid cultures were routinely grown with shaking at 240 rpm. *E. coli* cultures were incubated at 37°C while *P. putida* S12 cultures were incubated at 30°C.

2.1.1.4 Preparation of glycerol stocks

All strains were grown overnight on LB plates with the appropriate antibiotics and culture conditions. Cells were then suspended in 1 ml LB broth containing 20% (v/v) glycerol and stored at -80°C.

2.1.2 DNA isolation and transformation

2.1.2.1 Transformation of *E. coli*

Chemically competent *E. coli* DH5α cells (Invitrogen Corp., Carlsbad, CA) were transformed according to the manufacturer's instructions with slight modifications. Briefly, a 50 μl cell aliquot was thawed on ice before the plasmid DNA or the ligation mix (1-20 μl) was added and mixed gently with the cell suspension. The mixture was then incubated on ice for 30 minutes, followed by a brief heat shock at 42°C for 30 seconds and returned to ice for 2 minutes. Subsequently, 1 ml SOC medium

(Sambrook *et al.*, 1989) was added and the culture was incubated on a shaker (240 rpm) at 37°C for 1 hour. Following the incubation, 100 µl aliquots were spread plated on LB plates containing the appropriate antibiotic. When required for blue-white screening, plates were also supplemented with 100 mM IPTG and 40 µg/ml X-gal. All plates were incubated at 37°C overnight and checked for colony formation.

2.1.2.2 Isolation of plasmid DNA from *E. coli*

Plasmid isolations from *E. coli* cells were performed using the QIAprep Spin Miniprep Kit (QIAGEN Inc., Mississauga, ON) according to the manufacturer's instructions.

2.1.2.3 Electroporation of *P. putida* S12 strains

For electroporation of *P. putida* S12 strains, a single colony was used to inoculate 10 ml LB broth and the culture was grown overnight at 30°C. The overnight culture was diluted 1:50 in fresh LB and was grown until an OD_{600} of approximately 0.6 was reached. Cells were harvested from a 1 ml sample by centrifugation at 4200 rpm for 4 minutes using a microcentrifuge. The supernatant was discarded and the cells were washed with 1 ml 10% (v/v) glycerol and centrifuged as before. This wash step was repeated two more times and the cells were resuspended in 100µl 10% (v/v) glycerol before plasmid DNA (2-10 µl) was added and mixed gently. The mixture was then transferred to a 0.1 cm Gene Pulser Cuvette (Bio-Rad Laboratories, Hercules, CA) and electroporated using a Bio-Rad MicroPulser. Immediately afterwards, 1 ml Terrific broth (Sambrook *et al.*, 1989) was added to the cuvette and mixed with the cell suspension. Subsequently, the

mixture was transferred to a 1.5 ml microfuge tube prior to incubation on a shaker (240 rpm) at 30°C for 1 hour. Following the incubation, 20-100 µl aliquots were spread plated on LB plates containing the appropriate antibiotic. The plates were then incubated at 30°C overnight and checked for colony formation.

2.1.2.4 Isolation of chromosomal DNA from the *P. putida* S12 strains

Chromosomal DNA was isolated from the *P. putida* S12 strains using the procedure described by Ausubel *et al.* (1991). Briefly, a few single colonies from an overnight culture were suspended in 567 µl TE (10 mM Tris HCl [pH 8.0], 1 mM EDTA). Subsequently, 30 µl 10% (w/v) SDS and 3 µl 20 mg/ml proteinase K were added to the cell suspension, mixed by pipetting gently and incubated at 37°C for 60 minutes. The cell suspension was then mixed with 100 µl 5 M NaCl and 80 µl of CTAB buffer (10% hexadecyltrimethyl ammonium bromide [CTAB], 0.7 M NaCl) and incubated at 55°C for 10 minutes to allow the selective precipitation of proteins, polysaccharides, and cell wall debris. Following the incubation, 700 µl 24:1 chloroform:isoamyl alcohol was added, mixed gently and centrifuged for 5 minutes at 13 200 rpm. The top aqueous phase was subsequently extracted once with 400 µl phenol and once with 400 µl 24:1 chloroform:isoamyl alcohol. The aqueous phase recovered was then mixed with 450 µl isopropanol to precipitate the DNA and centrifuged for 5 minutes at 13 200 rpm. The DNA pellet obtained was washed with 500 µl 75% ethanol, vacuum dried for 30 minutes and redissolved in 100 µl TE (10 mM Tris HCl [pH 8.0], 1 mM EDTA).

2.1.3 DNA analysis and purification

2.1.3.1 Restriction digestion and cloning of DNA

Restriction endonuclease digestions of plasmid DNA and PCR products were performed according to the manufacturer's instructions (Invitrogen). Ligation reaction mixtures were prepared in volumes of 10-20 µl and consisted of a 3:1 ratio of the digested insert to vector DNA, 1x ligation buffer (300 mM Tris-HCl [pH 7.8], 100 mM $MgCl_2$, 100 mM DTT and 10 mM ATP) and 1 U of T4 DNA ligase according to the manufacturer's recommendations (Promega, Madison, WI). Ligation reaction mixtures were incubated at 16°C overnight followed by a 30 minute incubation at room temperature, prior to transformation into chemically competent *E. coli* DH5α cells. Typically all of the ligation mixture was transformed.

2.1.3.2 Polymerase chain reaction (PCR)

PCR was utilized to amplify the DNA fragments used in the cloning reactions as well as to obtain DNA to be used as probes for northern hybridization analysis and electrophoretic mobility shift assays. In addition, PCR was used to check for the presence of the correct insert after construction of recombinant plasmids and to check for the integration of the vector DNA into the chromosome of *P. putida* S12 strains. Routinely reactions were performed in 50 µl volumes in 0.2 ml tubes using the Mastercycler gradient (Eppendorf, Hamburg, Germany). Generally PCR mixtures contained 1 µg chromosomal DNA or 10 ng plasmid DNA, 1x PCR buffer (200 mM Tris HCl [pH 8.4], 500 mM KCl) (Invitrogen), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 2.5 U Taq polymerase (provided by Dr. Pickard, University of Alberta) and sterile milli-Q

H_2O (Millipore, Billerica, MA) to adjust the volume. When necessary, the high fidelity Platinum *Pfx* DNA polymerase (Invitrogen) was used and the reaction mixtures contained 1 μg chromosomal DNA or 10 ng plasmid DNA, 1x *Pfx* amplification buffer (Invitrogen), 0.3 mM dNTPs, 1 mM $MgSO_4$, 0.5 μM of each primer, 2.5 U Platinum *Pfx* DNA polymerase (Invitrogen) and sterile milli-Q H_2O to adjust the volume. A general PCR program consisted of a 2 minute denaturation period at 94°C followed by 29 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 55.4°C and 30 seconds extension at 72°C and a final 2 minute extension period at 68°C at the end of the program. All the oligonucleotide primers together with their specific uses in this study are listed in Table 2.1.5.

2.1.3.3 PCR purification of the amplified DNA fragments

The DNA fragments amplified for use in sequencing reactions as well as those to be used as probes for northern hybridization analysis and electrophoretic mobility shift assays were purified using the QIAquick PCR Purification Kit (QIAGEN Inc.) as per the manufacturer's instructions. DNA was eluted in 30 μl sterile milli-Q H_2O or TE (10 mM Tris HCl [pH 8.0], 1 mM EDTA) as required. A portion of the PCR reaction was analyzed by agarose gel electrophoresis (section 2.1.3.4) prior to and following the purification procedure to check the recovery efficiency. The rest of the purified product was subsequently used in the respective procedures.

Table 2.1.5. Oligonucleotide primers used in this study

Primer	Sequence 5'-3' *	Use
SP1 AP2	AACCTGTTCTTTCTCACCAC TTCTTCCAGAGCGTTGATGA	Amplifies a 490bp fragment in the *srpS-srpA* intergenic region to be used as the probe for electrophoretic mobility shift assays
SF1 SR2	ATGTCGACTACAGTGGCGGC TTAAGCTTCTAGGGAGCTTTCTTC	Amplifies a 831 bp fragment encompassing the entire coding sequence of *srpS* to clone into pMAL-c2X
RF1 RR2	TAGTCGACATGGCTAGAAAGACG ATAAGCTTTACTCGAAGGATTTGACTT	Amplifies a 642 bp fragment encompassing the entire coding sequence of *srpR* to clone into pMAL-c2X
SFN1 SRN2	ATCCAGGTCATCGCCAG ACCACTCTGCCTCACTTCG	Amplifies a 538 bp fragment within the coding sequence of *srpS* to be used as the probe for northern hybridization analysis
RFN1 RRN2	GAGCCTGCTGGTCCTTGCT TCGTGGTGCTGTCTACTGGC	Amplifies a 404 bp fragment within the coding sequence of *srpR* to be used as the probe for northern hybridization analysis
JD567 JD568	AATGCGCTGTCTTGTCTCCT CCTCTTCGCTATTA	PCR confirmation of pJD500 integration in the chromosome of S12/*lacZ*, SrpS$^-$/*lacZ* and SrpR$^-$/*lacZ* strains
ST3	ATGCGGATGACTGCCAAGC	Primer extension and sequencing to determine the transcription start site of *srpS*

* Underlined bases are engineered restriction sites

2.1.3.4 Agarose gel electrophoresis of DNA

Large DNA fragments (1-10 kb) were analyzed on 0.7% (w/v) agarose 1x TAE (40 mM Tris-acetate, 1 mM EDTA) gels, while smaller fragments (0.5-1 kb) were separated on 1% (w/v) agarose, 1x TAE gels. Gel electrophoresis was conducted in 1x TAE at a voltage of 100 or 120 V depending on the size of the gel. The 1 Kb Plus DNA Ladder (Invitrogen) was used as the molecular weight marker for the DNA fragments. Prior to loading, 1x loading dye (0.25% bromophenol blue and 40% sucrose) was added to all samples. All gels were stained with ethidium bromide before visualizing using a UV transilluminator.

2.1.3.5 DNA purification from agarose gels

DNA fragments were agarose-gel purified when necessary, using a GeneClean II kit (Q. BIOgene, Carlsbad, CA) according to the manufacturer's instructions.

2.1.3.6 DNA sequencing

Manual DNA sequencing was performed using a chain termination method with the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (Unites States Biochemical Corp. [USB], Cleveland, OH) per the manufacturer's instructions. The sequencing reactions were carried out in 0.2 ml tubes using a Perkin Elmer GeneAmp PCR System 2400 Thermocycler. All sequencing reactions were separated by electrophoresis on a 6% polyacrylamide sequencing gel (6% [v/v] acrylamide [19:1 acrylamide: N,N'-methylenebisacrylamide (Bio-Rad)], 8.3 M urea, 1x TBE [90 mM Tris, 89 mM boric acid, 2.5 mM EDTA], 0.125% [w/v] APS and 0.5% [v/v] TEMED) for 2

hours at a constant power of 40W. Following electrophoresis the gel was dried under a vacuum for 2 hours at 80°C and exposed to X-ray film at -80°C and visualized by autoradiography.

Automated DNA sequencing was performed with the Amersham DYEnamic ET kit (Amersham Pharmacia Biotech, Piscataway, NJ). Reaction mixtures were prepared in 20 µl volumes in 0.2 ml tubes and contained 100-500 ng plasmid DNA or 1 ng/bp PCR product, 5 pmol primer, 8 µl sequencing buffer (20 mM Tris [pH 9.0], 5 mM $MgCl_2$. $6H_2O$), 4 µl ET mix and sterile milli-Q H_2O to adjust the volume. Sequencing reactions were performed using the Mastercycler gradient (Eppendorf) with the reaction program consisting of 24 cycles of 20 seconds denaturation at 95°C, 15 seconds annealing at 50°C and 1 minute elongation at 60°C. Upon completion of the reaction, samples were transferred to 1.5 ml tubes and precipitated on ice for 15 minutes by the addition of 2 µl NaOAc/EDTA and 80 µl of 95% ethanol. In order to avoid precipitation of free nucleotides, the samples were removed from ice promptly and centrifuged. The pellets obtained were washed with 500 µl 70% (v/v) ethanol, dried for 20 minutes in a vacuum chamber and provided to the Molecular Biology Services Unit (MBSU), University of Alberta, for automated sequencing using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.1.3.7 Radioactive labeling of DNA

The linear double stranded (ds) DNA used as probes for northern hybridization analysis were labeled by a random primer labeling technique using the Ready-To-Go DNA Labeling Beads (-dCTP) (Amersham Pharmacia Biotech) according to the

manufacturer's instructions. Each reaction mixture consisted of 50 pmol target DNA (denatured), 50 μCi [α-^{32}P]-dCTP (Amersham Pharmacia Biotech) and sterile milli-Q H_2O up to 50 μl final volume added to a tube containing the reaction mix bead. Following the labeling reaction 5 μl 0.2 M EDTA was added to stop the reaction and unincorporated nucleotides were removed by passage through a Probe Quant G-50 Micro Column (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

The 5' ends of the oligonucleotides used for primer extension analysis and the linear dsDNA used as probes for electrophoretic mobility shift assays were labeled using a 5' end-labeling procedure (Chaconas and van de Sande, 1980). The end-labeling reaction consisted of 50 pmol target DNA, 1x polynucleotide kinase (PNK) buffer (Roche Diagnostics Corp., Indianapolis, IN), 50 μCi [γ-^{32}P]-dATP (ICN or MP Biomedicals Corp., Irvine, CA), 0.01 units of PNK and sterile milli-Q H_2O to make up to 20 μl final volume. The reaction mixture was incubated at 37°C for 15 minutes followed by the addition of another 0.01 units of PNK. Incubation was continued for another 15 minutes at 37°C, after which the enzyme was inactivated by heating for 10 minutes at 65°C. The volume of the reaction mixture containing oligonucleotide probes was subsequently adjusted to 50 μl with sterile milli-Q H_2O and was precipitated on ice for 15 minutes by the addition of 5 μl 3 M sodium acetate, 100 μl 95% ethanol and 2 μl glycogen (Roche). The sample was promptly centrifuged and the pellet was washed with 500 μl 70% ethanol and redissolved in sterile milli-Q H_2O to a final concentration of 2.5 pmol/μl. Following the end-labeling reaction of the dsDNA probes, the reaction volume was adjusted to 50 μl with STE (150 mM NaCl, 10 mM Tris HCl [pH 8.0], 1 mM EDTA) and unincorporated nucleotides were removed by passing the mixture through a Probe

Quant G-50 Micro Column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. In order to determine the volume of the probe to be used in the proceeding experiments, 1 µl of the purified probe was quantified by Cerenkov counting in a Beckman LS 3801 scintillation counter.

2.1.3.8 DNA hybridizations

Northern hybridizations were performed in glass hybridization bottles (Fischer Scientific International Inc., Hampton, NH) with all incubations conducted in a hybridization oven (Fischer Scientific). Membranes were hybridized in 20 ml hybridization buffer consisting of 6x SSC (3 M NaCl, 0.3 M trisodium citrate), 5x Denhardt's solution (0.08% polyvinylpyrrolidone, MW 360 000) and 100 µg/ml salmon sperm DNA. All membranes were pre-hybridized for 4 hours at 62°C before the radiolabeled DNA probe (Section 2.1.3.7) was added ($2x10^6$-$4x10^6$ cpm). Probe denaturation occurred prior to addition by incubation at 95°C for 15 minutes. A temperature of approximately 25°C below the melting temperature (T_m) of the DNA-RNA hybrid was used as the hybridization temperature. The T_m of the hybrid was calculated using the formula: $T_m = 79.8°C + 18.5\log M + 58.4\ (\%G+C) + 11.8\ (\%G+C)^2 - 820/n$ where M is the ionic strength (0.9 for 6x SSC) and n is the length of the annealed product (Casey and Davidson, 1977). Hybridizations occurred for 16-24 hours and subsequently the membranes were washed at the hybridization temperature twice for 20 minutes with wash solution I (1x SSC, 0.1% (w/v) SDS) and twice for 20 minutes with wash solution II (0.2x SSC, 0.1% (w/v) SDS). Following the wash steps the membranes

were blotted dry on Whatman paper, wrapped in Saran wrap and exposed to X- ray film at -80°C and visualized by autoradiography.

2.1.4 RNA isolation and analysis

2.1.4.1 Isolation of RNA

RNA isolation from wild-type *P. putida* S12 was performed using a modified version of the procedure of Marques *et al.* (1993). A single colony of the culture was used to inoculate 25 ml M9 minimal medium containing 0.2% glucose and grown overnight at 30°C. The overnight culture was diluted 1:100 in fresh M9 glucose medium with and without toluene added to a final concentration of 6.0 mM, and grown while shaking (240 rpm) at 30°C until they reached an OD_{600} of approximately 1.0-1.1. Cells were harvested from 15 ml samples by centrifugation at 5000 rpm for 15 minutes using an Eppendorf 5810 R centrifuge. The supernatant was discarded and the cells were resuspended in 4 ml of an ice cold solution containing 10 mM sodium acetate (pH 4.8), 0.15 M sucrose and 100 U heparin. Subsequently, 100 µl 20% (w/v) SDS was added and the cell suspensions were vortexed briefly. Immediately 4 ml of pre-heated (65°C) phenol (equilibrated with Tris HCl [pH 4.3]) supplemented with 0.1% (w/v) 8-hydroxyquinoline and 0.2% (v/v) β-mercaptoethanol was added, vortexed and incubated at 65°C for 5 minutes with occasional mixing. The samples were then incubated on ice for 5 minutes and centrifuged at 9000 rpm for 10 minutes. The top aqueous phase was saved and 100 µl 20% (w/v) SDS was added and the process of hot phenol extraction was repeated two more times. Subsequently, the aqueous phase was extracted twice with chloroform by the addition of 4 ml chloroform at room temperature followed by vortexing and

centrifugation as above. The samples were then precipitated at -80°C for 60 minutes by the addition of 10% (v/v) 2.5 M sodium acetate and 2.2 volume 95% ethanol, and subsequently centrifuged at 9000 rpm for 30 minutes to obtain pellets. The supernatant was discarded and the pellets were resuspended in 400 µl of a solution containing 0.1 M Tris HCl, 50 mM NaCl, 10 mM EDTA, 0.2% (v/v) SDS; pH 7.4 supplemented with 20 µg/ml proteinase K and incubated at 37°C for 60 minutes. The samples were then extracted once with hot phenol, once with chloroform and the aqueous phase was precipitated and centrifuged as above. The pellets obtained were washed with 500 µl 70% ethanol, vacuum dried for 15 minutes and resuspended in 50 µl DEPC- treated milli-Q H_2O. To remove contaminating DNA, 200 µl DNase I solution (5 µl DNase I made up to 200 µl with DNase I buffer; Epicenter, Madison, WI) was added and incubated at 37°C for 30 minutes. This process was repeated one more time and the resulting mixture was extracted three times with phenol at room temperature and once with chloroform. The aqueous phase was precipitated at -80°C for 60 minutes by the addition of 10% (v/v) 2.5 M sodium acetate, 2.2 volume 95% ethanol and 1 volume isopropanol and subsequently centrifuged for 10 minutes at 9000 rpm. The pellets were washed with 500 µl 95% ethanol, vacuum dried for 15 minutes and redissolved in 20 µl TE (RNase free; Epicenter). The RNA samples were quantified by calculating the A_{260}/A_{280} values and stored at -80°C.

2.1.4.2 Northern hybridization analysis

Northern hybridization analysis was performed to detect the *srpS* and *srpR* mRNA transcripts from total RNA isolated from *P. putida* S12 after growth in the

presence or absence of toluene (Section 2.1.4.1). The RNA molecular weight marker (0.24-9.5 Kb RNA Ladder; Invitrogen) and 40 μg total RNA samples were denatured by heating at 55°C in a solution containing 1 M glyoxal, 50% dimethyl sulfoxide (DMSO) and 10 mM Na_2HPO_4-NaH_2PO_4 (pH 7.0) for 1 hour according to the procedure described by McMaster and Carmichael (1977). Samples were then loaded on a 1.2% (w/v) agarose 10 mM Na_2HPO_4-NaH_2PO_4 (pH 7.0) gel and separated by electrophoresis as described by Sambrook *et al.* (1989), with constant recirculation of the running buffer (10 mM Na_2HPO_4-NaH_2PO_4 [pH 7.0]) to prevent the creation of H^+ gradients. Electrophoresis occurred at 30 V for 3-4 hours and subsequently the RNA samples were transferred overnight to a nylon membrane (Hybond-N membrane, Amersham Pharmacia Biotech) by capillary elution as described by Sambrook *et al.* (1989). The RNA was then UV cross-linked to the membrane using a Bio-Rad GS Gene Linker on Program C3. Glyoxal was removed from the RNA by baking the membrane at 80°C under vacuum for 1 hour and the membrane was stored at room temperature until the hybridization procedure.

2.1.4.3 Primer extension analysis

Primer extensions were performed using Superscript II RNase H^- Reverse Transcriptase (Invitrogen) as per the manufacturer's instructions. Reactions were performed in 20 μl volumes and contained 10 μg total RNA, 5 pmol end-labeled oligonucleotide primer (Section 2.1.3.7), 40 U RNA Guard (Amersham Pharmacia Biotech), 4 μl 5 x First strand buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$), 2 μl 0.1 M DTT, 200 U Superscript II reverse transcriptase (Invitrogen) and sterile milli-Q H_2O to adjust the volume. Primer extension occurred at 42°C for 50

minutes and subsequently the enzyme was inactivated by incubation at 70°C for 15 minutes. The sample was then precipitated on ice for 30 minutes by the addition of 5 µl 3M sodium acetate, 100 µl 95% ethanol and 2 µl glycogen (Roche) followed by centrifugation to obtain a pellet. The pellet was then washed with 500 µl 70% ethanol and redissolved in 3 µl stop solution from the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB). The sample was denatured at 95°C for 5 minutes and subsequently a volume of 3 µl was loaded on a 6% polyacrylamide sequencing gel (6% [v/v] acrylamide [19:1 acrylamide: N,N'-methylenebisacrylamide (Bio-Rad)], 8.3 M urea, 1x TBE [90 mM Tris, 89 mM boric acid, 2.5 mM EDTA], 0.125% [w/v] APS and 0.5% [v/v] TEMED) alongside sequencing reactions (Section 2.1.3.6) generated using the same primer used for the primer extension reaction. The samples were electrophoresed for 2 hours at a constant power of 40 W. Following electrophoresis the gel was dried under a vacuum for 2 hours at 80°C and exposed to X-ray film at -80°C and visualized by autoradiography.

2.1.5 Protein overexpression, purification and analysis

2.1.5.1 Overexpression of MBP-SrpS and MBP-SrpR

The regulators SrpS and SrpR were overexpressed in *E. coli* DH5α as N-terminal fusion proteins with the maltose-binding protein (MBP). *E. coli* DH5α cells were transformed (Section 2.1.2.1) with the IPTG inducible plasmid pMAL-c2X-srpS or pMAL-c2X-srpR (Table 2.1.5) to overexpress MBP-SrpS or MBP-SrpR respectively. Pilot experiments were initially conducted as described in the pMAL-c2X instruction manual (Protocol A, New England Biolabs [NEB], Ipswich, MA) to determine if the

proteins were being expressed, their degree of solubility and to determine the optimum conditions to obtain maximum soluble fusion proteins. Large scale expressions of MBP-SrpS and MBP-SrpR were then performed accordingly. A single colony of *E. coli* DH5α containing the respective plasmids was used to inoculate 10 ml LB broth containing 100 μg/ml ampicillin and 0.2% (w/v) glucose. The culture was grown overnight at 37°C and was used to inoculate 1 L of LB broth containing the respective concentrations of ampicillin and glucose. This large-scale culture was grown while shaking (240 rpm) at 37°C until an OD_{600} of 0.5-0.6 was reached, and subsequently IPTG was added to a final concentration of 0.3 mM to induce the overexpression of the fusion protein. The culture was then allowed to grow at 37°C while shaking at 240 rpm for 6 hours before the cells were harvested by centrifugation at 4000x g for 20 minutes using a Beckman Model J2-21 centrifuge. The supernatant was discarded and the cells were resuspended in 30 ml column buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA) containing 0.01 mM PMSF (phenylmethylsulfonyl fluoride) (Sigma-Aldrich, St. Louis, MO) and stored overnight at -20°C.

2.1.5.2 Affinity purification of MBP-SrpS and MBP-SrpR

Purification of the overexpressed fusion proteins were performed according to the procedure described in pMAL-c2X instruction manual (Method I, NEB) with slight modifications. The harvested cells (section 2.1.4.1) were thawed on ice and lysed by sonication in 4 pulses of 15 seconds using a Branson Sonifier 450 with a 2.5 mm probe. Following sonication, the lysed cell suspension was centrifuged at 9000x g for 30 minutes at 4°C and the supernatant containing the soluble protein fraction (crude extract)

was saved and 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 amylose resin (NEB). The resin was initially washed with 8 column volumes of column buffer. Subsequently, the diluted crude extract was added and the resin was washed further with 12 column volumes of column buffer. The fusion protein bound to the amylose resin was eventually eluted in 3 ml fractions using column buffer containing 10 mM maltose. Samples were analyzed by measuring the A_{280} values and the fractions containing the purified fusion protein were pooled and quantified by using the Bradford Dye-binding assay (Section 2.1.4.3) and analyzed by SDS-PAGE (2.1.4.4). Quantified fusion protein was stored in aliquots of 50 µl at -80°C.

2.1.5.3 Protein quantification

The purified fusion protein was quantified using the Bio-Rad Protein Assay Kit according to the procedure described in Bradford (1976). Bovine serum albumin was used as a standard.

2.1.5.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Purified fusion proteins were analyzed by SDS-PAGE with slight modifications to the procedure described by Sambrook *et al.* (1989). The protein samples were separated on a 12.5% SDS-polyacrylamide gel using a Bio-Rad Mini-Protean 3 electrophoresis cell. The resolving gel consisted of 12.5% (v/v) acrylamide (19:1 acrylamide: N,N'-methylenebisacrylamide [Bio-Rad]), 375 mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS,

0.075% (w/v) APS and 0.08% (v/v) TEMED. Once the resolving gel was poured into the cast, the gel was overlaid with isopropanol and was allowed to polymerize for 30 minutes. Subsequently, the isopropanol was rinsed off with milli-Q H_2O and blotted dry prior to the addition of the stacking gel. The stacking gel consisted of 3.2% (v/v) acrylamide (19:1 acrylamide: N,N'-methylenebisacrylamide [Bio-Rad]), 125 mM Tris-HCl (pH 6.8), 0.068% (w/v) SDS, 0.21% (w/v) APS and 0.04% (v/v) TEMED. The stacking gel was allowed to polymerize for 30 minutes before the protein samples were loaded. Prior to loading, the protein samples were prepared by adding 1/4 volume of 2x SDS loading dye (100 mM Tris HCl [pH 6.8], 200 mM DTT, 4% [w/v] SDS, 0.2% [w/v] bromophenol blue, 20% glycogen) followed by denaturation at 95°C for 10 minutes. Typically, a volume of 20 µl was loaded into the wells alongside the BenchMark Protein Ladder (Invitrogen). Tris-glycine-SDS buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) was used as the running buffer and the samples were electrophoresed at a constant voltage of 200 V. Following the electrophoresis, the gel was stained in Coomassie Brilliant Blue protein stain (10% [v/v] glacial acetic acid, 50% [v/v] methanol, 0.001-0.002% [w/v] Coomassie Brillian Blue) for 30 minutes at 37°C with gentle agitation. The gel was then rinsed with milli-Q H_2O and destained while rocking in a solution comprised of 25% (v/v) methanol and 10% (v/v) acetic acid for 2-4 hours at 37°C or overnight at room temperature. The destained gel was then rinsed with milli-Q H_2O and dried overnight at room temperature clamped in between two sheets of Gel Drying Film (Promega) presoaked in a solution consisting of 40% (v/v) methanol, 10% (v/v) glycerol and 7.5% (v/v) acetic acid.

2.1.5.5 Non-denaturing polyacrylamide gel electrophoresis (PAGE)

The reactions for electrophoretic mobility shift assays (Section 2.1.5.1) were separated on 4% non-denaturing polyacrylamide 1x TAE gels using a Bio-Rad Mini-Protean 3 electrophoresis cell. Each gel was comprised of 4% (v/v) acrylamide (19:1 acrylamide: N,N'-methylenebisacrylamide [Bio-Rad]) 1x TAE (40 mM Tris-acetate, 1 mM EDTA), 0.07% (w/v) APS and 0.035% (v/v) TEMED. The cast gel was allowed to polymerize for 30 minutes and subsequently electrophoresed using 1x TAE buffer at a constant voltage of 150 V for 30 minutes prior to loading the samples. Following the pre-electrophoresis, samples were loaded into the wells and the gel was electrophoresed at a constant voltage of 150 V for 60 minutes. After electrophoresis the gel was dried under a vacuum at 80°C for 45 minutes and exposed to X-ray film at -80°C and visualized by autoradiography.

2.1.6 Protein-DNA interactions and enzyme assays

2.1.6.1 Electrophoretic mobility shift assays (EMSA)

Electrophoretic mobility shift assays were performed to study the protein-DNA interactions of the SrpS and SrpR proteins within the *srpS* - *srpA* promoter region. A 490-bp DNA fragment within the *srpS* – *srpA* intergenic region was PCR amplified (Table 2.1.5) (Section 2.1.3.2), PCR purified (Section 2.1.3.3) and end-labeled with [γ- ^{32}P]-dATP (Section 2.1.3.7). Binding reactions were carried out in 20 µl volumes and consisted of increasing concentrations of purified MBP-SrpS or MBP-SrpR, 1x binding buffer (65 mM Tris-HCl [pH 7.8], 0.2 M KCl, 25 mM $MgCl_2.6H_2O$, 5 mM DTT, 0.25% Tergitol [NP-40], 12.5% glycerol, 1µg of poly [dI-dC]), labeled target DNA ($2x10^6$-

$4x10^6$ cpm) and sterile milli-Q H_2O to adjust the volume. The reaction mixtures were incubated for 30 minutes at 30°C to allow the binding reaction to occur, followed by the addition of 1/5 volume of 5x loading buffer prior to loading on a 4% non-denaturing polyacrylamide 1x TAE gel. The samples were electrophoresed and analyzed as described in Section 2.1.4.5. Competition assays were performed using the same conditions with the addition of competitive or non-competitive DNA. Similar experiments were also performed to determine if toluene affected the binding of SrpS and SrpR within the *srpS – srpA* intergenic region. Increasing concentrations of toluene and a fixed concentration of the protein of interest were added into the reaction mixtures simultaneously, followed by a 30 minute incubation at 30°C prior to the addition of the labeled target DNA. In a second set of samples protein-DNA binding reactions were allowed to occur prior to the addition of increasing concentrations of toluene and subsequently the samples were incubated at 30°C for 30 minutes before electrophoresis.

EMSAs were also performed to determine if SrpR, with or without toluene affected the binding of SrpS to the *srpS – srpA* intergenic region. The reaction mixtures consisted of a fixed concentration of MBP-SrpS, 1x binding buffer, labeled target DNA ($2x10^6$-$4x10^6$ cpm) and sterile milli-Q H_2O to adjust the volume. The binding reactions occurred at 30°C for 30 minutes as above. Toluene was then added to one set of samples to a final concentration of 1 mM. Subsequently, increasing concentrations of MBP-SrpR was added to the samples with the SrpS to SrpR ratio ranging from 1:1 to 1:14. The volumes of the initial binding reactions were scaled down accordingly such that the addition of MBP-SrpR brings the final volume up to 20 µl. Samples were then incubated at 30°C for 30 minutes followed by electrophoresis and autoradiography as above.

Another set of EMSAs were performed with the addition of both SrpS and SrpR fusion proteins simultaneously into the reaction mixtures with and without toluene added to a final concentration of 1 mM, followed by a 30 minute incubation at 30°C prior to the addition of the labeled target DNA ($2x10^6$-$4x10^6$ cpm). The reaction mixtures were subsequently incubated at 30°C for 30 minutes to allow the binding reaction to occur, after which the samples were electrophoresed and analyzed as before.

2.1.6.2 β-galactosidase assays

β-galactosidase assays were performed to determine the *srpABC* promoter activity in the S12/*lacZ*, SrpS$^-$/*lacZ* and SrpR$^-$/*lacZ* strains (Table 2.1.1) during growth in the presence and absence of toluene. A single colony of each culture was used to inoculate 10 ml LB broth and the cultures were grown overnight at 30°C. The overnight cultures were diluted 1:50 in fresh LB with and without toluene added to a final concentration of 6.0 mM and were grown while shaking (240 rpm) at 30°C until they reached an OD_{600} of approximately 0.2-0.5. β-galactosidase assays were performed according to the procedure described in Slauch and Silhavy (1991). Formation of *o*-nitrophenol (ONP) was monitored by measuring the A_{420} values using a Wallac 1420 Multilabel Counter (Perkin-Elmer Life Sciences). Readings were taken for an approximately 60 minute period and the β-galactosidase activity was determined by calculating the A_{420}/ OD_{600} ratios. Five different samples were analyzed per strain per assay and the highest and lowest ratio values were discarded and statistical analysis was performed on the remaining three values to determine error bars.

2.2 Isolation and characterization of novel organic solvent tolerant bacteria

2.2.1 Soil samples and growth conditions

2.2.1.1 Soil samples

Soil samples were obtained from various sites planted to marigolds as normal soil environments and oil contaminated locations as more extreme environments.

2.2.1.2 Media and Growth conditions

LB medium (Section 2.1.1.3) was routinely used as complete medium for growth. Solid medium contained 1.5% (w/v) agar. Where indicated, *Pseudomonas* Isolation Agar (PIA) (Difco) was used to select for pseudomonads. When necessary, cultures were supplemented with organic solvents at the indicated concentrations and the flasks were sealed with foil-covered stoppers. The organic solvents used in this study, together with their purity and source are presented in Table 2.2.1. Where indicated, cultures were grown in Nephelo flasks (Alcan-Wheaton Science Products, NJ). All liquid cultures were routinely grown while shaking at 240 rpm and all cultures were incubated at 30°C.

2.2.1.3 Enrichment and isolation of solvent tolerant bacteria

Each soil sample (1 g) was inoculated in 25 ml LB medium supplemented with 5 mM toluene and was allowed to grow overnight while shaking (240 rpm) at 30°C. A 1 ml sample of the culture was subsequently transferred to fresh LB medium supplemented with an additional 5 mM toluene. Enrichment was continued for several days with repeated subcultures in increasing concentrations of toluene. Cultures that grew in

Table 2.2.1 Organic solvents used in this study

Solvent	Purity (%)	Supplier
Hexane	95+	Sigma
Cyclohexane	99.9	Sigma
p-Xylene	99+	Sigma
Toluene	99.8	Sigma
1-Heptanol	98	Acros organics (Geel, Belgium)
Dimethylphthalate	99+	Sigma
Fluorobenzene	>99.5	Fluka Chemical Corp. (Milwaukee, WI)
Benzene	99.9	Sigma
Phenol	99	Fisher Scientific (Nepean, ON)

20 mM toluene and higher were plated without toluene on PIA to allow preferential growth of pseudomonads, and on LB to allow non-selective growth of all types of bacteria. All isolated strains were stored as frozen stocks at -80°C in LB with 20% (v/v) glycerol as described in Section 2.1.1.4.

2.2.2 Solvent tolerance characteristics of the isolated strains

2.2.2.1 Growth in organic solvent two-phase systems

The isolated strains were tested to determine their survival in the presence of high concentrations of a range of organic solvents in an attempt to compare their level of solvent tolerance. A single colony of each culture was used to inoculate 2 ml of LB broth and the cultures were grown on a shaker at 240 rpm at 30°C for 8 hours. Each culture was diluted 50-fold in 25 ml of fresh LB supplemented with a given volume of organic solvent (See Results). The cultures were sealed with foil-covered stoppers and were allowed to grow overnight at 30°C while shaking at 240 rpm and checked for growth.

2.2.2.2 Growth after solvent shock

The isolated strains were subjected to solvent shock conditions to determine their survival without pre-adaptation. A single colony of each culture was used to inoculate 10 ml LB broth and the cultures were grown overnight at 30°C. The overnight cultures were diluted 1:50 in fresh LB in Nephelo flasks and were grown at 30°C while shaking (240 rpm) until they reached an OD_{600} of approximately 0.5. Subsequently, a set volume of toluene was added to each culture and the flasks were sealed with foil-covered stoppers. Growth following toluene addition was monitored by measuring OD_{600} readings using a

Spectronic 20D+ spectrophotometer without opening and re-sealing the flasks. While OD_{600} readings above the valid measurement range (0.2 - 0.5) on the Spectronic 20D+ spectrophotometer will not give true OD_{600} readings, it allowed to obtain relative OD_{600} measurements.

2.2.3 Morphological characterization

2.2.3.1 Gram staining and cell morphology

The isolated strains were Gram stained and examined via light microscopy using a Zeiss KF-2 light microscope.

2.2.3.2 Examination of the ultrastructure

The ultrastructure of a solvent tolerant isolate was examined after growth in the presence and absence of toluene. A single colony of each culture was used to inoculate 2 ml of LB broth and the culture was grown on a shaker at 240 rpm at 30°C for 8 hours. The culture was diluted 1:50 in fresh LB with and without toluene added to a final concentration of 100 mM and the cultures were allowed to grow overnight at 30°C while shaking at 240 rpm. Initially a drop of each culture was applied to copper grids, the samples were allowed to adsorb for 10 seconds, blotted dry and were subjected to negative staining by applying a drop of 0.2% phosphotungstic acid. The stain was left on the samples for a few seconds before blotting dry and the cells were visualized using a Philips / FEI (Morgagni) Transmission Electron Microscope with a CCD camera (Microscopy Unit, University of Alberta). Capsular ultrastructure was observed following polycationic ferritin labeling and ruthenium red staining according to the procedure

described in Vanrobaeys *et al.* (1999) with slight modifications. A sample of each culture (1 ml) was harvested by centrifugation at 4200 rpm for 4 minutes using a microcentrifuge. The supernatant was discarded and the cells were washed with 1 ml 0.01 M PBS (137 mM NaCl, 7 mM K_2HPO_4, 2.5 mM KH_2PO_4; pH 7.3) and centrifuged as before. The washed cells were then fixed for 2 hours at room temperature in 1 ml 0.1 M cacodylate buffer (pH 7.0) supplemented with 5% (v/v) glutaraldehyde and 0.15% (w/v) ruthenium red (Sigma). The cell suspensions were then centrifuged, the supernatant was discarded and the fixed cells were treated with 150 µl 0.1 M cacodylate buffer (pH 7.0) containing 1 mg/ml polycationic ferritin (Sigma) for 30 minutes at room temperature to allow capsular stabilization. Following the stabilization reaction, the cells were diluted 1:10 in 0.1 M cacodylate buffer (pH 7.0) containing 0.05% ruthenium red (Sigma) and subsequently washed three times with 1 ml of the same buffer and provided to the Microscopy Unit, University of Alberta, where the cells were postfixed for 2 hours at room temperature in 1% (w/v) osmium tetroxide and 0.05% (w/v) ruthenium red and embedded in Spurr resin prior to processing for thin sectioning electron microscopy. The samples were visualized as above using a Philips / FEI (Morgagni) Transmission Electron Microscope with a CCD camera (Microscopy Unit, University of Alberta).

2.2.4 Taxonomic classification

2.2.4.1 Isolation of chromosomal DNA from the isolated strains

Chromosomal DNA was isolated from the solvent tolerant bacterial strains as described previously in Section 2.1.2.4.

2.2.4.2 PCR amplification of the 16S rRNA genes

The 16S rRNA genes of all isolated strains were amplified using PCR. PCR was performed using the high fidelity Platinum *Pfx* DNA polymerase (Invitrogen) as described previously in Section 2.1.3.2. Standardized primers designed for 16S rRNA gene sequence amplification (Johnson, 1990) were employed in all PCR reactions.

2.2.4.3 16S rRNA gene sequencing

All 16S rRNA gene fragments PCR amplified were purified (Section 2.1.3.3) and sequenced by automated DNA sequencing as described previously in Section 2.1.3.6. Sequencing of fragments between 1000 and 1500 bp in both directions was attempted for each isolate. The sequences were edited and assembled using Editview and Autoassembler programs respectively (Applied Biosystems). All sequences were compared to the sequence data in the GenBank database using BLASTN (Altschul *et al.*, 1990).

2.2.4.4 Carbon source utilization profile

The range of carbon sources utilized by the isolated strains was determined using Biolog Microplates (Biolog Inc., Hayward, CA) according to the manufacturer's recommendations. Fresh cultures of all strains were obtained by repeated subcultures on recommended media and growth conditions. All Gram negative bacteria and Gram positive non-spore-forming bacteria were grown at 30°C on Biolog Universal Growth (BUG) Agar supplemented with 5% sheep's blood. All Gram positive spore-forming bacteria were grown at 30°C on BUG Agar containing 0.25% maltose with a thin film of

thioglycolate spread on the surface of the agar plates. Cell suspensions of all Gram negative bacteria and Gram positive spore forming bacteria were made in GN/GP-IF (0.4% NaCl, 0.03% Pluronic F-68, 0.02% Gellum gum). Cell suspensions of Gram positive non-spore forming bacteria were made in GN/GP-IF supplemented with thioglycolate to a final concentration of 5 mM to prevent capsule formation. The density of the inoculum was adjusted to the recommended range using a turbidimeter (Biolog Inc.). Cell suspensions were then added into the wells of the respective test plates at a volume of 150 µl per well. All Gram positive bacteria were inoculated into GP2 MicroPlates and all Gram negative bacteria were inoculated into GN2 MicroPlates (Biolog Inc.). All plates were incubated at 30°C. The carbon source utilization was determined by reading the level of tetrazolium dye reduction in the wells (purple colour change) using MicroLog3 4.20 software after 4 and 24 hours of incubation and the results were compared with the GP 6.0 and GN 6.0 databases (Biolog Inc.).

Chapter 3:

Results

3. Results

3.1 Characterization of SrpS and SrpR, the regulators of the SrpABC solvent efflux pump in *Pseudomonas putida* S12

Although the expression of many RND-type multidrug and solvent efflux systems is controlled by complex regulatory networks, most RND-type efflux systems are typically regulated by one local repressor, the gene for which is situated upstream and transcribed divergently from the efflux pump genes. However, unlike most RND-type efflux pump operons the genes encoding the SrpABC system in *P. putida* S12 have been observed to have two upstream divergently transcribed regulatory genes (Dennis and Zylstra, 1999; Wery *et al.*, 2001) (Figure 3.1.1). Interestingly, the SrpABC efflux system has been shown to be involved exclusively in organic solvent efflux (Isken and De Bont, 2000) and moreover, organic solvents have been shown to induce the expression of this system (Kieboom *et al.*, 1998b). The unorthodox characteristics of the SrpABC system have made it an attractive system for study and the aim of this project was to determine how the two regulators SrpS and SrpR are involved in the expression of the *srpABC* genes.

3.1.1 RNA transcription analysis

3.1.1.1 Northern hybridization analysis

Northern hybridization analyses were performed in order to determine the transcription level of the *srpS* and *srpR* genes in the presence and absence of toluene and also to determine the transcript sizes. Total RNA was isolated from the wild-type

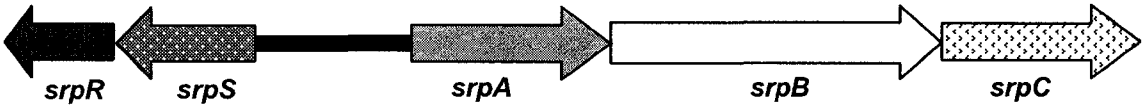


Figure 3.1.1. Schematic representation of the *srpABC-srpSR* cluster. The genes encoding the regulator proteins SrpS and SrpR are transcribed divergently from the efflux pump genes *srpABC*. The black bar represents the intergenic region between *srpS* and *srpA*.

P. putida S12 strain grown in M9 glucose minimal medium in the presence or absence of 6.0 mM toluene. Toluene at a concentration of 6.0 mM has been shown to induce the *srpABC* gene expression to a maximum (Kieboom *et al.*, 1998b). RNA samples (40 μg) were separated by electrophoresis after denaturation with glyoxal and DMSO and subsequently transferred to a nylon membrane. Hybridization was then performed overnight at 62°C with a probe specific to either *srpS* or *srpR*. Both probes were generated by PCR amplification and labeled with [α-^{32}P]-dCTP by a random primer labeling technique. The *srpS* specific probe was a 538 bp DNA fragment within the *srpS* coding sequence, amplified from the *P. putida* S12 chromosome using the primers SFN1 and SRN2. The *srpR* specific probe was a 404 bp DNA fragment within the *srpR* coding sequence, amplified from the *P. putida* S12 chromosome using the primers RFN1 and RRN2. It was observed that transcription of both *srpS* and *srpR* genes occurred constitutively but transcription of *srpS* was observed to increase about sixfold in the presence of toluene, while transcription of *srpR* was observed to increase only about twofold in the presence of toluene (Figure 3.1.2). The difference in the level of transcription of the two genes in the presence of toluene suggests that the two genes may not be transcribed as a single transcriptional unit. Determination of the transcript sizes to confirm a difference in size was problematic due to the high degree of smearing of the bands possibly caused by RNA degradation. However, it was estimated that the transcripts were about 1.4 kb, which is the expected size of the polycistronic mRNA, suggesting that the difference in trancription levels observed may be the result of post-transcriptional regulation.

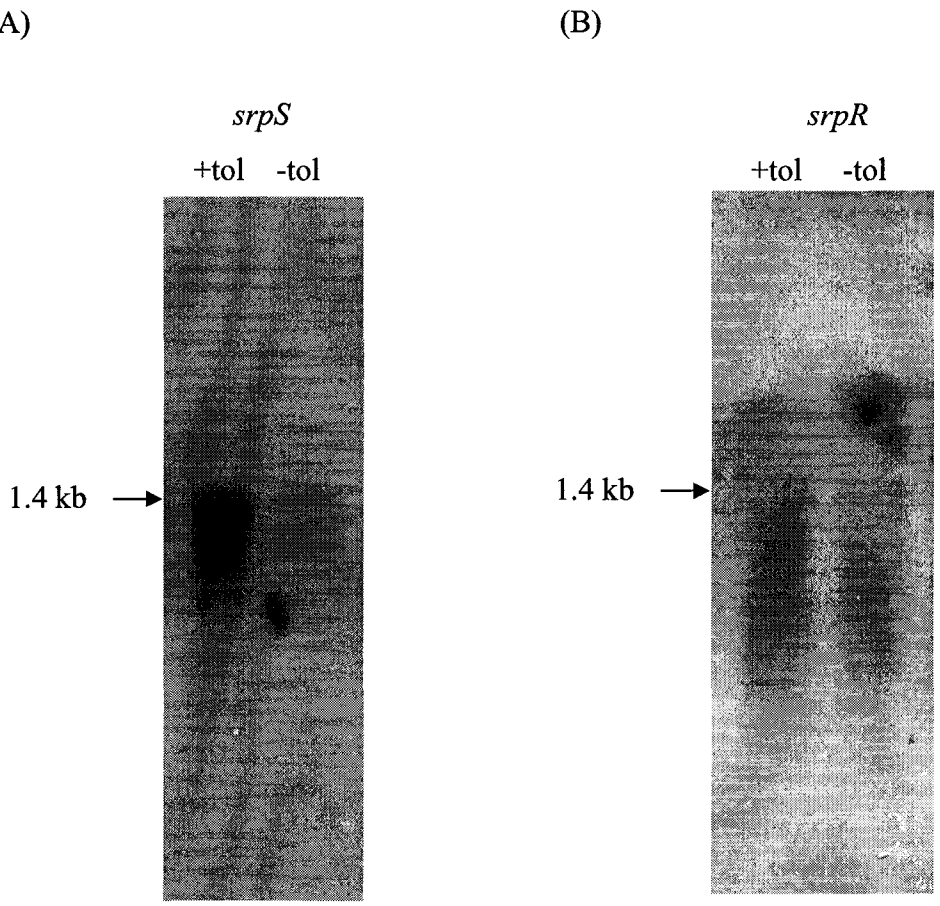


Figure 3.1.2. Transcription analysis of *srpS* and *srpR* by northern hybridization. Samples of total RNA (40 μg) isolated from *P. putida* S12 cells grown in M9 glucose minimal medium in the presence (+ tol) or absence (- tol) of 6.0 mM toluene were separated by electrophoresis after denaturation with glyoxal and DMSO and transferred to a nylon membrane. (A) The RNA samples hybridized with the 538 bp *srpS* specific DNA probe. (B) The RNA samples hybridized with the 404 bp *srpR* specific DNA probe. Arrows indicate the estimated transcript sizes.

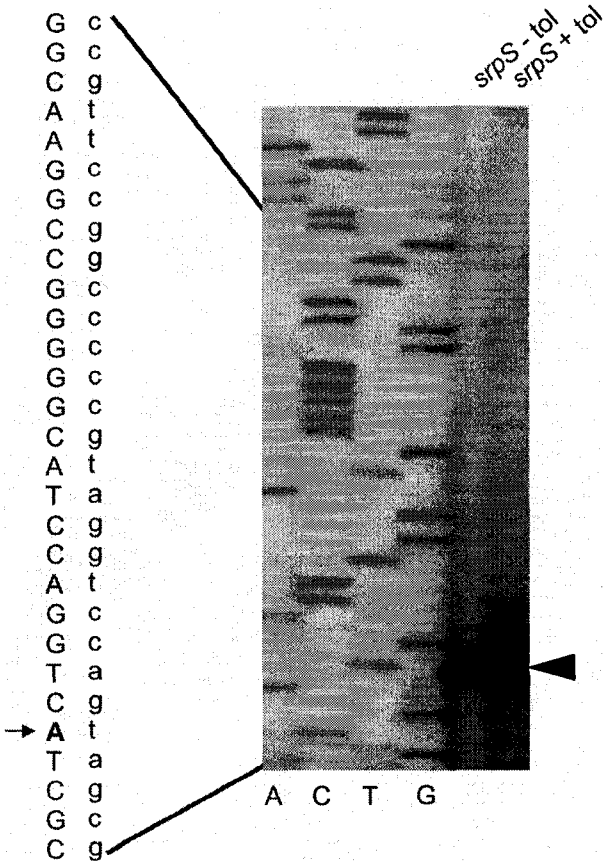
3.1.1.1 Primer extension analysis

It has been previously shown that organic solvents induce the expression of the *srpABC* genes (Kieboom *et al.*, 1998b). Since northern hybridization analysis revealed that the transcription of *srpS* also increased in the presence of toluene, it was of interest to determine if the transcription of *srpS* and the *srpABC* operon is coordinately regulated as has been observed in many RND-type efflux pump operons and their corresponding regulator genes (see Introduction). Thus, attempts were made to map the transcription start sites of *srpS* and *srpA* by performing primer extension analysis. Total RNA was isolated from the wild-type *P. putida* S12 strain grown in M9 glucose minimal medium in the presence or absence of 6.0 mM toluene. In order to determine the transcription start site of *srpS*, 10 μg samples of total RNA were hybridized to the oligonucleotide primer ST3 which was end-labeled with [γ-^{32}P]-dATP. Primer extension was achieved using Superscript II reverse transcriptase (Invitrogen) at 42°C for 50 minutes and subsequently, the enzyme was inactivated by incubation at 70°C for 15 minutes. The radioactive cDNA products synthesized were then separated on a 6% polyacrylamide sequencing gel alongside sequencing reactions generated using the primer ST3 with the plasmid p1B1 (Table 2.1.2) used as the template. It was observed that *srpS* is transcribed from a single promoter regardless of whether the cells were grown in the presence or absence of toluene (Figure 3.1.3A). Consistent with the results of the northern hybridization analysis, transcription of *srpS* was observed to increase in the presence of toluene as revealed by the increased intensity of the cDNA band (Figure 3.1.3A).

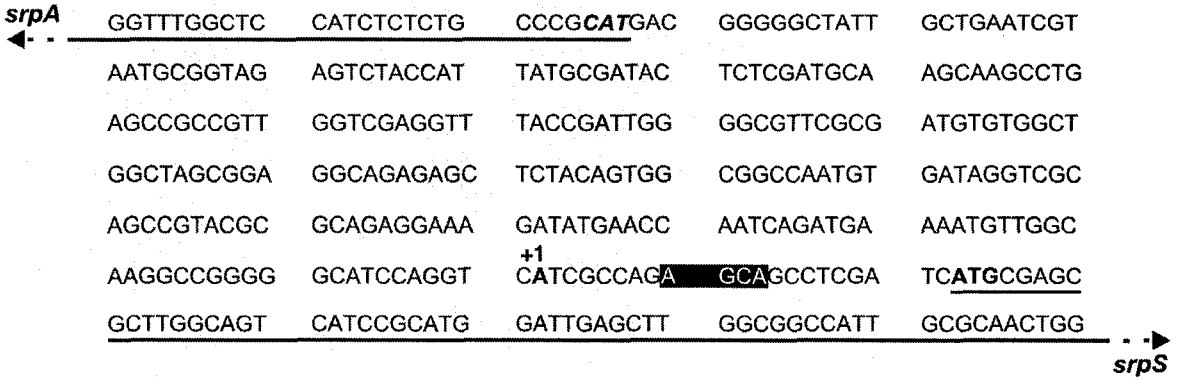
Attempts were made to determine the transcription start site of *srpA*, but no bands could be detected after trying a variety of end-labeled oligonucleotide primers

Figure 3.1.3. Transcript analysis. (A) Primer extension analysis to determine the transcription initiation site of *srpS*. Total RNA was isolated from *P. putida* S12 cells grown in M9 glucose minimal medium in the presence (*srpS* + tol) or absence (*srpS*- tol) of 6.0 mM toluene. RNA samples (10μg) were hybridized to the oligonucleotide primer ST3, which was end-labeled with [γ-^{32}P]-dATP and primer extension was carried out by using Superscript II reverse transcriptase (Invitrogen). The products were analyzed on a 6% denaturing polyacrylamide gel run alongside sequencing reactions generated using the primer ST3 (Lanes A, C, T, G). Arrow-head indicates the cDNA bands corresponding to the transcription initiation site. The transcription initiation site of *srpS* is indicated in boldface and marked with an arrow. (B) Mapping of the nucleotide sequence of the *srpA* and *srpS* promoter region. The transcription initiation site of *srpS* is indicated in boldface and marked +1. The putative Shine-Dalgarno sequence of *srpS* is in white letters in a black box and the *srpS* start codon is in boldface. The putative start codon of *srpA* is in boldface italics. The straight lines with the dotted arrows indicate the directions of transcription.

(A)



(B)



complementary to different regions downstream of the putative start codon of *srpA* (shown in boldface italics in Figure 3.1.3B). It is possible that the *srpABC* transcript forms complex secondary structures that inhibit primer binding and cDNA synthesis, or alternatively the transcript could be highly unstable and processed immediately. Unfortunately, it could not be determined if the promoters of *srpS* and *srpA* overlapped.

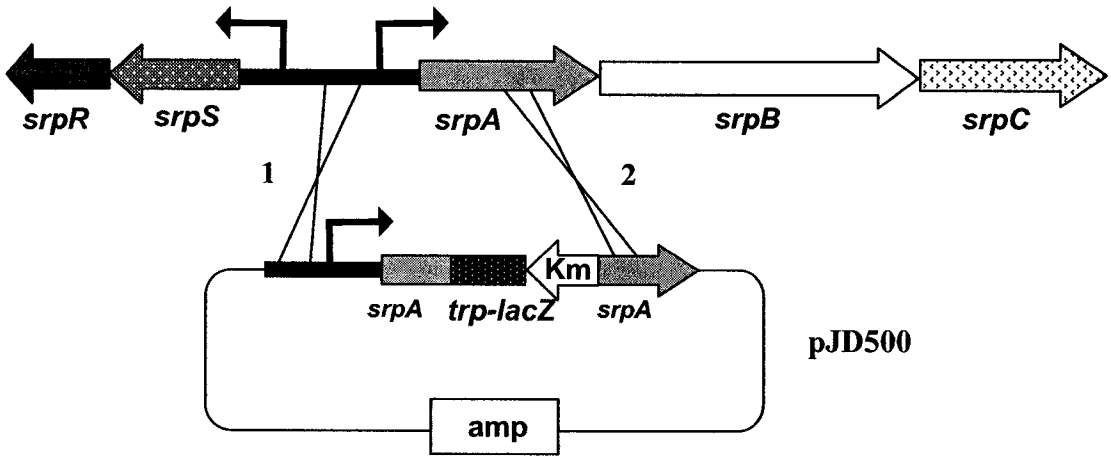
3.1.2 Effects of regulator deletion on the *srpA* promoter activity

3.1.2.1 Creation of *srpA-lacZ* reporter gene fusion strains

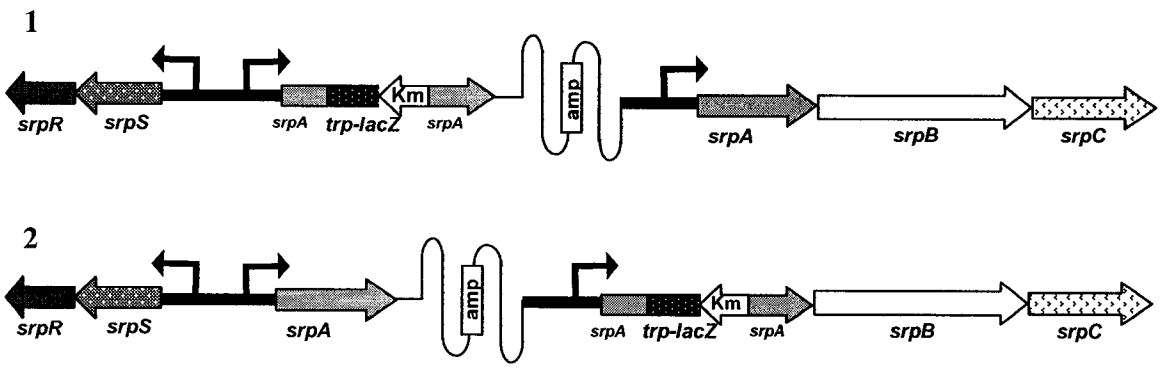
The plasmid pJD500 (Table 2.1.2) contains a promoterless *trp-lacZ* gene fused into the *srpA* gene downstream of the *srpA* promoter. This transcriptional fusion construct enables the *srpA* promoter to run into the *trp-lacZ* gene, which allows the *lacZ* gene to use the ribosome binding site and the start codon from *trp*. In order to create the *srpA-lacZ* reporter gene fusion strains S12/*lacZ*, SrpS$^-$/*lacZ* and SrpR$^-$/*lacZ*, the plasmid pJD500 was electroporated into the wild-type *P. putida* S12, SrpS$^-$ and the SrpR$^-$ strains respectively. Following electroporation, the cells were spread plated on LB plates containing kanamycin to select for kanamycin resistant colonies resulting from the chromosomal incorporation of the *srpA-lacZ* fusions by single or double cross-over events (Figure 3.1.4). It should be noted that the plasmid does not have a suitable origin of replication for propagation in *P. putida* S12 strains and also *P. putida* S12 strains are resistant to ampicillin and thus, the ampicillin resistant marker in the parent plasmid cannot be used to select for plasmid integration. Plasmid integration at only one position (position 1(A), Figure 3.1.4), leaves the strains with functional *srpABC* efflux pump genes to allow survival in the presence of organic solvents. Thus, several

Figure 3.1.4. Schematic illustration of the incorporation of the *srpA-lacZ* fusion construct into chromosome of the wild-type *P. putida* S12 strain. The plasmid pJD500 containing the *srpA-lacZ* fusion construct was electroporated into *P. putida* S12 cells. Following electroporation the plasmid could undergo a single or double homologous recombination events. (A) A single homologous recombination event at position 1 would incorporate the *srpA-lacZ* fusion construct in the chromosome, leaving the *srpABC* efflux pump operon functional. However, a single homologous recombination event at position 2 would result in the incorporation of the *srpA-lacZ* fusion construct in the chromosome with the accompanied disruption of the *srpABC* operon. (B) Occurrence of double cross-over events would result in the replacement of the functional *srpA* gene in the chromosome with the *srpA-lacZ* fusion construct. The angled black arrows represent the promoters of *srpS* and *srpA* indicating the direction of transcription. All three possible events would leave the recombinant strains resistant to kanamycin, thus in order to select for the occurrence of a single homologous recombination event at position 1, kanamycin resistant colonies were further screened for survival in the presence of toluene as a result of a functioning SrpABC efflux system.

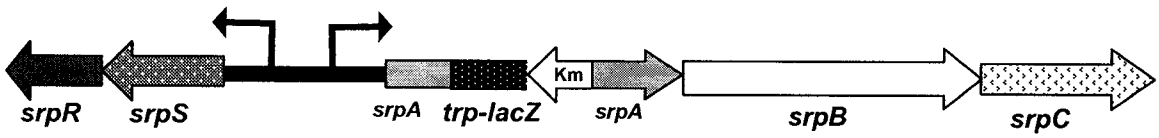
P. putida S12 chromosome



(A) Single homologous recombination



(B) Double homologous recombination



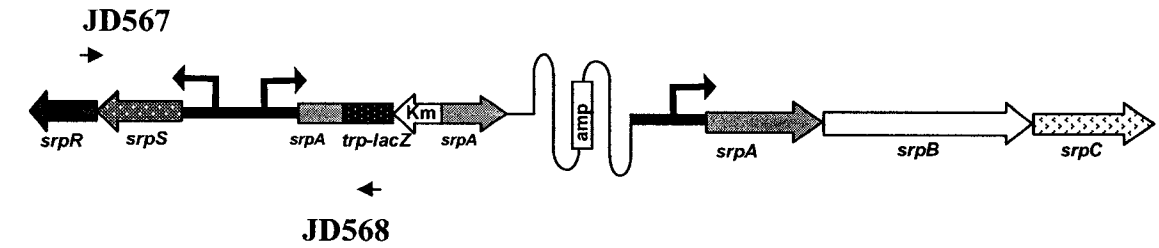
kanamycin resistant colonies obtained were screened for the presence of functional *srpABC* efflux pump genes by testing for survival in LB supplemented with 6.0 mM toluene. Subsequently, one kanamycin resistant, toluene tolerant colony from each strain was further screened to confirm the integration of the plasmid at the correct position. Chromosomal DNA was isolated and a region encompassing the *srpR* and *lacZ* genes was PCR amplified using the primers JD567 and JD568. Figure 3.1.5A shows a schematic view of the *srpA-lacZ* fusions integrated in the preferred position in the chromosome of the S12/*lacZ*, SrpS$^-$/*lacZ* and SrpR$^-$/*lacZ* strains, together with the locations of the primer annealing sites. The amplified DNA products were separated on a 0.7% TAE gel (Figure 3.1.5B). All three putative strains amplified DNA fragments of the desired sizes, confirming the creation of the correct *srpA-lacZ* reporter gene fusion strains. The strains S12/*lacZ* and SrpR$^-$/*lacZ* amplified a 2.1 kb fragment consistent with the size of the DNA fragment within the *srpR* and *lacZ* genes. The strain SrpS$^-$/*lacZ* amplified a 3.1 kb fragment due to the inserted 1 kb Gm^R cassette in the *srpS* gene in this strain. Even with the presence of a Sm^R cassette inserted in the *srpR* gene in the SrpR$^-$ /*lacZ* strain, the size of the amplified product remains the same as that of the S12/*lacZ* strain since the primer JD567 anneals upstream of the Sm^R cassette (Figure 3.1.5A).

3.1.2.2 β-galactosidase assays

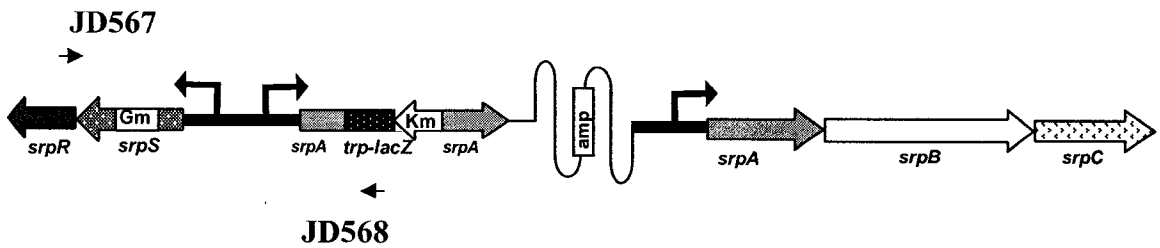
In an effort to elucidate the involvement of SrpS and SrpR in regulating the transcription of the *srpABC* efflux pump operon, the effect of *srpS* deletion or *srpR* deletion mutations on the *srpA* promoter activity was studied using the *srpA-lacZ* reporter gene fusion strains S12/*lacZ*, SrpS$^-$/*lacZ* and SrpR$^-$/*lacZ* (Section 3.1.2.1). The presence of the

Figure 3.1.5. Confirmation of plasmid integration in the desired position in the chromosome of S12/*lacZ*, SrpS$^-$/*lacZ* and SrpR$^-$/*lacZ*. (A) Schematic representation of the *srpA-lacZ* fusion constructs incorporated in the correct position in the chromosome of the (i) S12/*lacZ*, (ii) SrpS$^-$/*lacZ* and (iii) SrpR$^-$/*lacZ* strains. The strains contain transcriptional fusions of *srpA-lacZ* genes as well as functional *srpABC* efflux pump genes. The angled black arrows represent the promoters of *srpS* and *srpA* indicating the direction of transcription. To confirm the correct position of plasmid integration, PCR was performed using the primers JD567 and JD568. Primer positions are indicated with small black arrows. (B) Agarose gel electrophoresis. The amplified DNA fragments were electrophoresed on a 0.7% agarose 1x TAE gel, stained with ethidium bromide and visualized by using a UV transilluminator. The strains S12/*lacZ* and SrpR$^-$/*lacZ* amplified a 2.1 kb DNA fragment. The strain SrpS$^-$/*lacZ* amplified a 3.1 kb DNA fragment.

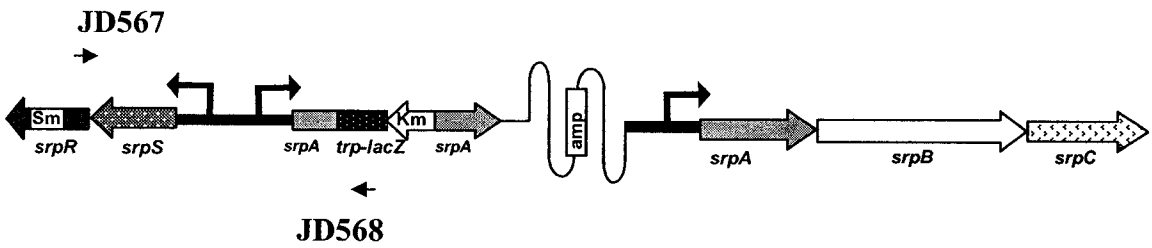
(A)(i)



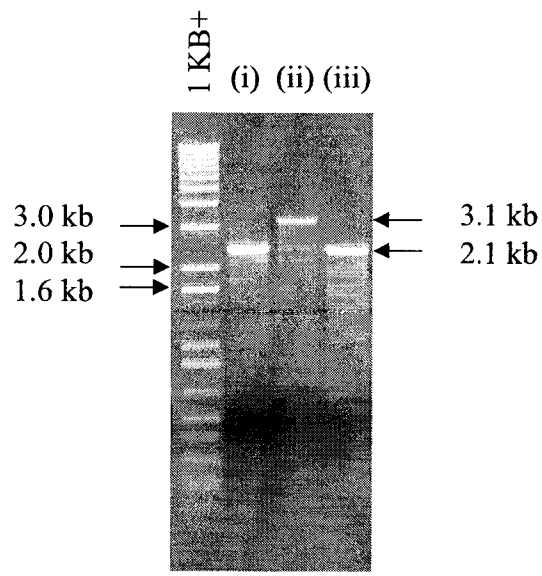
(ii)



(iii)



(B)



srpA-lacZ transcriptional fusion construct in the chromosome of these strains results in the expression of the β-galactosidase enzyme from the *lacZ* gene whenever the *srpA* gene is transcribed. Thus, β-galactosidase assays were performed according to the method of Slauch and Silhavy (1991) in order to determine the level of transcription from the *srpA* promoter, as measured by the formation of *o*-nitrophenol (ONP) as a result of the hydrolysis of *o*-nitrophenyl β-D-galactopyranoside (ONPG) by the expressed β-galactosidase enzyme. Assays were performed in the presence or absence of 6.0 mM toluene, and Figure 3.1.6 shows a representation of at least three independent assays. In the wild-type background, expression from the *srpA* promoter was observed even in the absence of toluene. However, the expression was increased to about sixfold in the presence of toluene. Expression from the *srpA* promoter was observed to increase about 5.5-fold in the *srpS* deletion background when compared to the wild-type background in the absence of toluene. This observation suggests that SrpS represses the expression of the *srpABC* genes, such that in the absence of SrpS the promoter is derepressed to allow almost full expression. In the presence of toluene, expression from the *srpA* promoter in the *srpS* deletion background was comparable to that in the wild-type background, suggesting that toluene may contribute to directly inhibit the repressor behavior of SrpS in the wild-type cells. Deletion of the *srpR* gene was observed to cause the expression from the *srpA* promoter to decrease about fivefold in the absence of toluene and about threefold in the presence of toluene when compared to the wild-type background. These results suggest that SrpR may be involved in the activation of the SrpABC efflux pump gene expression both in the presence and absence of toluene. In addition, the observation that toluene causes an increase in the expression of the *srpABC* operon in the

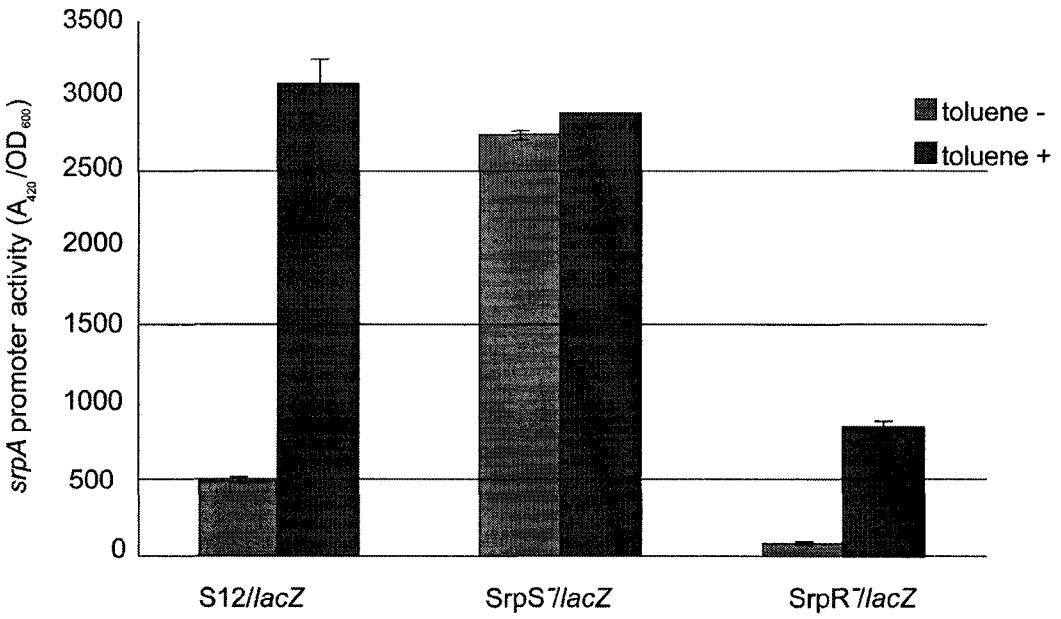


Figure 3.1.6. Transcription from the *srpA* promoter in the presence and absence of toluene in *srpS* deletion or *srpR* deletion backgrounds when compared to the wild-type background. β-galactosidase assays were performed on the *srpA-lacZ* reporter gene fusion strains SrpS$^-$/*lacZ*, SrpR$^-$/*lacZ* and S12/*lacZ*, in order to determine the level of expression from the *srpA* promoter in the presence and absence of toluene in *srpS* deletion and *srpR* deletion backgrounds when compared to the wild-type background, respectively. The y-axis shows the *srpA* promoter activity as determined by the A_{420}/ OD_{600} ratios. The light gray bars represent the expression in the absence of toluene and the dark gray bars represent the expression in the presence of 6.0 mM toluene.

srpR deletion background, but does not have a significant effect in the *srpS* deletion background, further suggests that toluene interacts directly with SrpS.

In order to verify the results of the above β-galactosidase assays, numerous attempts were made to complement the *srpS* deletion and the *srpR* deletion phenotypes of the SrpS$^-$/*lacZ* and SrpR$^-$/*lacZ* strains, respectively. Several sets of recombinant plasmids were constructed and used in attempted complementation experiments. Initially, *srpS*, *srpR*, or both *srpSR* genes were PCR amplified from the wild-type *P. putida* S12 genome and cloned downstream of the *lacZ* promoter in the high copy number plasmid pUCP26. Following restriction digestion and confirmation of the correct constructs, the recombinant plasmids were electroporated into the SrpS$^-$/*lacZ* and SrpR$^-$/*lacZ* strains. β-galactosidase assays were then performed using the same method as described above. Unfortunately, complementation was not observed and it was presumed that the high copy number plasmid may be causing regulator titration effects. A second attempt at gene complementation was performed using the low copy number broad host-range promoterless vector pKRZ-1. PCR was performed to amplify the *srpS* gene or both *srpSR* genes from the wild-type *P. putida* S12 genome including the region of about 100 bp upstream of the transcription start site identified in Figure 3.1.3. This was to ensure that the promoter region for *srpS* was included in order to allow for the expression of the regulator genes when cloned into the promoterless plasmid. The plasmid constructs were electroporated into the SrpS$^-$/*lacZ* and SrpR$^-$/*lacZ* strains and β-galactosidase assays were performed. Unfortunately, the constructs failed to complement the gene deletion phenotypes. To ensure that the whole promoter region was included, and that no mutations were introduced, a 2.3 kb DNA fragment encompassing the region between

about 70 bp downstream of *srpR* and about 680 bp into the *srpA* gene was digested from a genomic DNA fragment and cloned directly into pKRZ-1. However, these constructs when electroporated into SrpS$^-$/*lacZ* and SrpR$^-$/*lacZ* strains again showed no complementation. As a final attempt, a 4.4 kb genomic DNA fragment encompassing the region between 420 bp downstream of *srpR*, through the partial open reading frame of *srpB* was cloned downstream of the *lacZ* promoter in the medium copy number plasmid pBBR-MCS1. Nonetheless, β-galactosidase assays performed on the SrpS$^-$/*lacZ* and SrpR$^-$/*lacZ* strains following electroporation of these constructs showed no complementation of the gene deletion phenotypes.

3.1.3 MBP- SrpS and MBP-SrpR overexpression and *in vitro* protein-DNA binding studies

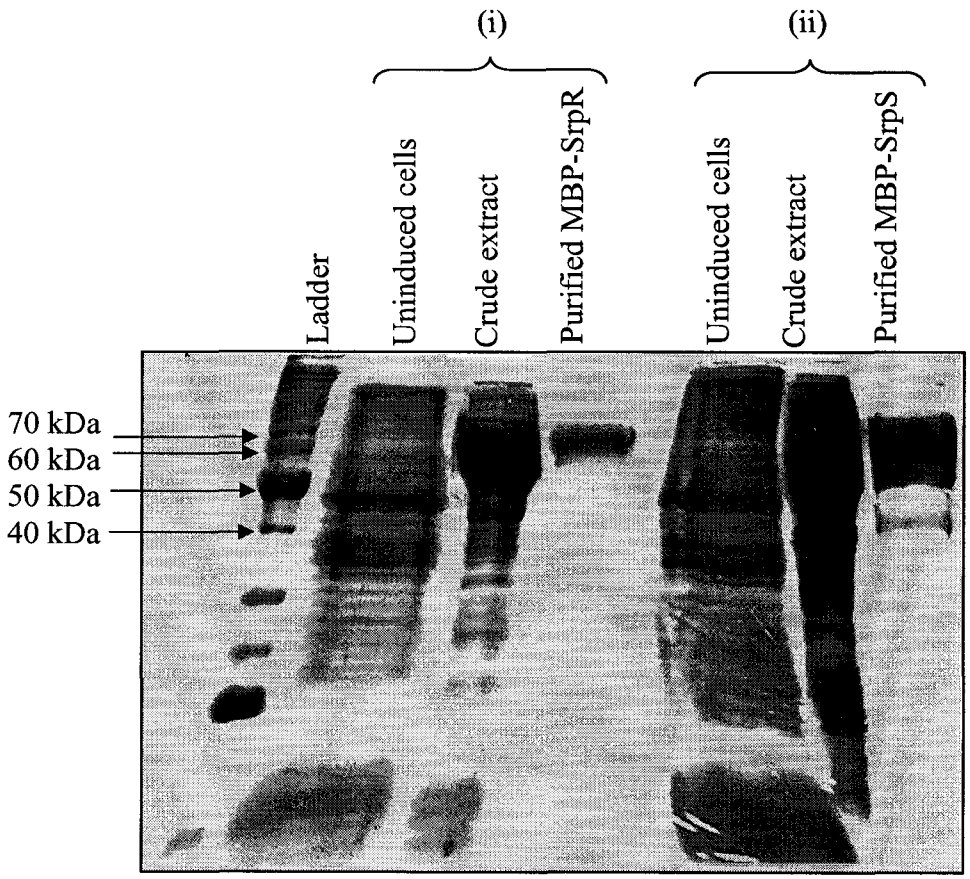
3.1.3.1 Protein overexpression and purification

The regulators SrpS and SrpR were overexpressed in *E. coli* DH5α as N-terminal MBP fusion proteins for use in *in vitro* protein-DNA binding studies. The fusion protein overexpression was achieved by cloning the gene of interest into the vector pMAL-c2X downstream of the *E. coli malE* gene that encodes the MBP. The *malE* gene is under the control of the strong IPTG inducible p*tac* promoter, which allows high levels of expression of the fusion protein. In order to construct the MBP-SrpS overexpression vector pMAL-c2X-srpS, an 831 bp region encompassing the entire coding sequence of *srpS* was PCR amplified from the *P. putida* S12 chromosome using the primers SF1 and SR2. The primers SF1 and SF2 contain engineered *Sal*I and *Hind*III sites respectively, and the amplified fragment was digested accordingly and cloned in-frame, downstream of

the *malE* gene in the plasmid pMAL-c2X. Likewise, in order to construct the vector pMAL-c2X-srpR for the overexpression of MBP-SrpR, the primers RF1 and RR2 containing engineered *Sal*I and *Hind*III sites respectively, were used to PCR amplify a 642 bp fragment encompassing the entire coding sequence of *srpR* from the *P. putida* S12 chromosome. Subsequently, the amplified fragment was digested and cloned in-frame, downstream of the *malE* gene in the plasmid pMAL-c2X. Following the cloning step, the inserts were sequenced from the recombinant plasmids by automated sequencing to ensure that the genes have been accurately cloned.

In order to overexpress the fusion proteins, *E. coli* DH5α cells transformed with the respective plasmids were grown at 37°C until an OD_{600} of 0.5-0.6 was reached and subsequently, the p*tac* promoter was induced with the addition of IPTG to a final concentration of 0.3 mM. Following induction, expression occurred at 37°C for 6 hours to obtain maximum soluble fusion proteins. Cells were harvested and frozen at -20°C overnight to promote rapid lysis. Subsequently, the cells were thawed, sonicated, and centrifuged to obtain the crude cell extract. The soluble fusion protein was purified from the crude extract by amylose resin affinity chromatography. The crude extract was introduced into the resin, followed by several washes with column buffer to remove any proteins non-specifically bound to the resin. The fusion protein bound to the amylose resin was eventually eluted using column buffer containing maltose, which competitively inhibits the binding of the MBP to the amylose resin. Eluted fractions were initially analyzed by measuring the A_{280} values, and the fractions containing the purified fusion protein were pooled and analyzed by electrophoresis on a 12.5% SDS-polyacrylamide gel (Figure 3.1.7). Both purified fusion proteins were observed to be of the expected

Figure 3.1.7. Overexpression, purification and analysis of MBP-SrpR and MBP-SrpS. The fusion proteins MBP-SrpR and MBP-SrpS were overexpressed in *E. coli* DH5α transformed with (i) pMAL-c2X-srpR or (ii) pMAL-c2X-srpS respectively, by inducing the P*tac* promoter with 0.3 mM IPTG. Prior to induction a 1 ml sample was withdrawn from each culture and centrifuged to obtain a cell pellet which was mixed with 50 µl 2x SDS loading dye (uninduced cells). Following induction and fusion protein overexpression, cells were lysed, sonicated and centrifuged to obtain the supernatant containing the crude soluble protein fraction. A 20 µl sample from the crude extract was mixed with 5 µl 2x SDS loading dye (crude extract). Subsequently, the soluble fusion protein was purified from the crude extract by amylose resin affinity chromatography. Fusion protein was eluted from the amylose resin in 3 ml fractions using column buffer containing 10 mM maltose, and initially analyzed by measuring the A_{280} values. Fractions containing the purified fusion protein were pooled and a 20 µl sample from each pooled sample was mixed with 5 µl 2x SDS loading dye (purified MBP-SrpS or purified MBP-SrpR). A volume of 25 µl from each prepared sample was separated on a 12.5% SDS-polyacrylamide gel alongside the BenchMark Protein Ladder (Invitrogen). The gel was stained with Coomassie Brilliant Blue protein stain.



sizes (MBP-SrpR is approximately 66 kDa and MBP-SrpS is approximately 69 kDa) and free of contaminating proteins. The faint extra protein band of about 42 kDa observed together with MBP-SrpS is about the size of the MBP and thus, was believed to be the result of spontaneous cleavage of MBP from the MBP-SrpS fusion.

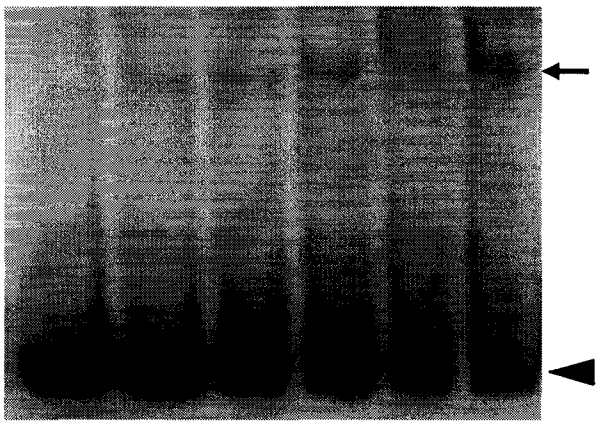
3.1.3.2. *In vitro* protein-DNA binding studies

Electrophoretic mobility shift assays (EMSAs) were performed to study the interactions of the SrpS and SrpR proteins within the *srpS - srpA* intergenic region. These assays are based on the rationale that protein-DNA complexes migrate slower than free DNA when analyzed by non-denaturing PAGE. A 490-bp DNA fragment within the *srpS – srpA* intergenic region was PCR amplified from the plasmid p1B1 using the primers SP1 and AP2. The amplified fragment was PCR purified and end-labeled with [γ- ^{32}P]-dATP. Increasing concentrations of the purified MBP-SrpS or MBP-SrpR were allowed to bind to the labeled DNA fragment in 1x binding buffer at 30°C for 30 minutes. The 20µl reaction mixtures were then electrophoresed on a 4% non-denaturing polyacrylamide 1x TAE gel. Incubation of increasing concentrations of MBP-SrpS with the labeled DNA was observed to result in a single shifted band (Figure 3.1.8A). The shifted band was observed even with 10 pmol of MBP-SrpS and the intensity of the shifted band increased with increasing concentrations of MBP-SrpS, suggesting that SrpS binds with high affinity to the target DNA within the *srpS – srpA* intergenic region. Competition assays were performed using the same conditions in a fixed concentration of MBP-SrpS to determine if the binding reaction was specific. When the same DNA fragment in the unlabeled form was added into the reaction mixtures as competitive

Figure 3.1.8. Electrophoretic mobility shift assays to determine 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 concentrations was allowed to bind to the labeled DNA fragment ($2x10^6$-$4x10^6$ cpm) in 1x binding buffer at 30°C for 30 minutes. (B) The 490-bp DNA fragment within the *srpS – srpA* intergenic region in the labeled and unlabeled form, with the ratio of unlabeled to labeled DNA ranging from 1:1 to 750:1 were mixed with 100 pmol samples of MBP-SrpS. The reaction mixtures were analyzed on a 4% non-denaturing polyacrylamide 1x TAE gel. The black arrows indicate the shifted band caused by the binding of SrpS to the target DNA. The arrow-heads indicate the unbound DNA.

(A)

MBP-SrpS (pmol) 0 10 50 80 120 150

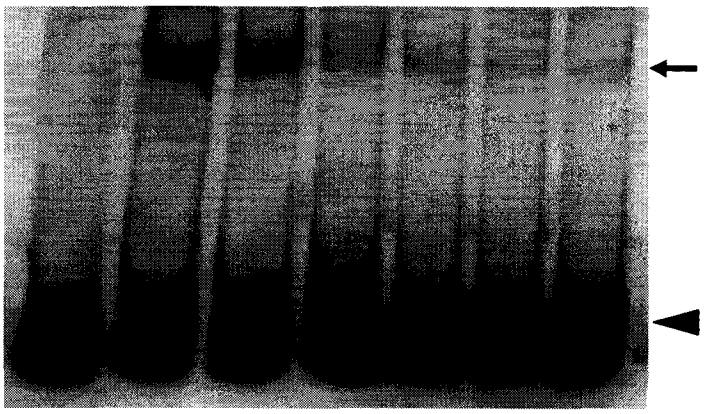


(B)

Ratio of Labeled to unlabeled target DNA

1:1 1:750

MBP-SrpS (pmol) 0 100 100 100 100 100 100



DNA, with the unlabeled to labeled DNA ratio ranging from 1:1 to 750:1, a gradual decrease in intensity of the shifted band was observed (Figure 3.1.8B). However, the intensity of the shifted band remained the same in a similar experiment where non-competitive DNA (poly [dI-dC]) was added into the reaction mixtures with the ratio of poly (dI-dC) to labeled DNA ranging from 1:1 to 750:1 (data not shown). These results together with the results of the β-galactosidase assays (Figure 3.1.6) suggest that SrpS functions as a specific repressor, which binds to an operator in the promoter region to inhibit the activity of RNA polymerase and repress the expression of the *srpABC* operon.

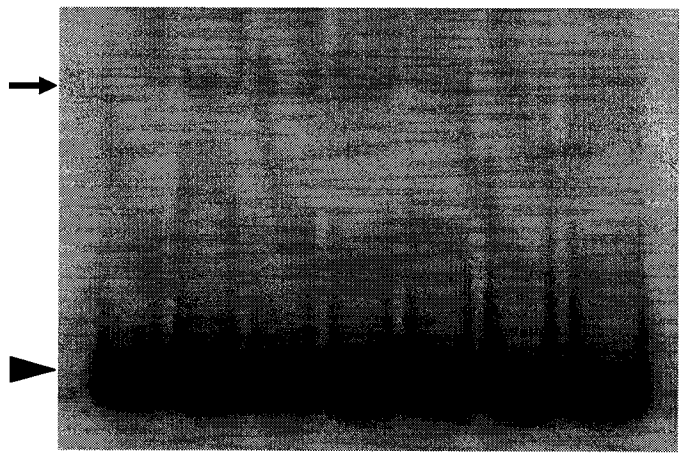
Several attempts were made to determine if SrpR binds to the same DNA fragment. EMSAs were initially performed using the same buffer and binding conditions as for SrpS, after which a number of variations of buffer compositions and different temperatures were tried. Additional attempts were also made by adding toluene into the reaction mixtures prior to or following the binding reactions, but none of the experiments showed a band shift with MBP-SrpR. Since the results of the β-galactosidase assays (Figure 3.1.6) suggest that SrpR may be involved in the activation of the *srpABC* efflux pump gene expression, it was originally assumed that SrpR binds to the promoter DNA to facilitate the transcription from the *srpA* promoter. But, because SrpR does not bind to the *srpS – srpA* intergenic region, it was hypothesized that SrpR binds to the repressor SrpS to somehow inhibit SrpS from binding to the promoter DNA and thus derepress the transcription from the *srpA* promoter. In order 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. All binding reactions were allowed to proceed at 30°C for 30 minutes in 1x binding buffer. Initially, assays were performed such that a fixed

concentration of MBP-SrpS was allowed to bind to the labeled target DNA, followed by the addition of increasing concentrations of MBP-SrpR, with the ratio of MBP-SrpS to MBP-SrpR ranging from 1:1 to 1:14, with or without toluene added to a final concentration of 1 mM. Subsequently, the samples were incubated at 30°C for 30 minutes to allow SrpR to react with SrpS and the reactions were electrophoresed on a 4% non-denaturing polyacrylamide 1x TAE gel. Another set of EMSAs was performed such that both MBP-SrpS and MBP-SrpR, with the ratio of MBP-SrpS to MBP-SrpR ranging from 1:1 to 1:14, were added simultaneously into the reaction mixtures with or without toluene added to a final concentration of 1 mM. The samples were incubated at 30°C for 30 minutes to allow SrpR time to react with SrpS, followed by the addition of the labeled target DNA to allow the binding reaction to occur. Interestingly, it was observed that regardless of whether MBP-SrpR was added to the reaction mixtures after SrpS was allowed to bind to the DNA or whether MBP-SrpR and MBP-SrpS were added simultaneously into the reaction mixtures, the intensity of the shifted band caused by SrpS gradually decreased as the concentration of MBP-SrpR increased (Figure 3.1.9A). Also, the intensity of the SrpS-mediated band shift was observed to decrease more dramatically as the concentration of MBP-SrpR increased in the presence of toluene (Figure 3.1.9B). These observations suggest that SrpR prevents the binding of SrpS to the *srpS – srpA* intergenic region and is capable of dissociating SrpS pre-bound to the *srpS – srpA* intergenic region. In addition, the results indicate that toluene enhances the inhibitory effects of SrpR on SrpS. Moreover, the decrease in intensity of the shifted band caused by SrpS in lane 2 compared to lane 3 in Figure 3.1.9B suggests that toluene also has a direct inhibitory effect on the binding of SrpS to the target DNA in the

Figure 3.1.9. Electrophoretic mobility shift assays to determine if SrpR, with or without toluene affected the binding of SrpS within the *srpS – srpA* intergenic region. The 490-bp DNA fragment within the *srpS – srpA* intergenic region was end-labeled with [γ- ^{32}P]-dATP. Samples of MBP-SrpS at a fixed concentration of 50 pmol were allowed to bind to the labeled DNA fragment ($2x10^6$-$4x10^6$ cpm) in 1x binding buffer at 30°C for 30 minutes (A) Following the binding reaction, purified MBP-SrpR was added into the reaction mixtures at the indicated concentrations and incubated at 30°C for 30 minutes. (B) Purified MBP-SrpR at the indicated concentrations and 1 mM toluene were added simultaneously into the reaction mixtures following the binding reaction, and incubated at 30°C for 30 minutes. The reaction mixtures were analyzed on a 4% non-denaturing polyacrylamide 1x TAE gel. The black arrows indicate the shifted band caused by the binding of SrpS to the target DNA. The arrow-heads indicate the unbound DNA.

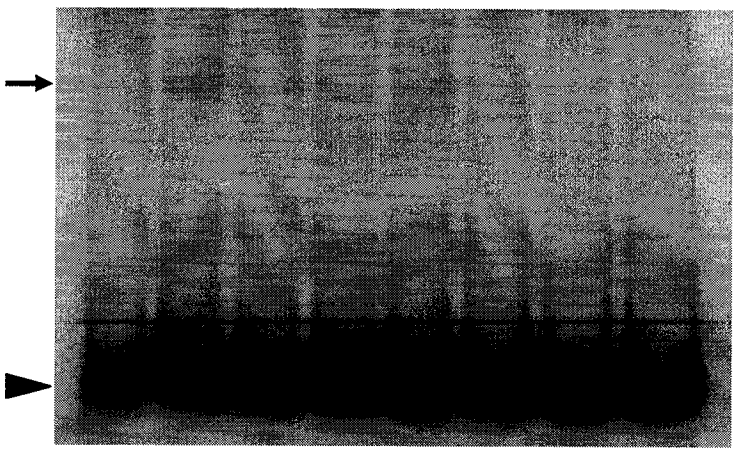
(A)

MBP-SrpS (pmol)	0	50	50	50	50	50	50
MBP-SrpR (pmol)	0	0	50	150	280	500	700



(B)

Toluene (1 mM)	-	-	+	+	+	+	+	+
MBP-SrpS (pmol)	0	50	50	50	50	50	50	50
MBP-SrpR (pmol)	0	0	0	50	150	280	500	700



absence of SrpR, which is consistent with the observations of the β-galactosidase assays (Figure 3.1.6). Additionally, the 1.5-fold increase in *srpA* promoter activity in the *srpR* deletion background in the presence of toluene (bar 1, Figure 3.1.6) relative to the wild-type background in the absence of toluene (bar 6, Figure 3.1.6) suggest that the inhibitory effects of toluene alone on SrpS is about 1.5-fold higher than that of SrpR alone.

3.2 Isolation and characterization of novel organic solvent tolerant bacteria

A number of organic solvent tolerant bacteria have been previously isolated from normal soil environments, while some have also been isolated from more extreme environments like oil contaminated sites and deep sea sediments. Organic solvent tolerant bacteria have high potential in environmental applications like soil remediation and wastewater treatment as well as in biocatalytic applications for the production of fine chemicals in two-phase systems. Thus, the aim of this project was to isolate and characterize new organic solvent tolerant bacteria from normal and extreme soil environments.

3.2.1 Enrichment and isolation of solvent tolerant bacteria

Soil samples were obtained from various sites planted to marigolds as normal soil environments. These soils have the potential presence of thiophenes (Christensen and Lam, 1990), which are sulfur containing heterocyclic aromatic organic compounds. It was anticipated that the exposure to thiophenes could result in enrichment of bacteria that have evolved mechanisms to tolerate organic solvents. Soil samples were also obtained

from oil contaminated locations as examples of more extreme environments. In order to enrich for solvent tolerant bacteria, the soil samples (1 g) were subcultured for several days in LB medium with increasing concentrations of toluene. Most microbes are killed at toluene concentrations as low as 10 mM, thus in an attempt to isolate toluene tolerant bacteria, cultures that grew in 20 mM toluene and higher were plated. The cultures were plated without toluene on PIA to select for pseudomonads, and plated without toluene on LB to allow for the non-selective growth of all types of bacteria. The strains that exhibited the highest toluene tolerance were selected for further screening. Initially, all eight isolates were Gram stained and examined via light microscopy to determine the Gram reaction and cell morphology (Table 3.2.1). The four strains isolated on LB were observed to be Gram positive and were designated ZZ1-ZZ4, while (as expected) the four strains isolated on PIA were observed to be Gram negative, presumably pseudomonads, and were designated ZZ5-ZZ8.

3.2.2 Taxonomic classification

3.2.2.1 16S rRNA gene sequence comparisons

In order to determine the taxonomic classifications of the isolated strains, chromosomal DNA was isolated from all strains and the 16S rRNA genes were PCR amplified using standardized primers designed for 16S rRNA gene amplification (Johnson, 1990). Subsequently, the amplified gene fragments were sequenced by automated sequencing and all sequences were compared to the sequence data in the GenBank database using BLASTN. Table 3.2.2 shows the isolated strains, the lengths of the 16S rRNA gene fragments sequenced, and the closest neighbours identified by

Table 3.2.1. Gram reaction and cell morphology of the isolated solvent tolerant bacteria*

Strain	Gram reaction	Cell morphology
ZZ1	Gram positive	Cocci arranged in irregular clusters
ZZ2	Gram positive	Bacilli arranged as single cells
ZZ3	Gram positive	Bacilli arranged in pairs
ZZ4	Gram positive	Bacilli arranged in regular clusters
ZZ5	Gram negative	Bacilli arranged in irregular clusters
ZZ6	Gram negative	Bacilli arranged in pairs or chains
ZZ7	Gram negative	Bacilli arranged in pairs
ZZ8	Gram negative	Bacilli arranged in pairs

* Visualized using a Zeiss KF-2 light microscope.

Table 3.2.2. Characteristics of the newly isolated solvent tolerant bacteria

Newly isolated strain	16S Comparison – Closest Neighbor (GenBank accession number)	Original Sample	Toluene Tolerance*
Staphylococcus sp. strain ZZ1	*Staphylococcus* sp. LMG-19417 (AJ276810) (1187/1187 bp identity)	Solvent contaminated media	100 mM (1% [v/v])
Bacillus cereus strain ZZ2	*Bacillus cereus* strain ATCC 10987 (AJ577290) (1166/1166 bp identity)	Marigold soil (U of A)	90 mM (0.96% [v/v])
Bacillus cereus strain ZZ3	*Bacillus cereus* strain 2000031513 (AY138279) (1414/1414 bp identity)	Marigold soil (Edmonton)	100 mM (1% [v/v])
Bacillus cereus strain ZZ4	*Bacillus cereus* strain LRN (AY138279) (1366/1366 bp identity)	Oil contaminated soil	100 mM (1% [v/v])
Pseudomonas sp. strain ZZ5	*Pseudomonas* sp. (biodegradation) (Y13246) (1482/1482 bp identity)	Marigold soil (Southern Alberta)	20 mM (0.21% [v/v])
Pseudomonas citronellolis strain ZZ6	*Pseudomonas citronellolis* (Z76659) (1156/1160 bp identity; 99%)	Marigold soil (Southern Alberta)	20 mM (0.21% [v/v])
Stenotrophomonas maltophilia strain ZZ7	*Stenotrophomonas maltophilia* (AB180661) (1487/1498 bp identity; 99%)	Marigold soil (U of A)	20 mM (0.21% [v/v])
Burkholderia cepacia complex strain ZZ8	*Burkholderia cepacia* complex strain ATCC 49709 (AY741349) (1435/1468 bp identity; 97%)	Marigold soil (Southern Alberta)	25 mM (0.27% [v/v])

*Toluene tolerance level obtained during enrichment

sequence comparison. The Gram positive strain ZZ1 isolated from solvent contaminated media was identified as a *Staphylococcus* species with 99% identity to a *S. warneri* strain (GenBank accession no. L37603) and 100% identity to a *Staphylococcus* species LMG-19417 (GenBank accession no. AJ276810). The strains ZZ2 and ZZ3 isolated from marigold soil, and strain ZZ4 isolated from oil contaminated soil were identified with 100% identity as *Bacillus cereus* strains. Interestingly, the Gram positive strains isolated displayed higher levels of toluene tolerance when compared to the Gram negative strains. The *Bacillus cereus* strain ZZ2 showed slightly lower levels of toluene tolerance (survival in up to 90 mM) when compared to the rest of the Gram positive strains, which were all observed to survive in 100 mM toluene (quantity of toluene is expressed both in mM and % (v/v) in Table 3.2.2). The Gram negative strain ZZ5 was identified as a *Pseudomonas* species based on its high sequence homology (99% identity) to a *Pseudomonas* species (GenBank accession no. Y13246) involved in solvent biodegradation. Strain ZZ6 was identified with 99% sequence homology as a *Pseudomonas citronellolis* strain, an organism frequently isolated from oil contaminated soils and involved in solvent biodegradation (Bhattacharya *et al.*, 2003). Strain ZZ7 was identified with 99% sequence identity as a *Stenotrophomonas maltophilia* strain, a nosocomial pathogen with high levels of multiple antibiotic resistance mediated by RND-type efflux systems (Alonso and Martinez, 2000; Li *et al.*, 2002). Finally, strain ZZ8 was identified with 97% sequence identity as a *Burkholderia cepacia* complex strain, an important opportunistic pathogen, resistant to multiple antibiotics partially due to the expression of RND-type efflux systems like CeoAB-OpcM (Burns *et al.*, 1996). The *B. cepacia* complex strain ZZ8 was observed to survive a slightly higher concentration of

toluene (25 mM) when compared to the rest of the isolated Gram negative strains (20 mM) (Table 3.2.2).

3.2.2.2 Carbon source utilization profile

In order to determine the range of carbon sources utilized by the isolated strains, all strains were inoculated into appropriate Biolog Microplates (GP2 MicroPlates for Gram positives and GN2 MicroPlates for Gram negatives) and analyzed after 4 and 24 hours of incubation at 30°C to determine the level of tetrazolium dye reduction in the wells (purple colour change) using the MicroLog3 4.20 software. The strains were identified by comparing the reaction profiles to the GP 6.0 database for the Gram positive strains and GN 6.0 database for the Gram negative stains. Results were consistent following both 4 and 24 hours of incubation. According to the carbon source utilization profile, strain ZZ1 was identified with 100% probability as a *S. warneri* strain, consistent with the observation of 99% 16S rRNA gene sequence identity to a *S. warneri* strain (GenBank accession no. L37603). Strain ZZ2 was identified as a *Bacillus* species, with a similarity index of 0.32 to *Bacillus subtilis*, indicating that the strain is a *B. cereus* strain (based on 16S rRNA gene sequence comparison) with metabolic capabilities similar to *B. subtilis*. In agreement with the results of the 16S rRNA gene sequence comparison, the strains ZZ3 and ZZ4 were identified with 100% probability as *B. cereus* or *Bacillus thuringiensis* strains, which are indistinguishable based on their metabolic capabilities. Confirming the results of the 16S rRNA gene sequence comparison, strain ZZ5 was identified as a *Pseudomonas* strain with a similarity index of 0.43 to *P. citronellolis*. Strain ZZ6 was identified with 100% probability as a *P. citronellolis* strain and strain

ZZ7 was identified with 100% probability as a *S. maltophilia* strain. Finally, strain ZZ8 was confirmed to be a member of the *B. cepacia* complex with 100% probability as a *Burkholderia multivorans* strain.

3.2.3 Solvent tolerance characteristics of the isolated strains

3.2.3.1 Growth in organic solvent two-phase systems

Since the toluene tolerance levels of the isolated Gram positive strains were observed to be much higher than those of the isolated Gram negative strains (Table 3.2.2), it was hypothesized that the isolated Gram positive strains are more tolerant to toxic organic solvents in general. In an attempt to test this hypothesis, experiments were performed to determine the survival of the isolates in the presence of high concentrations of a range of organic solvents. Each strain was tested by growing overnight (16 hours) in LB medium supplemented with one of several organic solvents. The quantity of solvents used, expressed both in mM and % (v/v) is included in Table 3.2.3 and Table 3.2.4. The isolated Gram positive strains exhibited tolerance to high concentrations of toxic organic solvents with log P_{OW} values ranging from 1.5 to 3.5. Of all the isolated strains, *Staphylococcus* sp. strain ZZ1 was revealed to be the most tolerant to organic solvents, as growth overnight was observed in the presence of all the solvents tested except 1-heptanol. The solvent tolerance profile of the *B. cereus* strain ZZ3 was similar to that of strain ZZ1 except that it exhibited a lower level of tolerance to 100 mM *p*-xylene. The *B. cereus* strain ZZ4 grew similarly, with survival observed in the presence of all solvents with the exception of 100 mM *p*-xylene and 100 mM 1-heptanol. The *B. cereus* strain ZZ2 was observed to be the least tolerant to organic solvents of all the Gram positive

Table 3.2.3 Growth of the Gram positive strains in organic solvent two-phase systems

Solvent (concentration)	log P_{OW}	*Staphylococcus* sp. strain ZZ1	*B. cereus* strain ZZ2	*B. cereus* strain ZZ3	*B. cereus* strain ZZ4
Hexane (100 mM - 1.3% [v/v])	3.5	+++	+++	+++	+++
Cyclohexane (100 mM - 1%[v/v])	3.2	+++	+++	+++	+++
p-Xylene (100 mM - 1.2% [v/v])	3.0	+++	-	+/-	-
Toluene (100 mM - 1%[v/v])	2.5	+++	+/-	+++	+++
1-Heptanol (100 mM - 1.4% [v/v])	2.4	-	-	-	-
Dimethylphthalate (100 mM - 2% [v/v])	2.3	+++	-	+++	+++
Fluorobenzene (100 mM - 1%[v/v)	2.2	+++	+++	+++	+++
Benzene (100 mM - 1%[v/v])	2.0	+++	+++	+++	+++
Phenol (20 mM - 0.18% [v/v])	1.5	+++	-	+++	+++

+++ = Growth overnight (16 hours)
+/- = Minimal growth overnight
- = No growth

Table 3.2.4 Growth of the Gram negative strains in organic solvent two-phase systems

Solvent (concentration)	log P_{OW}	*Pseudomonas* sp. strain ZZ5	*P. citronellolis* strain ZZ6	*S. maltophilia* strain ZZ7	*B. cepacia* complex strain ZZ8	*P. putida* strain S12
Hexane (100 mM - 1.3% [v/v]))	3.5	+++	+++	+++	+++	+++
Cyclohexane (100 mM - 1%[v/v])	3.2	+++	+++	+++	+/-	+++
p-Xylene (100 mM - 1.2% [v/v])	3.0	+/-	+/-	-	-	+++
Toluene (100 mM - 1%[v/v])	2.5	-	-	-	-	+++
1-Heptanol (100 mM - 1.4% [v/v])	2.4	-	-	-	-	+/-
Dimethylphthalate (20 mM - 0.33% [v/v])	2.3	+++	+++	+/-	+/-	+++
Dimethylphthalate (100 mM - 2% [v/v])	2.3	+++	+++	-	-	+++
Fluorobenzene (100 mM - 1%[v/v])	2.2	-	-	-	-	-
Benzene (100 mM - 1%[v/v])	2.0	-	-	-	-	-
Phenol (20 mM - 0.18% [v/v])	1.5	-	-	-	-	-

+++ = Growth overnight (16 hours)
+/- = Minimal growth overnight
- = No growth

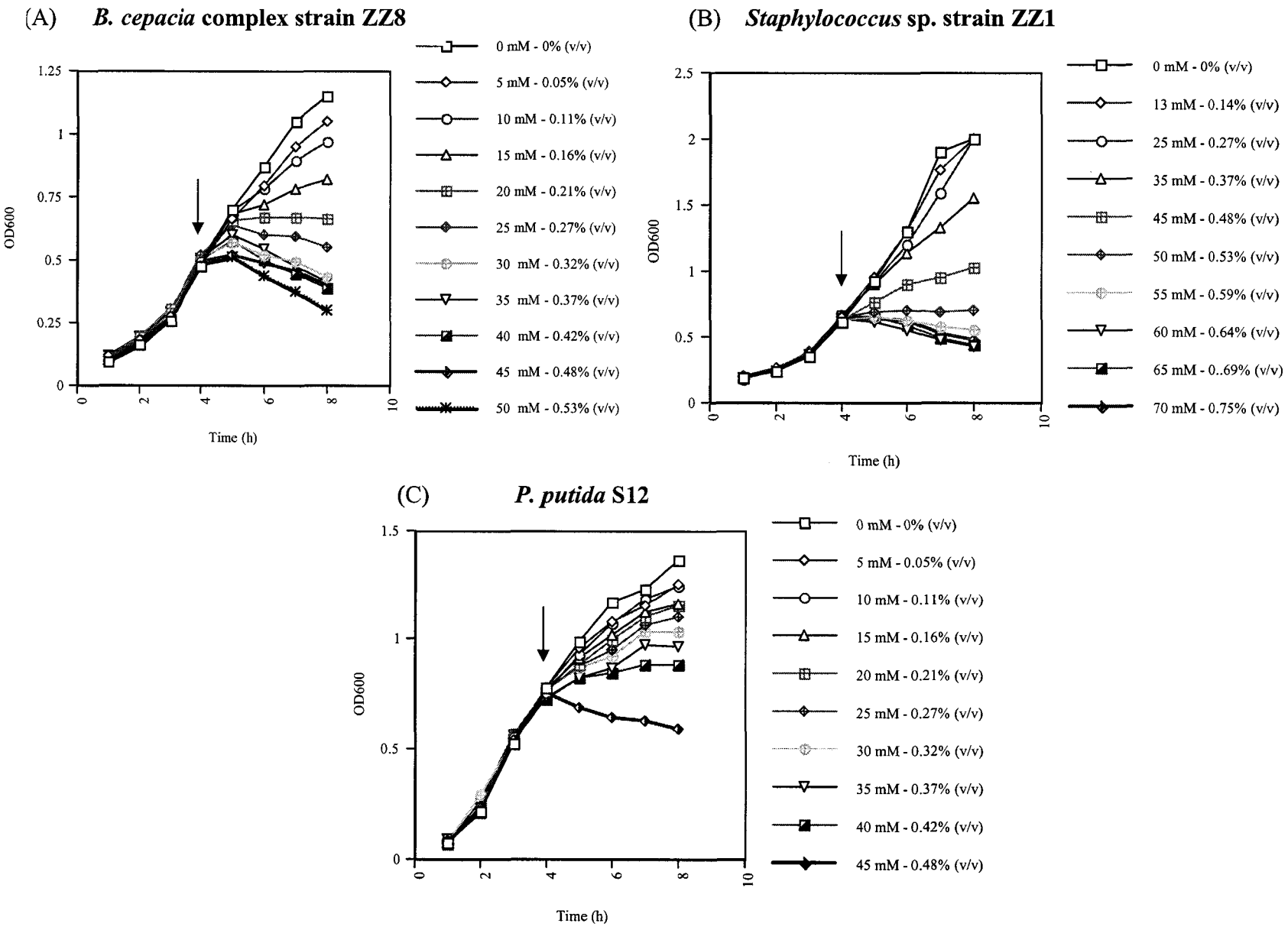
isolates, but this strain still displayed tolerance to high concentrations of toluene, benzene, and fluorobenzene (Table 3.2.3). In contrast, none of the Gram negative isolates were observed to survive in the presence of high concentrations of any of the highly toxic solvents including toluene, 1-heptanol, fluorobenzene, benzene, or phenol when tested under the same conditions (Table 3.2.4). However, all the Gram negative strains were observed to survive in the presence of high concentrations of less toxic solvents including hexane and cyclohexane, although the *B. cepacia* complex strain ZZ8 showed slight sensitivity to 100 mM cyclohexane. Interestingly, the *Pseudomonas* sp. strain ZZ5 and the *P. citronellolis* strain ZZ6 displayed some level of tolerance to 100 mM *p*-xylene and complete tolerance to 100 mM of the highly toxic solvent dimethylphthalate. The *S. maltophilia* strain ZZ7 and the *B. cepacia* complex strain ZZ8 also showed some level of tolerance to 20 mM dimethylphthalate.

As a control, similar experiments were also performed to determine the level of solvent tolerance of the previously tested solvent tolerant bacterium *P. putida* S12 when compared to the newly isolated strains. Consistent with the previous observations (Kieboom *et al.*, 1998a), the *P. putida* strain S12 showed high levels of tolerance to most organic solvents tested (Table 3.2.4). Interestingly, much like the isolated Gram negative strains, *P. putida* S12 was observed to be sensitive to fluorobenzene, benzene, and phenol. This is in contrast to the isolated Gram positive strains, which showed high levels of tolerance to these solvents (except *B. cereus* strain ZZ2, which showed sensitivity to 20 mM phenol). This pattern of tolerance of Gram positive bacteria to high concentrations of more toxic solvents suggests the possibility of the involvement of a specialized solvent tolerance mechanism in Gram positive bacteria.

3.2.3.2 Growth following sudden solvent shock

During the enrichment procedure where the cells were subcultured in increasing concentrations of toluene (Section 3.2.1) as well as during the experiments where the cells were grown overnight in the presence of the different organic solvents (Section 3.2.3.1), cells were given the opportunity to adapt to the presence of the toxic organic solvents. In contrast, experiments were also performed in an attempt to compare the innate abilities of the isolates to survive sudden solvent exposure without adaptation. Aliquots of an overnight culture of each strain were diluted 1:50 in fresh LB in Nephelo flasks and were grown until they reached an OD_{600} of approximately 0.5. Subsequently, a set quantity of toluene (expressed in mM and % [v/v] in Figure 3.2.1) was added to each culture and the flasks were sealed with foil-covered stoppers and the relative cell density was monitored following toluene addition by measuring OD_{600} readings without opening and re-sealing the flasks. Figure 3.2.1 shows a representation of at least two independent experiments performed on the *Staphylococcus* sp. strain ZZ1 and the *B. cepacia* complex strain ZZ8 as an illustration of the characteristics of the Gram positive isolates as compared to the Gram negative isolates. In all strains, growth rate was observed to decrease when shocked with increasing concentrations of toluene until shocked with a concentration that caused the cell density to plateau. Addition of higher concentrations of toluene was observed to cause a gradual increase in cell death. In general, the Gram positive isolates were observed to survive sudden exposure to much higher concentrations of toluene when compared to the Gram negative strains. The *B. cepacia* complex strain ZZ8 was observed to survive the sudden addition of toluene up to a concentration of 15 mM. Cell density was observed to reach a plateau with the addition

Figure 3.2.1. Growth assays comparing the survival efficiency of the *B. cepacia* complex strain ZZ8, *Staphylococcus* sp. strain ZZ1 and the *P. putida* strain S12 following sudden solvent shock. Overnight cultures of each strain were diluted 1:50 in fresh LB in a set of Nephelo flasks and were grown until they reached an OD_{600} of approximately 0.5. Subsequently, a specific volume of toluene was added to each culture and the flasks were sealed with foil-covered stoppers. Growth was assessed following toluene addition by measuring OD_{600} readings without opening and re-sealing the flasks to prevent the loss of toluene by volatilization. The y-axis shows the OD_{600} values and the x-axis shows the time elapsed in hours. The quantity of toluene each subculture was exposed to is indicated in the graphs for each strain. The black arrows indicate the addition of toluene. (A) *B. cepacia* complex strain ZZ8. (B) *Staphylococcus* sp. strain ZZ1 (C) *P. putida* strain S12.



of 20 mM toluene, indicating that new cell growth did not occur, and the addition of 25 mM toluene was observed to result in cell loss (Figure 3.2.1A). However, the *Staphylococcus* sp. strain ZZ1 was observed to survive the sudden addition of toluene up to a concentration of 45 mM. A plateau was reached with the addition of 50 mM toluene, while a decrease in cell density was observed with the addition of 55 mM toluene, indicating cell death (Figure 3.2.1B). In contrast to these observations, in the adaptive growth condition the *B. cepacia* complex strain ZZ8 was observed to withstand toluene concentrations of up to 25 mM, while the *Staphylococcus* sp. strain ZZ1 was observed to withstand toluene up to a concentration of 100 mM (Table 3.2.2). Since adaptive growth conditions allowed cell survival in higher concentrations of toluene when compared to sudden exposure to toluene, it suggests that when allowed time, cells can at least partially adapt to grow in the presence of high concentrations of toxic organic solvents by undergoing mutations or expressing cellular proteins or structures.

Similar experiments were also performed to determine the survival efficiency of the *P. putida* strain S12 when compared to the newly isolated strains. The *P. putida* S12 strain was observed to survive a sudden addition of 35 mM toluene. A plateau was reached with the addition of 40 mM toluene and cell death occurred with the addition of 45 mM toluene (Figure 3.2.1C). Since adaptive growth conditions allowed this strain to survive in the presence of a 100 mM toluene (Table 3.2.4) it was again indicative that exposure to toxic solvents in adaptive conditions are less harsh to cells when compared to sudden solvent shock. Interestingly, even with the known presence of the SrpABC system in *P. putida* S12, which has been observed to be up-regulated about sixfold in the presence of toluene (Figure 3.1.6), the strain was observed to be more sensitive to sudden

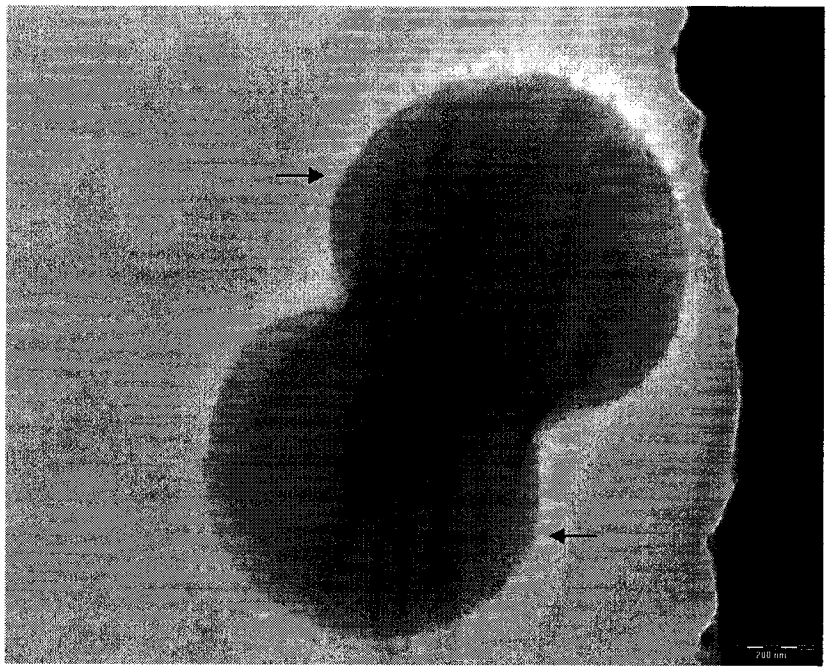
solvent shock when compared to the isolated Gram positive strains. However, the tolerance level of *P. putida* S12 was higher than the isolated Gram negative strains (Figure 3.2.1). The high tolerance of Gram positive bacteria to sudden solvent shock, suggests the possible involvement of a solvent tolerance mechanism in Gram positive bacteria that may be more efficient in providing immediate protection.

3.2.4 Examination of the cellular ultrastructure

In an attempt to identify a mechanism that contributes to the high solvent tolerance observed in the Gram positive bacteria, the ultrastructure of the *Staphylococcus* sp. strain ZZ1 was examined. The strain was grown overnight (16 hours) with or without toluene added to a final concentration of 100 mM. Initially, a sample of cells obtained from each growth condition was negatively stained using 0.2% phosphotungstic acid and examined by transmission electron microscopy. It was observed that cells when grown in the presence of 100 mM toluene produced an outer layer of an extracellular capsule-like structure, giving the cells a fuzzy appearance when compared to the control cells grown without toluene (Figure 3.2.2). In order to characterize this capsular structure, cell samples obtained from each growth condition were labeled with polycationic ferritin to allow capsular stabilization and stained with ruthenium red. The cells were subsequently thin-sectioned prior to visualization by transmission electron microscopy. A thick capsule of uniform and continuous appearance, stained strongly by the ruthenium red stain was observed beyond the cell wall of the cells grown in the presence of toluene. However, cells grown in the absence of toluene did not exhibit the capsules (Figure 3.2.3). These

Figure 3.2.2. Transmission electron micrographs of negatively stained *Staphylococcus* sp. strain ZZ1. Cell samples obtained following growth overnight in the presence or absence of 100 mM toluene were negatively stained using 0.2% phosphotungstic acid and visualized by transmission electron microscopy. (A) Cells grown in the presence of toluene. (B) Cells grown in the absence of toluene. Cells were observed at 44 000x magnification. Arrows indicate extracellular capsule-like material. Bar, 200 nm

(A)



(B)

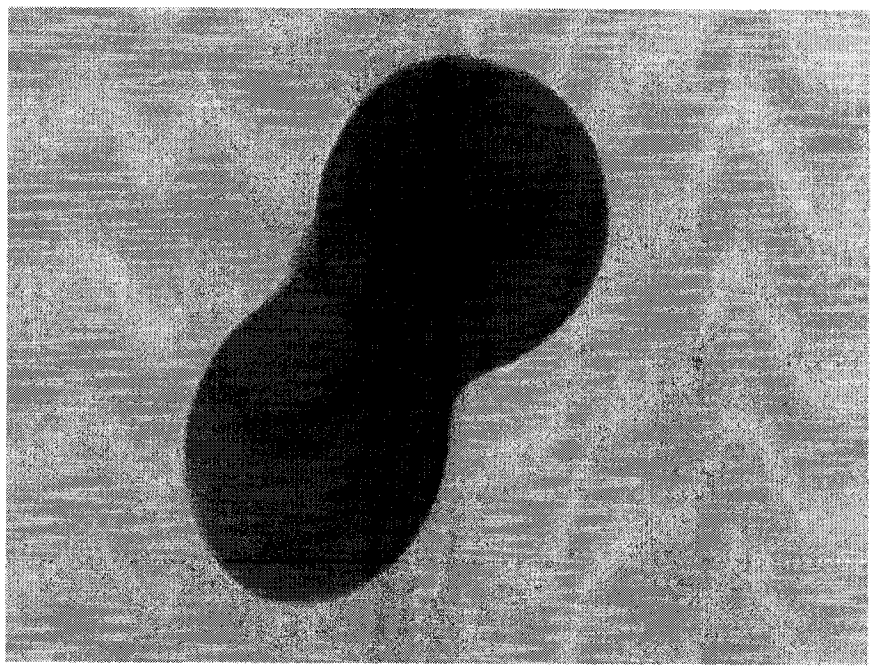
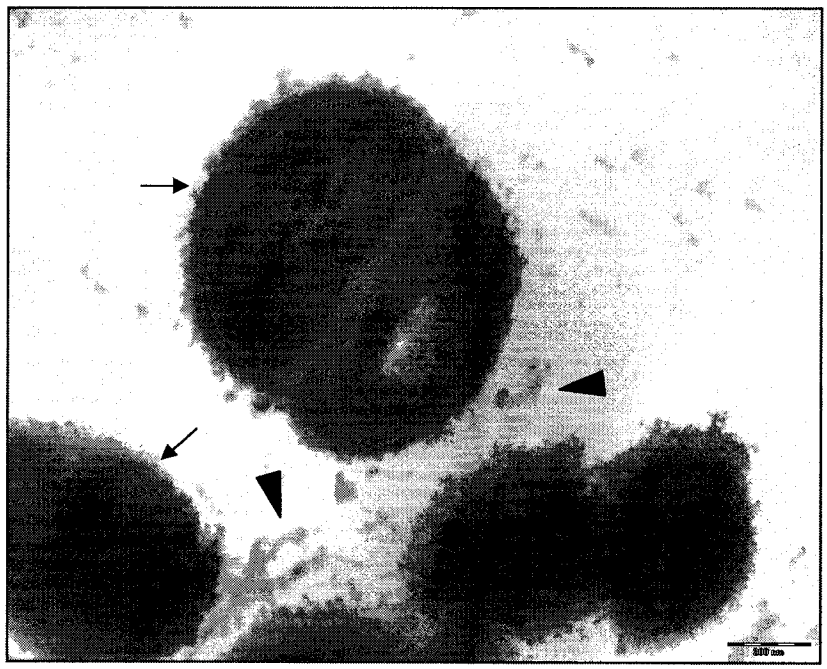
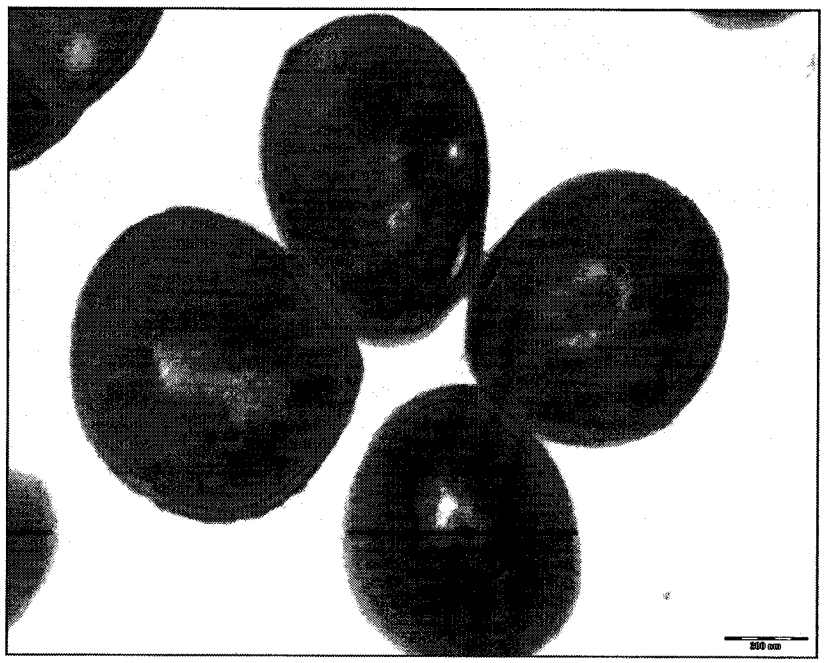


Figure 3.2.3. Transmission electron micrographs of thin sections of *Staphylococcus* sp. strain ZZ1. Cells were grown overnight in the presence or absence of 100 mM toluene. A sample of cells obtained from each growth condition was labeled with polycationic ferritin to allow capsular stabilization and stained with ruthenium red to demonstrate the capsule. The cells were subsequently thin-sectioned and visualized by transmission electron microscopy. (A) Cells grown in the presence of toluene. (B) Cells grown in the absence of toluene. Cells were observed at 44 000x magnification. Arrows indicate the extracellular capsule. Arrow-heads indicate fragments of capsular material. Bars, 200 nm.

(A)



(B)



observations strongly suggest that capsule production contributes to the extraordinary solvent tolerance observed in this strain.

Chapter 4:

Discussion

4. Discussion

4.1 Characterization of SrpS and SrpR, the regulators of the SrpABC solvent efflux pump in *Pseudomonas putida* S12

Interest in the RND-type efflux system SrpABC in *P. putida* S12 was originally stimulated by the specialized solvent efflux characteristic of this system (Isken and De Bont, 2000). In addition, the identification of two local regulatory genes, *srpS* and *srpR* situated upstream and transcribed divergently from the efflux pump operon indicated an unusual regulatory arrangement (Dennis and Zylstra, 1999; Wery *et al.*, 2001). The goal of this project was to elucidate the functions of SrpS and SrpR in the transcriptional regulation of the *srpABC* operon.

Initially, northern hybridization analyses were performed using *srpS*-specific and *srpR*-specific probes to determine the transcript sizes and the transcription level of the *srpS* and *srpR* genes in the presence and absence of toluene. The open reading frames of *srpS* and *srpR* are separated by 5 bp and it was presumed that the two genes were part of a single transcriptional unit. Results revealed that transcription of *srpS* and *srpR* occurred both in the presence and absence of toluene. However, transcription of *srpS* increased about sixfold in the presence of toluene, while transcription of *srpR* increased only about twofold in the presence of toluene. The observed difference in the level of transcription of the two genes in the presence of toluene suggests that the two genes may not be arranged as an operon. The bands obtained were highly smeared, possibly due to RNA degradation, which precluded the determination of accurate transcript sizes to confirm a difference in size of the *srpS* and *srpR* mRNA transcripts. However, it was estimated that

the transcripts were about the expected size of the polycistronic mRNA, suggesting that the difference in trancription levels observed might be the result of post-transcriptional regulation. Although working with RNA has proven to be difficult in this study, in order to determine if *srpS* and *srpR* genes are polycistronic, future attempts could be made to perform RT-PCR using one primer specific for *srpS* and one primer specific for *srpR*. Alternatively, primer extension analysis could be performed using primers that anneal downstream of the putative start codon of *srpR* to determine if a transcription start site can be identified for *srpR*.

Expression of many RND-type efflux pump operons and their local repressors are coordinately regulated due to the existence of promoters that overlap with the regulator binding site. For example, in *P. putida* DOT-T1E, it has been shown that the *ttgVW* and *ttgGHI* operons are transcribed coordinately from single overlapping promoters that also overlap with the TtgG binding site (Guazzaroni *et al.*, 2004; Rojas *et al.*, 2003). Likewise, the *ttgR* gene and *ttgABC* operon have been observed to have single overlapping promoters that also overlap with the TtgR binding site (Duque *et al.*, 2001; Teran *et al.*, 2003). Because northern hybridization analysis revealed a sixfold increase in transcription of *srpS* in the presence of toluene, while it has been previously shown that expression of the *srpABC* efflux pump operon is induced in the presence of toluene (Kieboom *et al.*, 1998b), it was presumed that the transcription of *srpS* and the *srpABC* operon is coordinately regulated. In an attempt to prove this, primer extension analyses were performed to identify the transcription start sites of *srpS* and *srpA*. Regardless of whether the cells were grown in the presence or absence of toluene, transcription of *srpS* was observed to occur from a single promoter. Comparison of the intensities of the

cDNA bands obtained from each growth condition showed that expression of *srpS* increased in the presence of toluene, supporting the results obtained from northern hybridization analysis. Attempts to determine the transcription start site of *srpA* were unsuccessful, perhaps the *srpABC* mRNA transcript forms complex RNA secondary structures that inhibit primer binding or it is possible that the transcript is highly unstable and is processed immediately. However, identification of the transcription start site of *srpA* is crucial to determine if there is an overlap between *srpS* and *srpA* promoters. Perhaps in the future, S1 nuclease protection assays might prove more effective in surpassing the difficulties encountered in this study.

In order to characterize the functions of SrpS and SrpR in regulating the transcription of the *srpABC* efflux pump operon, studies were performed to determine the effect of *srpS* deletion or *srpR* deletion mutations on the *srpA* promoter activity. Initially, the plasmid pJD500, containing a transcriptional fusion construct of *srpA-lacZ*, was integrated into the chromosome of the wild-type *P. putida* S12, SrpS$^-$ and the SrpR$^-$ strains without disrupting the parental copy of the *srpABC* operon. The chromosomal location of a single copy of the reporter gene reduces regulator titration effects. Following PCR confirmation of the plasmid's integration in the desired position in the chromosome, β-galactosidase assays were performed to determine the *srpA* promoter activity in the presence and absence of toluene. In the wild-type background, expression of the *srpABC* operon occurred even in the absence of toluene, while addition of toluene caused the expression to increase to about sixfold. Since northern hybridization analysis revealed a similar pattern of expression for the *srpS* gene, it is plausible that the promoters of *srpS* and the *srpABC* operon overlap and are coordinately regulated.

Deletion of *srpS* allowed a 5.5-fold increase in expression of the *srpABC* operon when compared to the wild-type background in the absence of toluene. Moreover, in the presence of toluene, expression of the *srpABC* operon in the *srpS* deletion background was similar to that in the absence of toluene, suggesting that deletion of *srpS* results in a constitutively active state of the *srpA* promoter. These results indicate that SrpS functions as a transcriptional repressor of the *srpABC* operon. In addition, expression of the *srpABC* operon was similar in the *srpS* deletion background and the wild-type background in the presence of toluene, suggesting that toluene may directly inhibit the repressor activity of SrpS in the wild-type background. Deletion of *srpR* caused the expression of the *srpABC* operon to decrease about fivefold in the absence of toluene and about threefold in the presence of toluene when compared to the wild-type background. This suggests that SrpR may function to activate the transcription of the *srpABC* operon. The observation that toluene causes an increase in the expression of the *srpABC* operon in the *srpR* deletion background, but does not have a significant effect in the *srpS* deletion background, supports the deduction that toluene interacts directly with SrpS.

Attempts to complement the *srpS* deletion and the *srpR* deletion phenotypes of these mutants were unsuccessful. It is probable that the regulator genes were not expressed from the expression vector constructs. In the future, total RNA could be isolated from the regulator deletion mutants in the presence and absence of toluene and northern hybridization analysis or RT-PCR could be performed to examine the expression of the regulator genes. This would prove useful in determining if SrpR is still expressed in the *srpS* deletion mutant and would help confirm whether or not the genes are polycistronic. Similar experiments could be performed with the mutant strains

transformed with the sets of expression vector constructs to determine whether the regulator genes are in fact being expressed in *trans*. To examine the regulator expression at the protein level, western analysis could be performed on total cell extracts from the regulator deletion mutants and the mutants transformed with the complementation vector constructs. Further experimentation could be performed by creating transcriptional fusions of *srpS-lacZ*. These constructs could be integrated in the chromosome of the wild-type strain as well as the regulator deletion mutants and would be helpful to compare the activity of the *srpS* and *srpA* promoters as well as to determine if SrpS affects its own transcription.

The tentative conclusions deduced were strengthened by the results obtained from electrophoretic mobility shift assays. SrpS was observed to bind with high affinity to the *srpS – srpA* intergenic region. Binding of SrpS to the intergenic region possibly hinders access of RNA polymerase to the *srpA* promoter, preventing transcription of the *srpABC* operon. DNase I footprint analysis should be performed in the future to identify the exact region in which SrpS binds. EMSAs showed that SrpR is unable to bind to the *srpS – srpA* intergenic region, ruling out the possibility that SrpR binds to the promoter DNA to facilitate the access of RNA polymerase to the *srpA* promoter. Interestingly, SrpR was observed to prevent the binding of SrpS to the target DNA as well as dissociate SrpS pre-bound to the target DNA. These observations indicate the possible role of SrpR as an anti-repressor, which functions to allow the derepression of the *srpA* promoter. Results of the northern hybridization analyses are in agreement with these conclusions. Increased expression of SrpR in the presence of toluene would allow increased derepression of the *srpA* promoter. The higher level of expression of SrpS in the presence of toluene when

compared to SrpR is possibly to ensure that the efflux system is not overexpressed to harmful levels. Additionally, EMSAs showed that toluene enhances the inhibitory effects of SrpR on SrpS, and confirmed that toluene can also directly inhibit the binding ability of SrpS to the operator DNA in the absence of SrpR.

The proposed mechanism for the transcriptional regulation of the *srpABC* operon in the wild-type *P. putida* S12 is presented in Figure 4.1.1. The data obtained suggest that under normal conditions presumably SrpR binds to SrpS and causes a slight change in the conformation of SrpS. This reduces the binding ability of SrpS to the operator DNA and at the same time increases dissociation of the SrpS proteins pre-bound to the DNA to allow a low level expression of the *srpABC* operon. The possible overlap of the *srpA* and the *srpS* promoters results in a low level expression of the *srpS* gene as well (Figure 4.1.1A). If toluene is present, toluene possibly binds to SrpS in the SrpS-SrpR complex, causing a change in the conformation of SrpS. This prevents SrpS from binding to the operator DNA and at the same time relieves the SrpS proteins pre-bound to the DNA, to allow full expression of the *srpABC* operon as well as the *srpS* gene (a sixfold increase in expression observed in both cases). It is as yet unclear if the *srpR* gene has its own promoter and if so, how it might be regulated (Figure 4.1.1B). If the *srpS* gene is deleted, the repressor protein is not expressed to bind to the operator DNA and repress the expression of the *srpABC* operon. Therefore, the efflux pump operon is fully expressed both in the presence and absence of toluene. Since SrpR does not bind to the *srpS-srpA* intergenic region, this phenotype would be observed regardless of whether *srpS* and *srpR* genes are polycistronic and both the regulators are disrupted in this mutant or if *srpR* has its own promoter and is expressed even if *srpS* is disrupted (Figure 4.1.2). In the

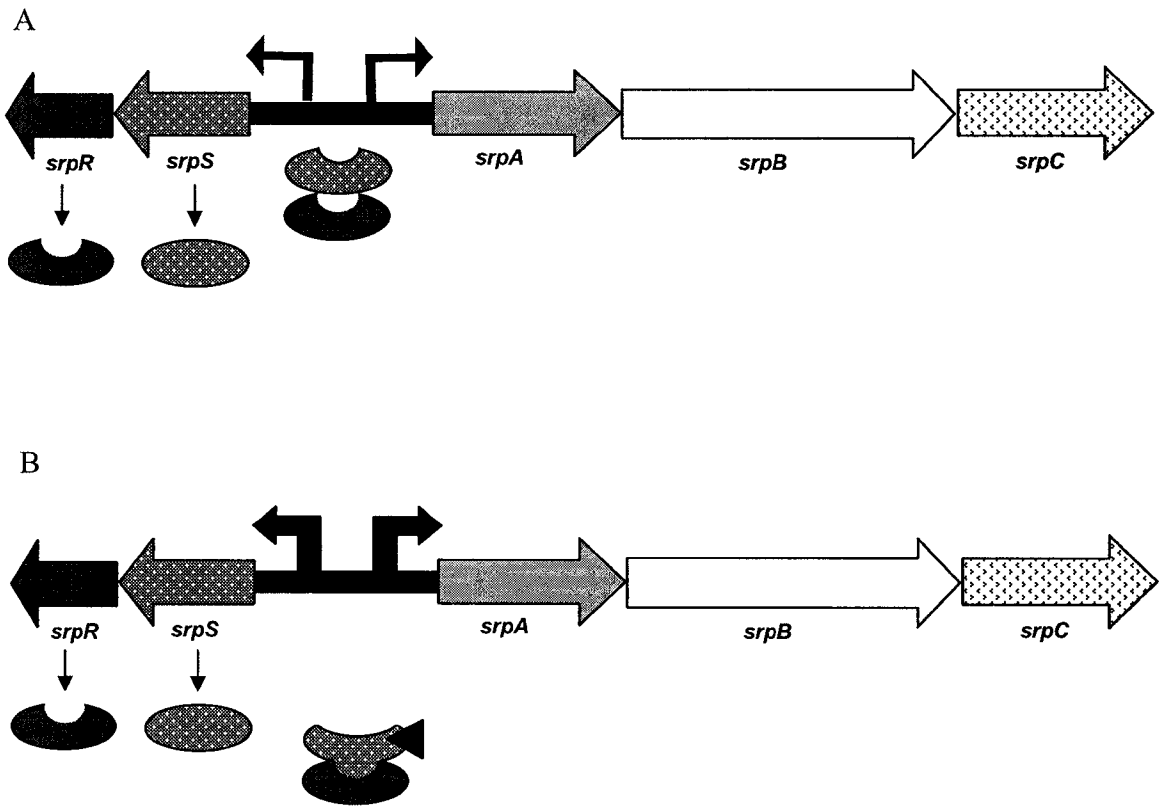


Figure 4.1.1. Proposed mechanism of transcriptional regulation of the *srpABC* operon in *P. putida* S12. (A) In the absence of toluene, SrpR binds to SrpS and changes the conformation of SrpS. This reduces the binding ability of SrpS to the operator DNA and causes a slight increase in dissociation of the SrpS proteins pre-bound to the DNA, allowing some derepression of the presumably overlapping *srpS* and *srpA* promoters. (B) In the presence of toluene, toluene binds to SrpS in the SrpS-SrpR complex and alters the conformation of SrpS to inhibit the binding of SrpS to the operator DNA. This relieves the SrpS proteins pre-bound to the DNA, allowing a sixfold increase in expression of the *srpS* gene and the *srpABC* operon. The angled 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.

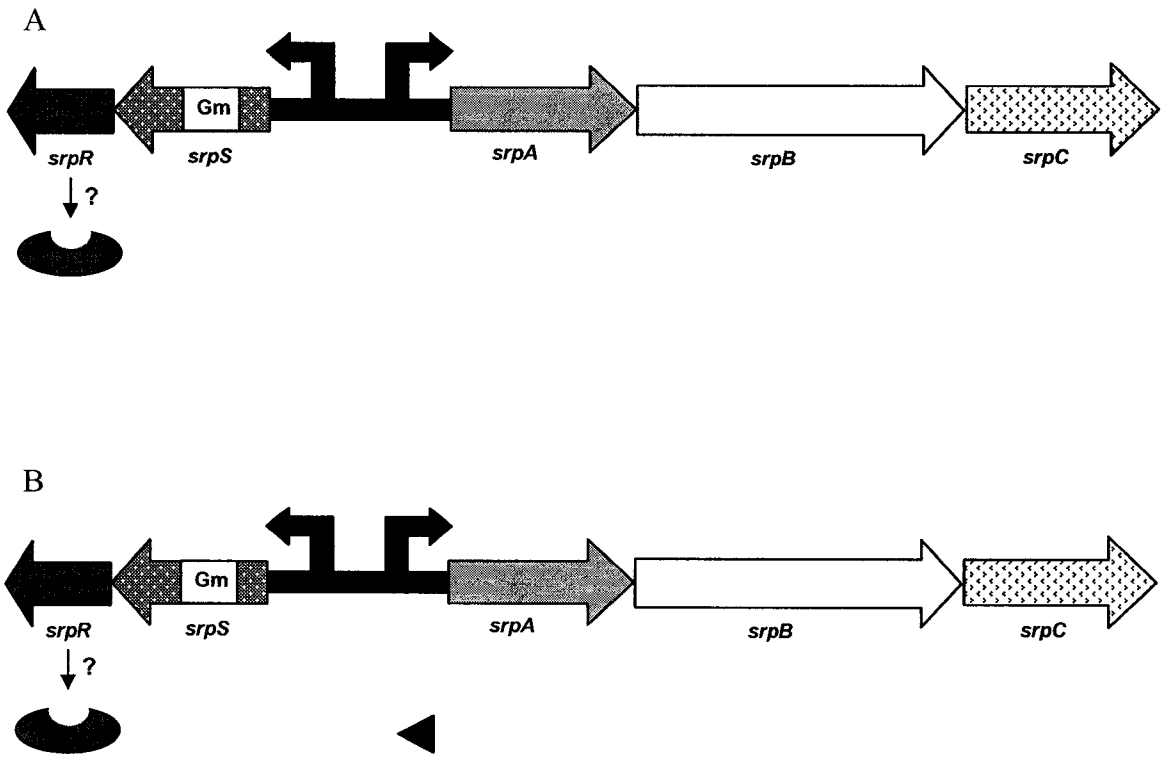


Figure 4.1.2. Proposed mechanism of transcriptional regulation of the *srpABC* operon in the *srpS* deletion mutant. Both in the absence of toluene (A) and in the presence of toluene (B), full expression of the *srpS* gene and the *srpABC* operon occurs, since the repressor SrpS is not expressed to bind to the operator DNA. It is not clear if expression of *srpR* occurs in this mutant strain. The angled 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.

srpR deletion mutant the anti-repressor protein is not expressed, so there is no reduction in the binding ability of SrpS to the operator DNA or dissociation of SrpS pre-bound to the DNA. Therefore, in the absence of toluene, SrpS binds to the operator DNA with higher avidity than the wild-type cells, resulting in a lower level of expression of the efflux pump operon when compared to wild-type cells. However, in the presence of toluene, the interaction of toluene with SrpS changes the conformation of SrpS. This causes the dissociation of SrpS from the operator DNA to some extent to allow a higher level of expression of the efflux pump operon when compared to the absence of toluene (Figure 4.1.3). The derepression caused by toluene is higher than that caused by SrpR alone.

Many eukaryotic systems exhibit regulatory mechanisms that involve interactions between repressor and anti-repressor proteins. Several examples of bacterial systems that utilize similar regulatory mechanisms have also been reported. By virtue of electrophoretic mobility shift assays it was shown that in *Myxococcus xanthus*, CarA, the transcriptional repressor of the carotenoid biosynthesis operon *crtEBDC*, is prevented from binding to the operator DNA by the anti-repressor protein CarS. Additionally, CarS was observed to dissociate CarA pre-bound to the operator DNA (Whitworth and Hodgson, 2001). In *Bacillus subtilis*, it was observed that the inhibitor of sporulation, SinR, binds to the operator DNA and represses the transcription of the *aprE* gene which encodes the protease subtilisin. The anti-repressor protein of this system, SinI was shown to inhibit the binding of SinR to the operator. However, SinI was observed to be less effective in relieving the repressor protein pre-bound to the operator DNA (Bai *et al.*, 1993). Similarly, in the photosynthetic bacteria *Rhodobacter sphaeroides*, PpsR, the

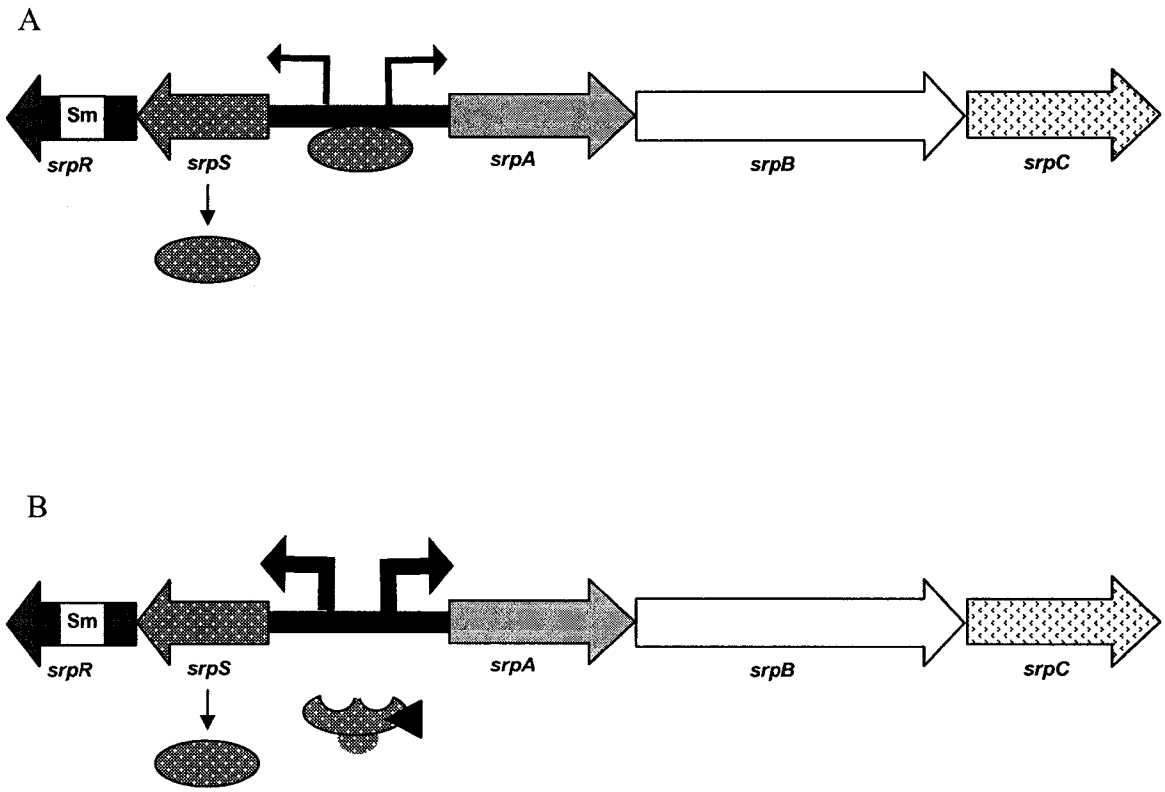


Figure 4.1.3. Proposed mechanism of transcriptional regulation of the *srpABC* operon in the *srpR* deletion mutant. (A) Since the anti-repressor protein SrpR is not expressed in this mutant strain, in the absence of toluene, SrpS is allowed to bind with high avidity to the operator DNA, repressing the transcription of the *srpS* gene and the *srpABC* operon more than in wild-type cells. (B) In the presence of toluene, toluene binds to SrpS and changes the conformation of SrpS to reduce the binding ability of SrpS to the operator DNA. This causes a slight derepression of the *srpS* and *srpABC* promoters when compared to the absence of toluene. The angled 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.

repressor of photosynthesis gene expression was observed to bind to the operator DNA. Co-incubation of PpsR with the anti-repressor AppA prior to the addition of target DNA was shown to inhibit PpsR from binding to the operator DNA (Masuda and Bauer, 2002). Interestingly, none of these pairs of bacterial repressor and anti-repressor proteins belong to the same family. The protein CarA belongs to the MerR family of transcriptional repressors, while CarS has no sequence homologues. The protein pair SinR and SinI shows homology to bacteriophage repressors. The repressor protein PpsR belongs to a family of proteins that regulate the transcription of genes in response to light or redox conditions, whereas AppA is a photoreceptor of the BLUF-domain family (Bai *et al.*, 1993; Gomelsky and Klug, 2002; Whitworth and Hodgson, 2001). The proteins under scrutiny in this study, SrpS and SrpR, are members of the IclR and TetR family, respectively.

Thus far, the data obtained from this study provide the first evidence of the involvement of a repressor and an antagonizing anti-repressor protein in the transcriptional regulation of an RND-type efflux pump operon. However, further experimentation should be undertaken to confirm a direct protein-protein interaction between SrpR and SrpS. An effective approach would be to raise antibodies against the fusion proteins MBP-SrpS and MBP-SrpR and perform co-immunoprecipitation experiments. Alternatively, far-western analysis could be performed where one of the proteins would be separated by native PAGE, transferred to a membrane and probed with the labeled form of the second protein. The amino acid sequences of SrpS and SrpR proteins can be examined for motifs that might be associated with protein-protein

interactions. Once interaction between the two proteins has been confirmed, it will be interesting to determine the stoichiometry of the interaction.

4.2 Isolation and characterization of novel organic solvent tolerant bacteria

The goal of this project was to isolate and characterize novel organic solvent tolerant bacteria from soil ecosystems. Soil samples were obtained from oil contaminated locations and from various sites planted to marigolds, which have the potential presence of thiophenes (Christensen and Lam, 1990). In order to enrich for solvent tolerant bacteria, the soil samples were subcultured for several days in LB medium with increasing concentrations of toluene. The toxicity of toluene kills most microbes at concentrations as low as 10 mM. The strains that survived in 20 mM toluene and higher were isolated and identified by comparison of 16S rRNA gene sequences and carbon source utilization profiles with the appropriate databases. The four Gram positive strains isolated were designated strain names ZZ1, ZZ2, ZZ3 and ZZ4, and were identified as a *Staphylococcus warneri* strain and three *Bacillus cereus* strains, respectively. The four Gram negative strains isolated were designated strain names ZZ5, ZZ6, ZZ7 and ZZ8, and were identified as a *Pseudomonas* sp., a *Pseudomonas citronellolis* strain, a *Stenotrophomonas maltophilia* strain and a *Burkholderia multivorans* strain, respectively.

The isolated Gram positive strains were observed to survive higher concentrations of toluene (90-100 mM) when compared to the isolated Gram negative strains (20-25 mM). In order to determine if the Gram positive strains are more tolerant to toxic organic solvents in general when compared to the Gram negative isolates, each strain was tested

for growth overnight in a range of organic solvent-LB two-phase systems. Tolerance of *P. putida* S12 was also tested similarly for comparison. Of all the isolated strains *Staphylococcus* sp. strain ZZ1 was revealed to be the most tolerant to organic solvents, as growth was observed in 100 mM concentrations of all the solvents tested except 1-heptanol. The Gram positive isolates were generally observed to be more tolerant to organic solvents when compared to the Gram negative isolates. Although some distinctions were observed in the solvent tolerance profile of each individual strain, all the Gram positive strains survived in 100 mM concentrations of less toxic solvents such as hexane and cyclohexane, as well as highly toxic solvents including toluene, fluorobenzene, and benzene. In comparison, all the Gram negative isolates survived in 100 mM of the less toxic solvents hexane and cyclohexane, but did not survive in 100 mM concentrations of toluene, fluorobenzene and benzene. The solvent tolerance profile of the *P. putida* S12 strain was consistent with the previous observations (Kieboom *et al.*, 1998a). In general, the Gram negative isolates were less tolerant to organic solvents when compared to *P. putida* S12. It is plausible that the new Gram negative isolates do not possess a solvent efflux system equivalent to the SrpABC system. *P. citronellolis* strains have been shown to be involved in solvent biodegradation (Bhattacharya *et al.*, 2003). It is possible that biodegradation contributes to confer some level of organic solvent tolerance in the *Pseudomonas* sp. strain ZZ5 and the *P. citronellolis* strain ZZ6, which survived in 100 mM of the highly toxic solvent dimethylphthalate. Interestingly, *P. putida* S12 and all of the isolated Gram negative strains were observed to be sensitive to fluorobenzene, benzene and phenol even at a concentration of 20 mM. This is in contrast to the isolated Gram positive strains which showed high level of tolerance to these

solvents (except *B. cereus* strain ZZ2, which showed sensitivity to 20 mM phenol). This pattern of tolerance to more polar highly toxic solvents has been recently reported in other Gram positive bacteria. For instance, the *Rhodococcus* species recently isolated from a chemical contaminated site in Australia showed tolerance to high concentrations of benzene and the *Bacillus* sp. strain SB-1 isolated from mangrove sediments showed tolerance to high concentrations of benzene and *n*-butanol (Paje *et al.*, 1997; Sardessai and Bhosle, 2002b). Although most organic solvent tolerant pseudomonads, for example *P. putida* S12, show tolerance to solvents like toluene, 1-heptanol and dimethylphthalate, thus far only one Gram negative isolate, a *Flavobacterium* sp. obtained from deep sea sediments has been reported to show tolerance to benzene (Moriya and Horikoshi, 1993). These observations indicate the possibility that perhaps solvent tolerant Gram positive bacteria have evolved mechanisms that differ considerably from the mechanisms involved in solvent tolerance in Gram negative bacteria. Interestingly, the *Bacillus cereus* strains isolated in this study showed sensitivity to *p*-xylene, and none of the Gram positive strains isolated in this study showed tolerance to 1-heptanol, which have log P_{OW} values higher than benzene. Similarly, the benzene tolerant *Flavobacterium* sp. was also observed to be sensitive to 1-heptanol and other organic solvents with log P_{OW} values higher than benzene (Moriya and Horikoshi, 1993). These observations indicate that there is no clear solvent tolerance limit *per se*, based on the parameter log P_{OW}. Instead, and as has been shown previously, toxicity of a solvent to an organism cannot be estimated solely on the basis of solubility and the log P_{OW} value of a solvent (see Introduction). Additionally, it is possible that the benzene tolerant Gram positive strains and the Gram negative *Flavobacterium* sp. exhibit a common mechanism that is specialized to function

against more polar organic solvents. Further experimentation could be performed to determine if the Gram positive isolates can survive higher concentrations of benzene and other organic solvents. In addition, experiments could be performed to determine if these Gram positive isolates use any of the organic solvents as a sole carbon source. Preliminary studies with toluene showed that none of the isolates utilized toluene as a sole carbon source.

The isolated strains were also tested to determine how well they can survive immediately after solvent shock. Growth was monitored by measuring OD_{600} readings following the addition of increasing concentrations of toluene. In general, the growth rates of all strains were observed to decrease when shocked with increasing concentrations of toluene, until a concentration was reached that caused the cell density to plateau. Perhaps somewhat unexpectedly, the addition of higher concentrations of toluene was observed to cause a gradual increase in cell death (see below). However, the Gram positive isolates showed higher tolerance to toluene shock when compared to *P. putida* S12 as well as the isolated Gram negative strains. These results suggest that the mechanisms of solvent tolerance in Gram positive bacteria are more efficient in providing immediate protection when compared to the Gram negative mechanisms. Accordingly, the observations support the presumption that the mechanisms of solvent tolerance in Gram positive bacteria differ from that of Gram negative bacteria. Nonetheless, all strains were observed to survive better if allowed time to adapt to grow in toluene when compared to survival immediately after sudden toluene shock. Possibly, cells require time to undergo mutations or establish long-term responses by expressing proteins or cellular structures. To determine the time entailed for the adaptation process, time course

experiments could be performed in the future by continuing the cell density measurements until cell growth starts to increase after the initial decline caused by solvent shock.

An interesting observation made during the course of this project was that the toxic effects of organic solvents increased with increasing concentrations of the solvents, even at concentrations above their maximum aqueous solubility limits. Excess insoluble solvent molecules exist on the surface of the aqueous phase, introducing an organic solvent phase in the system. It has been observed that agitation of yeast cells in a dodecanol-broth two-phase system resulted in increased cell death, while cell viability was not affected in stagnant systems (Bar, 1988). Light microscopic examination revealed that the yeast cells adhered to the dodecanol droplets. Since dodecanol when dissolved in the aqueous phase is not toxic to yeast cells, it was proposed that in an organic solvent-broth two-phase system, solvent toxicity is caused both by the organic solvent droplets present as a second phase in the system (termed phase toxicity) and by the solvent molecules that dissolve in the aqueous phase. It was suggested that phase toxicity might be caused from limited access to nutrients as a result of cell adhesion or entrapment in solvent emulsions. Additionally, it was proposed that organic solvent emulsions could cause the extraction of cell surface components causing lysis and cell death (Bar, 1988). Results of the present study support these conclusions. It is possible that direct contact of cells with the solvent emulsions may also cause increased partitioning of the solvent molecules directly into the cell membrane, resulting in increased toxic effects in the membrane. However, the molecular mechanisms of phase

toxicity have yet to be determined. Microscopic examination could be performed in the future to determine if the strains isolated in this study adhere to solvent emulsions.

In an attempt to identify a possible mechanism that contributes to the high solvent tolerance observed in the Gram positive bacteria, the ultrastructure of the *Staphylococcus* sp. strain ZZ1 was examined after growth in the presence and absence of toluene. Initial examination of negatively stained cells showed that the cells produced an extracellular capsule-like structure when grown in the presence of toluene but not when grown in the absence of toluene. Bacterial capsules are composed of highly hydrated polymers, usually polysaccharides, which are difficult to preserve during the dehydration and embedding procedures of thin-sectioning electron microscopy (Vanrobaeys *et al.*, 1999). Therefore, in order to characterize the capsular structure by thin-section electron microscopy, cells were initially treated with polycationic ferritin to stabilize the capsules and stained with ruthenium red to detect potential capsular polysaccharides. Subsequent examination of thin-sections of the cells confirmed that the cells produced a thick capsule of a uniform and continuous characteristic when grown in the presence of toluene. Because the capsule is observed only when the cells are grown in the presence of toluene and not in the absence, it is highly probable that the capsule is expressed as a long-term response to overcome the toxic effects of organic solvents. It is possible that the hydrophilic nature of the polysaccharide capsule protects the cells by reducing the access of organic solvent molecules into the target cell membrane. However, additional solvent tolerance mechanisms must be involved, since the *Staphylococcus* sp. strain ZZ1 showed high tolerance to toluene even under shock conditions where the adaptive response of capsule production would not have occurred.

To date, this is the first evidence of the possible involvement of a bacterial capsule in organic solvent tolerance. However, because the capsules were observed following cell growth overnight, at the stationary phase of growth, it is possible that capsule production is a general stress response rather than a specific response to the presence of organic solvents. In the future, experiments can be performed to determine whether other stress conditions such as high salt or heat shock induce capsule production. Nonetheless, determining the composition of the capsular material would help elucidate the role of the capsule in protecting the cell against organic solvents. Further electron microscopy analyses could be performed to determine if other organic solvents induce capsule production. At a genetic level, random gene deletion mutagenesis could be performed to identify the gene or genes involved in capsule production as well as to identify other genes that might be involved in organic solvent tolerance. Once the genes are identified, RNA transcript analysis could be performed to determine whether their expression occurs specifically in response to organic solvents. Identification and characterization of the molecular mechanisms responsible for bacterial solvent tolerance and elucidating their regulatory mechanisms are imperative in determining how the solvent tolerance properties of bacteria can be utilized in practical applications.

Chapter 5:

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