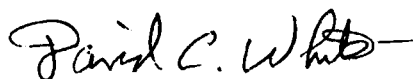


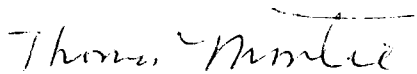
To the Graduate Council:

I am submitting herewith a dissertation written by Holly Chubb Pinkart entitled "Cell Envelope Dynamics and Solvent Tolerance in *Pseudomonas putida*". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

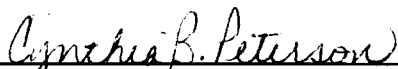


Dr. David C. White, Major Professor

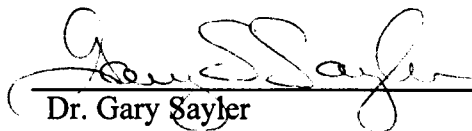
We have read this dissertation and
recommend its acceptance:



Dr. Thomas Montie



Dr. Cynthia Peterson



Dr. Gary Saylor

Accepted for the Council:



Associate Vice Chancellor and Dean
of the Graduate School

CELL ENVELOPE DYNAMICS AND SOLVENT TOLERANCE IN
PSEUDOMONAS PUTIDA

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Holly Chubb Pinkart
December 1996

DEDICATION

This dissertation is dedicated
to my parents,
Mr. Harold W. Chubb and Mrs. Patricia C. Chubb,
whose love and unwavering support
have made my graduate education possible.

ACKNOWLEDGMENTS

Although the Doctorate of Philosophy is a degree given to a single individual, it is in actuality a group effort designed to benefit that single individual. I would like to first and foremost thank my major professor, Dr. David C. White, for his guidance and support throughout my graduate career. I truly feel that the education I received in his laboratory is unrivaled in its ability to create an independent, critical scientist, with an appreciation for all science, not just the finer points of microbiology. I would like to thank my committee members, Dr. Gary Sayler, Dr. Cynthia Peterson, and Dr. Thomas Montie, for their help in focusing my research, and for valuable advice on technical matters.

I gratefully acknowledge the financial support of the Office of Naval Research grant no. N00014-94-1-0765. I would also like to thank the Microbiology Department for the teaching assistantship awarded to me during my first year as a graduate student. The teaching experience was a very rewarding one.

I would like to extend my sincere appreciation to my colleagues on campus and at the Center for Environmental Biotechnology, Bruce Applegate, Jennifer Black, Wade Johnston, Staci Kehrmeyer, Charles Phiefer, James Rice, Scott Rice, Julia Stair, and Dr. David Nivens for their help with experiments and papers. This was not only an exceptional group of colleagues, but an

exceptional group of friends as well. I would like to thank Tina Richey and Jennifer McGee their help with experiments, and David Ringelberg for his (frequently requested!) technical advice. I appreciate the efforts of Renee Thomas and Cheryl Owenby for their work on my behalf.

I would also like to thank those of you who didn't believe in my abilities (you know who you are). It gives me great pleasure to prove you wrong.

Finally, I would like to thank my family for their patience, understanding, and support during my tenure in graduate school. My husband Wes has been a rock in a sea of uncertainty, has reasonably tolerated my late nights and weekends at the lab, and has put up with life in the Buckle of the Bible Belt with extraordinary patience and remarkable good humor. I thank my parents for their support, both financial and emotional.

ABSTRACT

The role of the cell envelope in the solvent tolerance mechanisms of *Pseudomonas putida* was investigated. The responses of a solvent tolerant strain, *P. putida* Idaho, and a solvent sensitive strain, *P. putida* MW1200 were examined in terms of phospholipid and fatty acid content and composition, phospholipid biosynthetic rate, and alteration of lipopolysaccharide following exposure to a non-metabolizable solvent, *o*-xylene.

Following *o*-xylene exposure, *P. putida* MW1200 exhibited a decrease in total phospholipid content. In contrast, *P. putida* Idaho demonstrated an increase in phospholipid content 1-6 hours following *o*-xylene exposure. Both strains produced *trans*-unsaturated fatty acids in response within 5 minutes of xylene exposure. The Idaho strain exhibited an increase in saturated fatty acids, a response not seen in *P. putida* MW1200. These alterations serve to compensate for increased membrane fluidity induced by intercalation of *o*-xylene within the phospholipids.

Analysis of phospholipid biosynthesis showed *P. putida* Idaho to have a higher basal rate of phospholipid synthesis than MW1200. This rate increased significantly following exposure to xylene, and was independent of xylene concentration. Both strains showed little significant turnover of phospholipid in the absence of xylene. In the presence of xylene, both strains showed increased phospholipid turnover. The rate of turnover was significantly

greater in the *P. putida* Idaho strain than in the MW1200 strain. These results suggest that *P. putida* Idaho has a greater ability than the solvent sensitive MW1200 strain to repair damaged membranes through efficient turnover and increased phospholipid biosynthesis.

Surface structure of the two strains was also quite different, as evidenced by scanning electron microscopy, PAGE analysis, and chemical composition of LPS isolated from each strain. In the presence of xylene, the Idaho strain altered its LPS to include increased amounts of hydroxy fatty acids and charged sugar residues. These changes maintained a hydrophilic cell surface less permeable than the MW1200 strain to difloxacin and 1-*N*-phenylnaphthylamine.

A two-phase model for solvent tolerance was proposed. The first phase includes the fatty acid alterations, which allow for short term (1-2 hours) survival of high concentrations of solvent. The second phase, which includes increased phospholipid biosynthesis and cell surface alteration, allows for long term survival.

TABLE OF CONTENTS

CHAPTER		PAGE
PART I . INTRODUCTION		
BACKGROUND AND RESEARCH OBJECTIVES		2
LITERATURE REVIEW		5
Degradation of Aromatic Hydrocarbons		5
Gram Negative Bacterial Membrane Structure		8
Hydrocarbon Interaction with Lipids and Membranes		11
Compensation for Membrane Fluidity		17
Alterations of Surface Structure and Permeability		21
Resistance Pumps		23
Solvent Tolerant Organisms		24
PART II. PHOSPHOLIPID AND FATTY ACID ALTERATION OF <i>PSEUDOMONAS PUTIDA</i> STRAINS IN RESPONSE TO <i>O</i>-XYLENE.		
INTRODUCTION		28
MATERIALS AND METHODS		30
Strains		30
Culture Conditions and Doubling Time Determinations		32
Phospholipid Extraction		34
Separation of Inner and Outer Membranes		34
Identification of Phospholipid Class		35
Fatty Acid Analysis		36
Statistics		37
RESULTS		37
Growth Curves		37
Doubling Times		39

CHAPTER	PAGE
Xylene Kill Curves for <i>P. putida</i> MW1200	39
Phospholipid Composition	42
Fatty Acid Content and Composition	46
Kinetics of Phospholipid Fatty Acid Change	49
Location of Phospholipids	53
DISCUSSION	55

**PART III. PHOSPHOLIPID BIOSYNTHETIC RATE AND TURNOVER
RATE IN SOLVENT-TOLERANT AND SOLVENT-SENSITIVE
PSEUDOMONAS PUTIDA STRAINS**

INTRODUCTION	65
MATERIALS AND METHODS	66
Pulse-Chase Protocol	66
Statistics	68
RESULTS	69
DISCUSSION	82

**PART IV. OUTER MEMBRANE ALTERATIONS OF SOLVENT-
TOLERANT AND SOLVENT-SENSITIVE *PSEUDOMONAS PUTIDA*
STRAINS TO *O*-XYLENE**

INTRODUCTION	89
MATERIALS AND METHODS	91
Growth Conditions	91
Scanning Electron Microscopy (SEM)	92
Extraction and Purification of LPS	92
LPS Component Analysis	93
Cell Surface Hydrophobicity	94

CHAPTER	PAGE
Difloxacin Permeability Assay	95
NPN permeability Assay	95
Statistics	96
RESULTS	97
SEM	97
PAGE Analysis of LPS	102
Chemical Composition of LPS	102
Surface Hydrophobicity Assay	108
Difloxacin Permeability Assay	111
NPN Permeability Assay	111
DISCUSSION	113

PART V. SUMMARY AND CONCLUSIONS OF PARTS II-IV

SUMMARY AND CONCLUSIONS	118
-------------------------------	-----

PART VI. LITERATURE CITED AND VITA

LITERATURE CITED	123
VITA	150

LIST OF TABLES

TABLE	PAGE
1-1. Physical characteristics of monoaromatic hydrocarbons.	7
1-2. Differential partition coefficients of lipophilic compounds between synthetic membranes and aqueous phases based on membrane composition.	12
2-1. Growth of <i>P. putida</i> Idaho in the presence of saturating concentrations of organic solvents.	31
2-2. Mole percentages of each class phospholipids and total phospholipid obtained from <i>P. putida</i> MW1200 and <i>P.</i> <i>putida</i> Idaho in the presence and absence of <i>o</i> -xylene.	44
2-3. Molar percentages and concentrations (± 1 standard deviation, $n = 5$) of phospholipid fatty acids from <i>P. putida</i> MW1200 and <i>P. putida</i> Idaho grown in the presence or absence of <i>o</i> - xylene.	47
2-4. Changes in fatty acid composition of individual phospholipids of <i>P. putida</i> Idaho following exposure to <i>o</i> -xylene.	48
2-5. Molar percentages of phospholipid and phospholipid fatty acids associated with the inner membrane (IM) and outer membrane (OM) of each <i>Pseudomonas putida</i> strain in the absence and presence of <i>o</i> -xylene.	54

TABLE	PAGE
2-6. Membrane adaptations reported for various bacteria in response to exposure to organic solvents.	61
3-1. T-tests for phospholipid biosynthetic rate data ($\alpha = 0.01$).	71
3-2. T-tests for rate of loss (phospholipid turnover) data ($\alpha = 0.01$).	74
4-1. Fatty acid composition (mole percentage) of LPS Lipid A for <i>P. putida</i> MW1200 and <i>P. putida</i> Idaho in the presence and absence of <i>o</i> -xylene.	105
4-2. Difloxacin permeability assay for <i>P. putida</i> MW1200 and <i>P. putida</i> Idaho as measured by sensitivity to the hydrophobic antibiotic (MIC = minimum inhibitory concentration).	110
5-1. Summary of responses elicited by <i>P. putida</i> MW1200 and <i>P. putida</i> Idaho following <i>o</i> -xylene exposure.	119

LIST OF FIGURES

FIGURE	PAGE
1-1. Membrane structure of a typical gram-negative bacterium.	9
1-2. Monoaromatic hydrocarbon interaction with the gram-negative bacterial membrane, showing disorganization of phospholipid bilayers.	14
2-1. Growth curves of <i>P. putida</i> MW1200 and <i>P. putida</i> Idaho in the absence of <i>o</i> -xylene, and in the presence of 75 ppm and 200 ppm <i>o</i> -xylene as determined by optical density (wavelength = 600 nm).	40
2-2. Growth curves of <i>P. putida</i> MW1200 and <i>P. putida</i> Idaho in the absence of <i>o</i> -xylene, and in the presence of 75 ppm and 200 ppm <i>o</i> -xylene as determined by viable counts (CFU, colony forming unit).	41
2-3. Kill curve for <i>P. putida</i> MW1200 over various concentrations (in ppm) of <i>o</i> -xylene as determined by viable counts (CFU, colony forming unit).	43
2-4. Phospholipid composition of <i>P. putida</i> MW1200 and <i>P. putida</i> Idaho grown in the presence and absence of <i>o</i> -xylene.	45
2-5. Change in total phospholipid concentration in <i>P. putida</i> MW1200 over time following exposure to <i>o</i> -xylene.	51

FIGURE	PAGE
2-6. Increase in phospholipid in <i>P. putida</i> Idaho over time in the presence and absence of <i>o</i> -xylene.	52
3-1. Rates of ^{32}P -orthophosphate incorporation into phospholipid for <i>P. putida</i> MW1200 and <i>P. putida</i> Idaho in the absence and presence of <i>o</i> -xylene.	70
3-2. Rates of ^{32}P -orthophosphate loss from phospholipid for <i>P. putida</i> MW1200 and <i>P. putida</i> Idaho in the absence and presence of <i>o</i> -xylene.	73
3-3. Turnover of individual phospholipids of <i>P. putida</i> MW1200 in the absence of <i>o</i> -xylene.	76
3-4. Turnover of individual phospholipids of <i>P. putida</i> MW1200 in the presence of 75 ppm <i>o</i> -xylene.	77
3-5. Turnover of individual phospholipids of <i>P. putida</i> Idaho in the absence of <i>o</i> -xylene.	78
3-6. Turnover of individual phospholipids of <i>P. putida</i> Idaho in the presence of 75 ppm <i>o</i> -xylene.	79
3-7. Turnover of individual phospholipids of <i>P. putida</i> Idaho in the presence of 200 ppm <i>o</i> -xylene.	80

FIGURE	PAGE
4-1. SEM micrograph of <i>Pseudomonas putida</i> MW1200 grown in the absence of <i>o</i> -xylene.	98
4-2. SEM micrograph of <i>Pseudomonas putida</i> MW1200 exposed to 200 ppm <i>o</i> -xylene.	99
4-3. SEM micrograph of <i>Pseudomonas putida</i> Idaho grown in the absence of <i>o</i> -xylene.	100
4-4. SEM micrograph of <i>Pseudomonas putida</i> Idaho exposed to 200 ppm <i>o</i> -xylene.	101
4-5. Silver stained polyacrylamide gel of LPS from <i>P. putida</i> MW1200 and <i>P. putida</i> Idaho.	103
4-6. Carbohydrate composition of LPS from <i>P. putida</i> MW1200 and <i>P. putida</i> Idaho in the presence and absence of <i>o</i> -xylene.	104
4-7. A comparison of the fatty acid content of <i>P. putida</i> Idaho and <i>P. putida</i> MW1200 in the absence and presence of <i>o</i> -xylene.	107
4-8. Bacterial adhesion to hydrocarbons (BATH) assay performed on <i>P. putida</i> MW1200 and <i>P. putida</i> Idaho.	109
4-9. NPN permeability assay for <i>P. putida</i> MW1200 and <i>P. putida</i> Idaho in the absence and presence of <i>o</i> -xylene (given in concentrations of ppm).	112

PART I.

INTRODUCTION

BACKGROUND AND RESEARCH OBJECTIVES

In the past several years, many organic solvent-tolerant organisms have been discovered. The first report of toluene-resistant bacteria was by Inoue and Horikoshi (1989). Although these *Pseudomonas* strains could not grow on toluene, they tolerated very high concentrations of aromatic hydrocarbons. The phenomenon of solvent tolerance is an important one, because it allows microorganisms to grow in high concentrations of organic solvents. Organisms capable of surviving these conditions have great application in bioremediation of contaminated sites, "end of the pipe" biotransformation of industrial wastes, and many have potential for use in biphasic bioconversion systems. Solvent tolerant *Pseudomonas* strains capable of producing solvent-stable lipases and proteases have been isolated recently, further expanding the utility of these organisms (Ogino et al., 1994, 1995).

The toxic effects of aromatic hydrocarbons are associated with loss of cytoplasmic membrane integrity (de Smet et al., 1978; Sikkema et al., 1994, 1995). This loss of membrane integrity results in disruption of proton motive force, loss of membrane barrier functions, inhibition of membrane protein function, and subsequent cell lysis and death.

The mechanism for solvent tolerance is currently unknown. However, there are some conclusions from the current data that can be made. Solvent tolerance does not appear to be related to degradation, as most solvent tolerant

organisms tolerate a wide variety of aromatic hydrocarbons, alkanes and alcohols that they are incapable of degrading or transforming (Cruden et al., 1992; Inoue et al., 1991; Ramos et al., 1995; Weber et al., 1995). Most of these organisms do not harbor plasmids, indicating the resistance factors reside on the chromosome (Cruden et al., 1992; Ramos et al., 1995). Extrusion pumps have been postulated as having a role in solvent tolerance, but no data has been presented yet as to their existence.

Because of the membrane toxicity of monoaromatic hydrocarbons, it is likely that a mechanism exists in solvent tolerant strains that either prevents or compensates for membrane damage. The primary goal of this study is to determine the role of the cell envelope in the solvent tolerance phenomenon of *P. putida* Idaho and a similar, yet solvent sensitive control strain, *P. putida* MW1200. The goals of this study were the following:

- Determine differences in growth rate and levels of solvent tolerance between *P. putida* Idaho and *P. putida* MW1200
- Ascertain differences in phospholipid and fatty acid structure between the two strains in the absence and presence of *o*-xylene
- Determine the kinetics of phospholipid and fatty acid alteration in the two strains in the absence and presence of *o*-xylene
- Localize changes in phospholipid and fatty acid structure to inner and outer membranes

- Determine membrane biosynthesis rates and phospholipid turnover rates for each strain in the absence and presence of *o*-xylene
- Determine changes in LPS structure and composition of both strains in the absence and presence of *o*-xylene
- Relate the changes in LPS structure to cell surface hydrophobicity and permeability to hydrophobic compounds
- Incorporate the data obtained from the solvent sensitive and solvent tolerant strains into a model for the phenomenon of solvent tolerance

LITERATURE REVIEW

Degradation of Aromatic Hydrocarbons

The interaction of microorganisms with hydrocarbons is an important aspect of several disciplines in microbiology, including industrial microbiology, biotechnology, microbial ecology and bioremediation. Some of the common forms of hydrocarbons encountered in these applications are cycloalkanes, terpenes, and aromatic hydrocarbons. The aromatic hydrocarbons toluene, xylene and benzene are commonly found in fuels and industrial wastes. Because of their widespread use, these hydrocarbons have been released into the environment by a variety of means, causing contamination and pollution of water and soil.

Many aromatic hydrocarbons can be transformed into less harmful substances by microbial processes. The catabolic pathways for many of these transformations have been characterized for a wide variety of microorganisms (Gibson and Subramanian, 1984; Smith, 1990). The genetic determinants for these transformations are also well characterized (Assinder et al., 1990, Harayama et al., 1986, Harayama and Timmis, 1989). As understanding of the biodegradation processes grows, so does the potential to use this information in applications such as bioremediation and "end of the pipe" treatments of toxic and hazardous waste. One of the major stumbling blocks to the implementation

of this technology is that many of the compounds capable of being degraded are toxic to the bacteria that degrade them when the compound is present in high concentrations (Gibson et al., 1970; Jenkins et al., 1987).

The monoaromatic hydrocarbons are non-polar, and are lipophilic in nature. A summary of their physical characteristics is shown in Table 1.

Although somewhat limited by solubility in the aqueous phase, monoaromatic hydrocarbons are toxic to most bacterial cells at low concentrations. This toxicity was observed in early studies of biotransformation. Gibson et al. (1970) observed that the addition of benzene directly to the culture medium inhibited growth of *Pseudomonas putida*, but that addition via vapor phase could support growth. Toluene was also shown to inhibit growth under similar conditions (Jenkins et al., 1987). Aromatics are generally believed to enter the cell via passive diffusion. Naphthalene was shown to enter *Pseudomonas putida* in the absence of electrical potential and did not require ATP (Bateman et al., 1986). However, it should be noted here that plasmid-encoded uptake systems for toluene and biphenyl have recently been proposed (Lau et al., 1996). It is possible that active uptake systems also exist for the uptake of other hydrophobic compounds. Although utilization of substrates that enter the cell via passive diffusion have the advantage that no energy is required for uptake (thus increasing bioavailability), in the case of aromatic hydrocarbons it is the permeability to these compounds that facilitates hydrocarbon toxicity. In order to more fully understand this mechanism, an understanding of bacterial cell

Table 1. Physical Characteristics of Monoaromatic Hydrocarbons.

Compound	Formula	MW	Solubility ¹	Log P _{oct} ²
Benzene	C ₆ H ₆	78.11	22.9	2.13
Toluene	C ₇ H ₈	92.14	6.28	2.69
Ethylbenzene	C ₈ H ₁₀	106.17	1.27	3.15
Xylene	C ₈ H ₁₀	106.17	2.02	3.12

¹ mmol/L at 25°C

² octanol - water partition coefficient

structure is required.

Gram Negative Bacterial Membrane Structure

Because of their unique membrane structure, gram-negative bacteria are resistant to a large number of compounds toxic to other microorganisms (Nikaido and Vaara, 1985; Vaara, 1992). The outer membrane (OM) is impermeable to most macromolecules, and only allows limited diffusion of hydrophobic substrates (Hancock, 1987). The membrane structure of the gram-negative bacterium is well established (Jantzen, 1994; Labischinski et al., 1985; Seydel and Brandenburg, 1992). Its tri-laminar architecture consists of the outer membrane, the periplasm, and the inner cytoplasmic membrane (Figure 1-1). Both the outer and cytoplasmic membranes are arranged as a bilayer. The outer membrane is an asymmetric bilayer. The exterior leaflet of the outer membrane bilayer contains lipopolysaccharide (LPS), which consists of three regions, the O-antigen, the oligosaccharide core region, and Lipid A. The O-antigen region extends into the surrounding environment, while the negatively charged core regions of LPS are stabilized by magnesium and calcium divalent cations. The inner leaflet of the outer membrane is composed of phospholipid. Phospholipids are composed of a polar head region covalently bound to a glycerol backbone, and two fatty acids linked to the glycerol backbone via ester bonds. The outer membrane bilayer is interspersed with proteins known as porins, which form

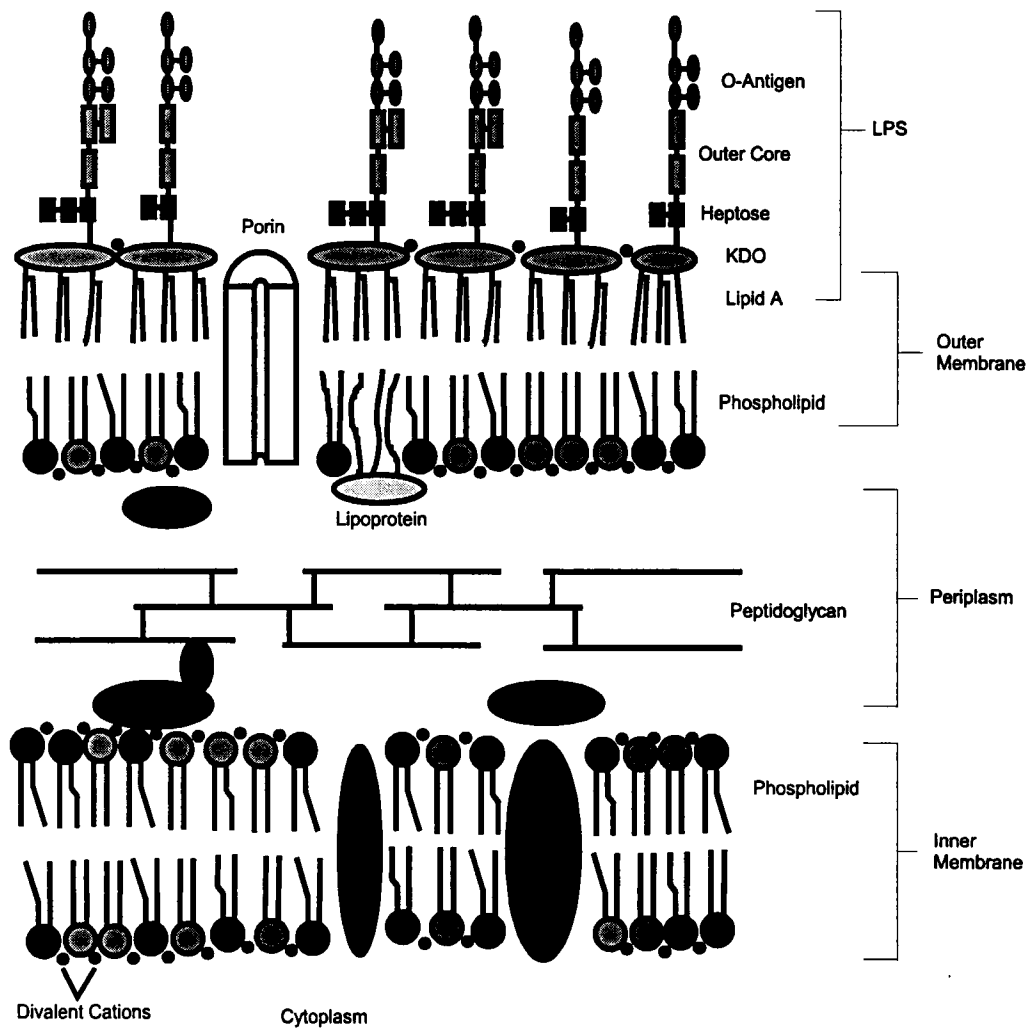


Figure 1-1. Membrane structure of a typical gram-negative bacterium. LPS, lipopolysaccharide; KDO, 2-keto-3-deoxyoctulosonic acid; divalent cations, Mg^{2+} and Ca^{2+} ; core, various oligosaccharides.

water-filled channels that span the outer membrane. Porins act as molecular sieves, permitting the passage of small hydrophilic molecules (such as cations) and to the periplasm.

The polar heads of the outer membrane phospholipid layer extend into the periplasm, intermittently bound to a matrix of highly hydrated peptidoglycan through an amide linkage to murein lipoprotein. The cytoplasmic membrane is a phospholipid bilayer. Each phospholipid molecule contains two fatty acids of variable chain length. The phospholipid fatty acids of both outer and inner membranes may be saturated or monounsaturated. Polyunsaturated fatty acids are not found in *Pseudomonas* species (Wilkinson, 1988). In both the inner leaflet of the outer membrane and the bilayer of the inner membrane, the negatively charged, polar heads of the phospholipids are stabilized by divalent magnesium cations.

The role of the bacterial cell envelope is to maintain cellular structure while supporting various transport activities and protein functions. These include solute transport, turgor pressure, signal transduction, and maintenance of membrane potential. The phospholipid bilayer of the cytoplasmic membrane has a characteristic ability to change its state from a gel to a liquid. This change of state is termed phase transition, and is generally associated with temperature. This is an important concept because cells require a certain degree of fluidity for optimal membrane function (Melchior, 1982; Sinesky, 1974). A membrane that is too fluid cannot maintain the structural integrity required for a unicellular

organism, while a membrane that is too rigid cannot create the correct environment for membrane protein function. Failure to maintain fluidity can result in the loss of transport properties and in leakage of proteins and other cellular components through "cracks" in the membrane (McElhaney, 1982; Sikkema et al., 1994).

Hydrocarbon Interaction with Lipids and Membranes

Several studies have examined the interactions of hydrocarbons with biological membranes. This relationship is usually expressed as a membrane-buffer partition coefficients or octanol-water partition coefficients. These coefficients are used to predict effects of lipophilic compounds on biodegradation, bioconcentration, toxicity and anesthetic effects. However, these are only broad estimations of toxicity, as partitioning of various lipophilic compounds is highly dependent on the lipid composition of the biological membrane. As shown in Table 1-2, variation of the fatty acid composition of the same phospholipid can have a significant effect on membrane partition coefficients (Antunes-Madeira and Madeira, 1984, 1985, 1986, 1987). The lipophilic nature of aromatic hydrocarbons allows partitioning within phospholipid membranes. Studies with anesthetics have shown that accumulation of lipophilic compounds substantially increases the volume of the membrane bilayer. This was supported by studies with lipid monolayers that show the surface pressure of the monolayer increases linearly with the amount

Table 1-2. Differential partition coefficients of lipophilic compounds between synthetic membranes and aqueous phases based on membrane composition ^{b, c}.

Lipophilic Compound	DMPC ^a	DPPC ^a	DSPC ^a
Malathion	225	135	48
Parathion	1,950	650	270
Lindane	2,250	600	50
DDT	336,000	180,00	88,000

a. DMPC, dimyristoylphosphatidylcholine (14:0); DPPC, dipalmitoylphosphatidylcholine (16:0); DSPC, distearoylphosphatidylcholine (18:0)

b. membrane - buffer partition coefficient data from Antunes-Madeira and Madeira, 1984-1987.

c. adapted from Sikkema et al., 1995

of solute that entered the monolayer. Hydrocarbons will generally partition within the aliphatic (fatty acid side chain) portion (Boden et al., 1991; King et al., 1985) as well as the interior bilayer portion of the phospholipid bilayer (Sikkema et al., 1995). Fluorescence polarization studies with *E. coli* (Sikkema et al., 1994; Cranney et al., 1983) showed that addition of cyclic hydrocarbons caused an increase in the bilayer surface area due to interaction of the hydrocarbons with the fatty acyl side chains of the phospholipids. The accumulation of hydrocarbons within phospholipid fatty acids and the resultant membrane expansion has several ramifications with regards to proper membrane function (Sikkema et al., 1995).

The first and most obvious consequence of hydrocarbon accumulation in phospholipid membrane is the swelling of the membrane to accommodate the lipophilic material (Antunes-Madeira and Madeira, 1989; McIntosh et al., 1980; Pope et al., 1986, Pope et al., 1989, Simon et al., 1984). This swelling expands the membrane, creating pores throughout the bilayer (Figure 1-2). Small molecules such as magnesium ions, potassium ions, sodium ions and protons leak through these pores, resulting in a loss of stability of the phospholipid bilayer and disruption of proton motive force (de Smet et al., 1978; Sikkema et al., 1994, 1995). The leakage of protons also interferes with the cell's ability to maintain intracellular pH essential for homeostatic processes. Pore formation also allows more hydrocarbon molecules to enter the membrane, thus perpetuating the problem. Following pore formation, it is also possible to lose

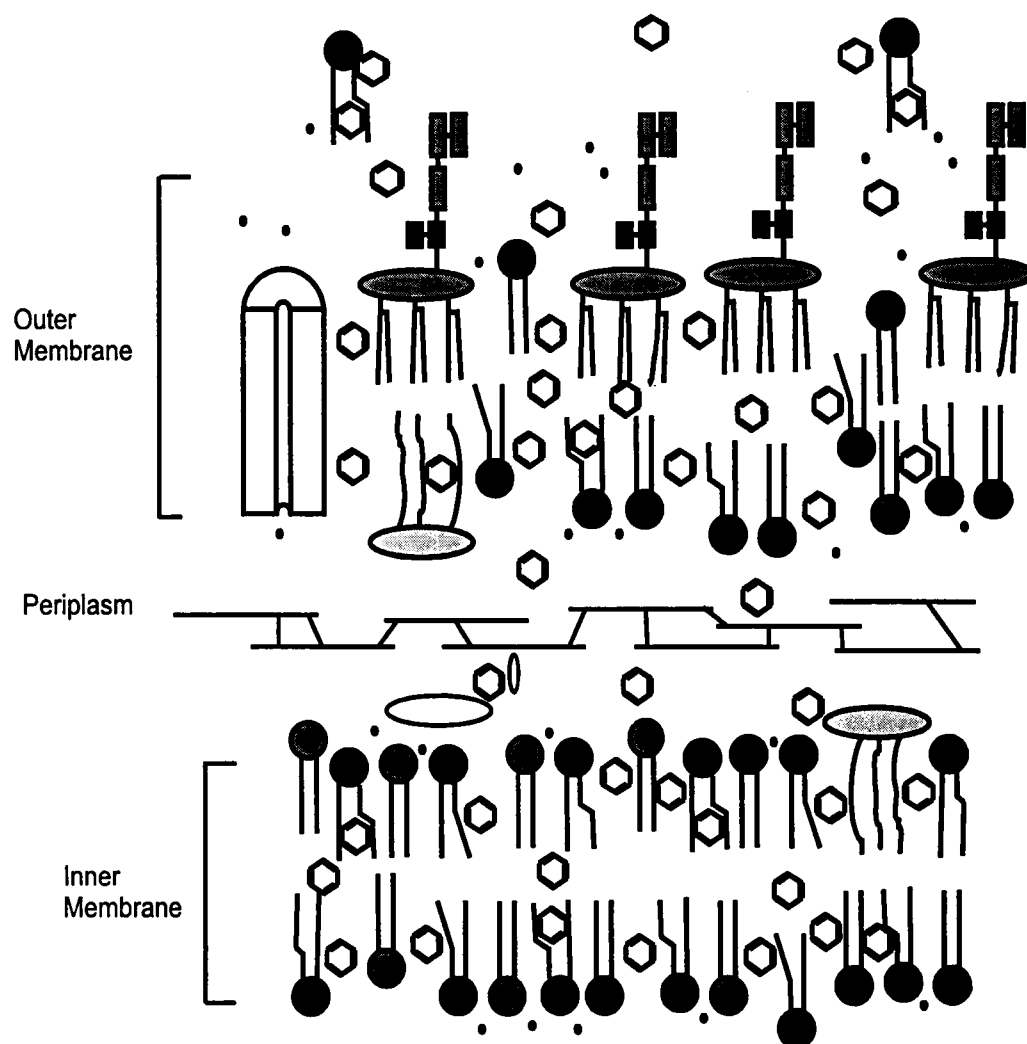


Figure 1-2. Monoaromatic hydrocarbon interaction with the gram-negative bacterial membrane, showing disorganization of phospholipid bilayers. Structures are the same as in Figure 1-1.

lipid material via extraction by solvent that is in the surrounding medium, creating further disruption of the cytoplasmic membrane (de Smet et al., 1978). At high concentrations of aromatic hydrocarbons, loss of membrane integrity leads to cell lysis.

The toxic effects of benzene and toluene have been studied for *E. coli* and *Pseudomonas* species. Jackson and DeMoss (1965) showed that the galactose permease system was completely inhibited in the presence of toluene. Electron micrographs show extensive cytoplasmic membrane damage, as well as some sloughing of the outer membrane (Woldringh, 1974; de Smet et al., 1978). Increased permeability of cells exposed to toluene and other hydrocarbons has been shown by several groups (de Smet et al., 1978; Heipieper et al., 1991; Sikkema et al., 1994; Silver, 1967).

Although most studies have not directly addressed the effect of toluene on membrane protein function, several inferences may be made about these effects. Most membrane-embedded proteins require intact boundary lipids for proper conformation and function. Disruption of this interaction can render the protein inactive. Hydrophobic regions of membrane-spanning proteins may interact with the hydrocarbon, thus obscuring active sites or preventing conformational changes required for protein function. Vercellone-Smith and Herson (1994) showed inhibition of protein synthesis in *P. putida* mt-2 following toluene exposure. Proteins requiring proton-motive force for their function would be indirectly inactivated by the presence of aromatic

hydrocarbons. Swelling of the cytoplasmic membrane could inactivate those proteins sensitive to membrane thickness (In't Veld, 1991), and could prevent the interactions of protein complexes that span the cytoplasmic membrane and periplasm, such as those involved in signal transduction. Finally, those membrane proteins sensitive to membrane fluidity (heat shock sensors, etc.) could also be adversely affected by the presence of hydrocarbons, which serve to increase membrane fluidity.

In some very recent work, Vercellone-Smith and Herson (1996) demonstrated shifts in protein synthesis by 2D PAGE in *P. putida* mt-2 exposed to sub-lethal and lethal concentrations of toluene. When exposed to sub-lethal concentrations of toluene (4 ppm), protein synthesis was inhibited by 70-90%, with a specific protein pattern expressed. The addition of *m*-toluate alleviated this response. At higher concentrations of toluene (130 ppm), the same response was noted, with the exception that the pattern expressed in the toluene exposed cells was maintained for a longer period of time, although this pattern of expression was again alleviated by the addition of *m*-toluate. At lethal concentrations (267 ppm), the some starvation proteins were observed prior to cell death. It was also found that addition of *m*-toluate hastened cell death. Surprisingly, the protein pattern showed little resemblance to heat-shock protein patterns. It did, however, show close resemblance the pattern of proteins expressed in carbon-starvation response. Identification of these proteins as carbon starvation proteins was supported by the data that addition of *m*-toluate

alleviated the carbon starvation response at 4 and 130 ppm toluene concentrations. The fact that the addition of *m*-toluate hastened cell death at 267 ppm toluene while suppressing carbon starvation response suggests that the carbon starvation response plays a protective role in cells exposed to starvation conditions (i.e. "leaky" membranes in the case of hydrocarbon exposure).

In summary, the toxic effects of aromatic hydrocarbons are associated with loss of cytoplasmic membrane integrity. This loss of membrane integrity results in disruption of proton motive force, loss of membrane barrier functions, inhibition of membrane protein function, and subsequent cell lysis and death.

Compensation for Membrane Fluidity

One of the immediate consequences of exposure of prokaryotes to monoaromatic hydrocarbons is an increase in membrane fluidity. Most eukaryotic cells can alter membrane fluidity by inserting cholesterol molecules within the phospholipid bilayers. This is not an option for bacteria because they lack the biosynthetic machinery to synthesize cholesterol. Despite this apparent handicap, bacterial alteration of membrane structure in response to changes in membrane fluidity is well documented (Guckert et al., 1986; Henderson et al., 1993; Langworthy, 1982; Miller, 1985; Nichols et al., 1994). For example, the phospholipid compositions of both gram-negative and gram-positive bacteria have been shown to undergo major shifts when exposed to increases in temperature. Elevated temperatures have been shown to cause an increase in the

fatty acid chain length (Lepage et al., 1987), increase the percentage of saturated fatty acids (Ingram, 1977), and cause shifts in the phospholipid classes (Benschoter and Ingram, 1986) in a variety of microorganisms.

Phosphatidylethanolamine has a higher melting point than phosphatidylglycerol, and is often found in higher concentrations at higher growth temperatures. Most growth temperature studies have been performed on enteric bacteria, but a few investigators have examined temperature response in *Pseudomonas* species. *P. aeruginosa* has demonstrated the ability to grow in temperatures ranging from 15° - 45° C. Like the enteric bacteria, it conforms to the rules of homeoviscous adaptation (Sinesky, 1974), changing membrane lipids of both the inner and outer membrane (Kropinski, 1987). As temperature increases, the percentage of saturated phospholipid fatty acids (dodecanoic, hexadecanoic and octadecanoic acid) increases, while the percentage of unsaturated fatty acids, hexadecenoic and octadecenoic acid, decreases. The fatty acids found in Lipid A of the outer membrane also change as temperature increases. Dodecanoic acid, hexadecanoic acid, and 3-hydroxydodecanoic acid increased, while 3-hydroxydecanoic and 2-hydroxydodecanoic acid decreased (Kropinski, 1987). An increase in the LPS:phospholipid ratio was noted as well. This increase in carbon chain length and saturation helps to maintain the proper phase transition state at increased temperature to maintain normal membrane permeability properties as well as providing the suitable environment for membrane proteins.

In addition to temperature, several other factors may also affect membrane integrity and fluidity. Decreasing pH results in an increase in the level of saturated and cyclopropyl fatty acids, and in the case of *Sarcina ventriculi*, the formation of long, 32 to 36 carbon dicarboxylic acids (Jung et al., 1993). Acidophilic bacteria have been shown to contain high concentrations of cyclopropyl and cyclohexyl fatty acids (Langworthy, 1982), which has been suggested as an advantageous adaptation for maintaining membrane integrity. Exposure to alcohols generally results in an increase in the relative percentage of saturated fatty acids to compensate for the fluidizing activity of the alcohols in *E. coli* and *Clostridium* species (Ingram, 1986; Lepage, 1987). Marine *Vibrio* species have demonstrated an increase in unsaturated fatty acids when subjected to increases in hydrostatic pressure (DeLong and Yayanos, 1985). In *Pseudomonas* and *Vibrio* species, the conversion of unsaturated fatty acids from *cis* to *trans* has been linked to combating membrane damage due to environmental stresses such as temperature shifts (Guckert et al., 1986; Morita et al., 1993) and the presence of phenol and toluene (Heipieper et al., 1991; Weber et al., 1994). The *trans* isomer of the unsaturated fatty acid is thermodynamically more stable than the *cis* isomer, which increases membrane phase transition temperature, and has been suggested as a mechanism for avoiding substrate toxicity. These changes maintain membrane integrity by regulating the degree of fluidity required for optimal membrane protein activity and membrane permeability.

Studies have shown that oxygen limitation can induce the formation of cyclopropyl fatty acids in *P. denitrificans* (Jaques and Hunt, 1980). Further studies have shown that it appears to be an indirect function of oxygen tension; the production of cyclopropyl fatty acids in *P. denitrificans* is directly related to the state of reduction of components of the respiratory system rather than oxygen tension per se (Jacques, 1981), and therefore is associated with starvation response.

P. aureofaciens lipid profiles were strongly influenced by moisture and nutrient availability (Kieft et al., 1994). The strain studied showed a marked increase in saturated/unsaturated fatty acid ratios, an increase in *trans*-unsaturated fatty acid to *cis*-unsaturated fatty acid ratios, and an increase in cyclopropyl fatty acids following desiccation. These changes in fatty acid profiles coincided with entry into the viable but non-cultureable state of the starved, desiccated cells. While the specific reason for these shifts aren't completely understood, it is generally believed that the increased saturation of the fatty acids, coupled to the modifications of the unsaturated fatty acids, create a membrane with a higher phase transition temperature. A membrane with a higher phase transition temperature is thought to create a more rigid, less permeable cell envelope capable of maintaining envelope integrity during environmental stress such as starvation.

Several studies involving the membrane alterations of *P. putida* strains resistant to high concentrations of phenol, toluene, ethanol and other organic

solvents have been conducted recently. One common response seen in cells exposed to toluene, xylene, and phenol is the formation of *trans*-unsaturated fatty acids (Heipieper et al., 1992; Pinkart et al., 1996; Weber et al., 1994). The mechanism for formation of *trans*-unsaturated fatty acids has not been extensively characterized, but evidence exists for both de novo synthesis of *trans*-unsaturated fatty acids (Guckert et al., 1986), as well as for an isomerization of the intact phospholipid (Heipieper and de Bont, 1994). Some strains demonstrated increase in cyclopropyl fatty acids when exposed to xylene, which is also a modification of the intact phospholipid. An increase in saturated fatty acids following exposure to organic solvents has been noted in some strains, but not in others. Exposure to ethanol causes a decrease in saturated fatty acids in *P. putida* S12. Ethanol also caused an increase in C₁₈ fatty acids relative to C₁₆, while toluene caused an opposite reaction. This unexpected response was postulated to be due to inhibition of fatty acid biosynthetic enzymes by ethanol (Heipieper and de Bont, 1994).

Alterations of Surface Structure and Permeability

Alterations in the outer membrane structure of gram-negative bacteria have the consequences of changing the permeability and surface charge of the cell. Changing these properties can result in antibiotic resistance, and changes in the ability of cells to adhere to surfaces and interact with hydrophobic compounds. In *Shewanella* species, reduction of O-antigen polysaccharide chain

length, or complete omission of the O-antigen region creates a much more hydrophobic cell surface (Pickard et al., 1993; Sledjeski et al., 1991). It was suggested that this adaptation allows better interaction with hydrophobic substrates such as oil droplets. *Thiobacillus* with altered outer membrane structure has better interaction with hydrophobic sulfur prills (Arredondo et al., 1994). *Aeromonas* species have shown the ability to alter cell surfaces and permeability properties by changing surface proteins in response to low pH, a response termed adaptive acid tolerance (Karem et al., 1994). Several *Pseudomonas* strains are antibiotic-resistant due to changes in the LPS and protein structure of the cell surface, which change the cell surface hydrophobicity and permeability properties (Arnoff et al., 1988; Legakis et al., 1989; Leying et al., 1992; Michea-Hamzehpour et al., 1991; Piddock et al., 1992).

Some *Pseudomonas* strains have developed antibiotic resistance through membrane alterations (Hancock, 1984). *P. aeruginosa* strains with increased amounts of KDO and Lipid A were found to be much less permeable to hydrophilic quinolones, and had much higher resistance to these antibiotics (Chamberland et al., 1990; Michea-Hamzehpour et al., 1991). In an experiment comparing four *P. aeruginosa* strains with respect to lipid content and antibiotic susceptibility, it was found that strains with a higher lipid content were considerably more resistant to ampicillin than strains containing less lipid

(Norris et al., 1985). Similar results were observed by Cox and Wilconson (1991).

Resistance Pumps

Many bacterial strains have acquired export pumps to actively remove antibiotics (Furukawa et al., 1993; Neyfakh et al., 1991; Levy, 1992), hydrophobic cations (Ahmed et al., 1994; Brozel and Cloete, 1993; Sakagami et al., 1989), and proton motive force uncouplers (Lewis, 1992; Lomovskaya et al., 1995) from the cell. The resistance to uncouplers results from a protein complex termed EmrAB, which pumps out the uncoupler (such as CCCP) from the periplasm into the external medium thus preventing the compound from entering the cytoplasmic membrane. The Emr protein complex also pumps out thiolactomycin, a hydrophobic antiobiotic. This type of resistance pump has relatively broad substrate ranges, so the possibility of a multidrug resistance pump that could extrude aromatic hydrocarbons is reasonable. Preliminary evidence by Ramos et al. (1996) and Isken and de Bont (1996) indicate that such systems may exist in their solvent-tolerant strains. Isken and de Bont showed that the presence of the uncouplers CCCP and potassium cyanide resulted in accumulation of toluene in their previously solvent-tolerant strain, concluding that an active export/efflux system must be responsible for the observations. Although no specific extrusion pumps have been definitively identified for solvent resistance, their existence cannot be ruled out at this time.

Solvent Tolerant Organisms

In the past several years, many organic solvent-tolerant organisms have been discovered. The first report of toluene-resistant bacteria was by Inoue and Horikoshi (1989). Although these *Pseudomonas* strains could not grow on toluene, they tolerated very high concentrations of aromatic hydrocarbons. Cruden et al. (1992) isolated a solvent-resistant *P. putida* strain which could grow on toluene, *p*-xylene and *m*-xylene, and tolerate these compounds in biphasic organic-aqueous media. *P. putida* Idaho could also tolerate *o*-xylene and a variety of other organic solvents, though it lacked the ability to degrade them. Another *P. putida* strain capable of degrading styrene and withstanding high concentrations of this organic compound (Weber et al., 1993) was isolated by styrene enrichment at low concentrations. The most recent organism discovered to be solvent tolerant was isolated by Ramos et al. (1995), and was also a *Pseudomonas putida* strain. This strain exhibited the ability to tolerate a wide range of organic solvents similar to the previously mentioned strains. Solvent tolerant *P. aeruginosa* strains have also been isolated (Aono et al., 1992). An interesting *P. aeruginosa* strain isolated by Ogino et al. (1994, 1995) has shown the ability to synthesize organic solvent-stable proteases and lipases. The enzymes were stable in the presence of saturating concentrations of linear alkanes, aromatic hydrocarbons, and ethanol, with only 50% loss of activity in the presence of chloroform (Ogino et al., 1994, 1995). To date, no gram-positive bacteria resistant to monoaromatic hydrocarbons have been reported.

As of yet, the mechanism for solvent tolerance is unknown. However, there are some conclusions from the current data that can be made. The first is that the determinants for solvent tolerance are probably not located on a plasmid. Efforts to isolate plasmids from the solvent tolerant strains have shown that these organisms do not harbor plasmids of any type (Cruden et al., 1992; Ramos et al., 1995). In those organisms with catabolic activity, the genes responsible for toluene degradation were located on the chromosome. The second conclusion that may be drawn is that the ability to degrade the hydrocarbon is not a factor in solvent tolerance. Since some solvent-tolerant strains do not have the ability to degrade the solvents that they can tolerate in high concentrations (Inoue et al., 1989; Cruden et al., 1992), it is unlikely that rapid substrate detoxification is a factor in solvent tolerance. Work by Wolfram and Rodgers has shown that *P. putida* Idaho does not transform *o*-xylene in any way (unpublished data). The third conclusion that may be drawn is that solvent-tolerant bacteria require elevated concentrations of magnesium cations to grow. This requirement was demonstrated by Ramos et al. (1995) and by Inoue et al. (1991). The fourth conclusion that can be made about these strains is that although they grow in high concentrations of aromatic hydrocarbons, the cytoplasmic membranes of these strains did not escape damage, as shown by electron micrographs. It should be noted, however, that these strains appeared somewhat less damaged than toluene-exposed *E. coli* pictured in the studies by Woldringh (1974) and de Smet et al. (1978).

At this time, no hydrocarbon extrusion pumps have been definitively described for these strains. The fact that the cytoplasmic membranes of solvent-tolerant strains show damage may indicate that if an extrusion system does exist, it is not very efficient. Ferrante et al. (1995) described an alkylperoxide reductase of an *E. coli* mutant which demonstrated increased activity for hydrophobic hydroperoxides. This mutant demonstrated increased resistance towards tetralin, cyclohexane, propylbenzene, and 1,2-dihydronaphthalene. This strain was still sensitive to toluene, benzene, and xylenes. Two genes in *E. coli*, designated *ostA* (Aono et al., 1994) and *robA* (Nakajima et al., 1995), have been suggested to confer hexane tolerance and cyclohexane tolerance, respectively. Regardless, the functions of the gene products are not yet known, and confer no resistance to monoaromatic hydrocarbons.

PART II.

PHOSPHOLIPID AND FATTY ACID ALTERATION OF *PSEUDOMONAS PUTIDA* IN RESPONSE TO *O*-XYLENE

INTRODUCTION

Because much of the initial damage caused by straight chain and aromatic hydrocarbons occurs at the phospholipid membranes (de Smet et al., 1978; de Smet et al., 1983; Heipieper et al., 1992; Sikkema et al., 1994; Sikkema et al., 1995; Woldringh, 1974), the membrane structure of solvent resistant strains warrants further study. *Pseudomonas putida* Idaho grows in high concentrations of organic solvents, so it is likely that it harbors some mechanism that either prevents or compensates for membrane damage. Several mechanisms for decreasing membrane fluidity (due to temperature increases, solvents, etc.) have been demonstrated in bacteria. Changes in polar head groups create increases in membrane phase transition state (McElhaney, 1982; Melchior, 1982; Sinesky, 1974). Increasing the relative percentage of saturated fatty acids will decrease membrane fluidity (Ingram, 1986) following ethanol exposure, and the conversion of *cis*-unsaturated fatty acids to the *trans*-isomer has been suggested as a mechanism for avoiding substrate toxicity in *P. putida*. (Heipieper et al., 1992; Weber et al., 1994). However, none of these studies have examined the response of solvent-tolerant organisms versus solvent-sensitive organisms in side-by side comparisons, so no definite conclusions can be made.

Pseudomonas putida Idaho (Cruden et al., 1992) grows in a biphasic organic-aqueous media with solvents such as xylene or toluene as the sole carbon and energy source. In a biphasic system, the organic compound is found

in saturating concentrations in the aqueous phase (the concentration is based on its solubility in water; for xylene this is 200 mg per liter). The ability of the Idaho strain to degrade toluene and xylene (*para* and *meta* isomers) is due to the presence of the *xyl*/CAB genes on its chromosome. It tolerates *o*-xylene, phenol, benzene and several other organic compounds in very high concentrations, although it lacks the ability to degrade these compounds. Solvent resistance is not altered when the surfactant Triton X-100 is added in concentration of 1000-3000 ppm (Jim Wolfram, personal communication).

The objectives of this study were to characterize response of two strains of *Pseudomonas putida* to various concentrations of *o*-xylene in terms of growth and with respect to membrane adaptation in the presence and absence of monoaromatic hydrocarbons. The first of the two strains is *P. putida* Idaho, a strain capable of thriving in aqueous solutions saturated with xylenes and toluene. The second strain is *P. putida* MW1200, a strain similar to Idaho in that it also carries the TOL genes on its chromosome (Sinclair et al., 1986), but lacks the ability to withstand high concentrations of monoaromatic hydrocarbons. The first goal was to establish the differences in growth rate and monoaromatic hydrocarbon tolerance between the solvent-tolerant and solvent-sensitive organisms. Following establishment of these differences (if any), the second goal of this study was to determine any differences in phospholipid structure and composition and phospholipid fatty acid structure in *P. putida* Idaho versus *P.*

putida MW1200 when each strain is exposed to increasing concentrations of *o*-xylene.

MATERIALS AND METHODS

Strains

For all experiments, two strains of *Pseudomonas putida* were used. *P. putida* Idaho was first isolated by Dr. James Wolfram and Dr. Robert Rogers at Idaho National Engineering Laboratories (U.S. Patent 4,863,872, September 1989). This strain was further characterized by Cruden et al. (1992). Table 2-1 shows the list of organic solvents *P. putida* Idaho is can tolerate in saturated aqueous solutions. The lowest Log P_{OCT} for this strain to withstand in saturating concentrations is 2.3. *P. putida* Idaho can also tolerate benzene (Log P_{OCT} 2.0) and fluorobenzene (Log P_{OCT} 2.2) in the aqueous phase, but at lower concentrations than saturation. Although it can tolerate many organic solvents in saturating concentrations, it only has the ability to degrade toluene, *m*-xylene and *p*-xylene. This is due to the presence of the *xylCAB* genes on its chromosome. These genes are homologous to the pWWO plasmid of *P. putida* mt-2, and encodes degradation of toluene via the benzyl alcohol pathway. No plasmids have been isolated from *P. putida* Idaho (Cruden et al., 1992; Pinkart, unpublished data). The second strain used for these experiments is *P. putida*

Table 2-1. Growth of *P. putida* Idaho in the presence of saturating concentrations of organic solvents^a.

Solvent	Log P _{OCT}	Growth ^b
<i>n</i> -Decane	5.6	+++
<i>n</i> -Octane	4.5	+++
Cyclooctane	4.1	+++
<i>n</i> -Heptane	4.0	+++
1-Octene	3.7	+++
Propylbenzene	3.6	+++
<i>n</i> -Hexane	3.5	+++
Diethylphthalate	3.3	+++
Cyclohexane	3.2	+++
Ethylbenzene	3.1	+++
<i>p</i> -Xylene	3.1	+++
<i>n</i> -Pentane	3.0	++
Styrene	3.0	+++
1-Octanol	2.9	+++
Cyclopentane	2.5	+++
Toluene	2.5	+++
1,5-Hexadiene	2.5	++
1-Heptanol	2.4	+++
Dimethylphthalate	2.3	+++

a. Adapted from Cruden et al., 1992.

b. Turbidity at 600 nm ; +++, >1 after 24h; ++, 0.7 - 1.0 after 24h.

MW1200. This strain has the ability to grow with toluene as the sole carbon source in both minimal and complex media at 28 or 37°C. No plasmids were isolated from this strain, indicating that the genes for toluene degradation are located on the chromosome. The location of these genes was mapped by Sinclair et al. (1986), and found to be linked to *vil* (valine-isoleucine-leucine) and *catB* (muconate lactonizing enzyme). The 56 kb region containing the toluene degradation genes is almost identical to the same region found on the TOL plasmid pWWO. This strain is not reported to be solvent-tolerant.

Culture conditions and doubling time determinations

Unless otherwise stated, for all experiments each strain was grown in a minimal salts medium with 10 mM sodium succinate as the carbon source. The minimal salts medium was formulated as follows: 0.7 g K₂HPO₄, 0.7g KH₂PO₄, 0.5 g (NH₄)₂SO₄, 0.3 g MgSO₄, and 1 ml Wolfe's Minerals (Balch et al., 1979) per liter of nanopure water. Most cultures were started using 5 mg lyophilized cells. Batch cultures, when used, were incubated at 25°C -27°C with rotary shaking at 200 rpm. In this section, batch cultures were used for growth curves and determination of doubling time. Succinate was used as a carbon source for these strains to avoid catabolite repression problems associated with other carbon sources (such as glucose). For the experiments utilizing organic solvents, *o*-xylene was added directly to the aqueous phase of the medium. The

concentration of *o*-xylene in the aqueous phase was verified by gas chromatographic analysis. Neither *Pseudomonas putida* strain had the ability to transform or mineralize *o*-xylene. The use of this compound had two advantages. The first was that it eliminated substrate utilization as a contributor to solvent tolerance. The second advantage was that this allowed for stable maintenance of xylene concentration in the medium, with no loss due to substrate consumption by the bacteria. For determination of doubling time, optical density of the cultures was monitored, with samples taken for viable counts at various time intervals. Doubling time was established by plate counts (that time in which the bacterial population has doubled in number) following entry into exponential growth. Optical density measurements were used in subsequent experiments to establish growth phase and to estimate cell number.

For continuous culture experiments, the medium described above was utilized. Continuous cultures were used to maintain each strain in exponential phase for analysis of cellular components. It was important to utilize cells in exponential phase because many of the changes bacterial cells experience upon entry into stationary phase could contribute to general starvation resistance, and could interfere with the observations of solvent tolerance mechanisms.

Conditions for continuous culture were established using the equation:

$$Fx/V + \mu x = dx/dt$$

where F = medium flow rate

V = liquid volume of the bioreactor

x = bioreactor cells

μ = specific growth rate

dx/dt = rate of change of cell mass

Phospholipid Extraction

Cells (100 ml) were removed from continuous culture (in the absence or presence of *o*-xylene), centrifuged, the supernatant discarded, and the cell pellet lyophilized. The pellet is then extracted using a modified Bligh and Dyer technique (Guckert et al., 1985). This method extracts cellular lipids by the use of the solvent system methanol:chloroform:phosphate buffer (2:1:0.8).

Following extraction, the total lipid portion was applied to a silicic acid column. The lipid samples were then fractionated into a neutral lipid fraction, a glycolipid fraction and a polar lipid fraction by elution with chloroform, acetone and methanol, respectively. The phospholipids were contained in the polar lipid fraction.

Separation of Inner and Outer Membranes

Some experiments required separation of the inner and outer membranes prior to lipid analysis. For these experiments, cells were harvested from the continuous culture in 100 ml samples, filtered, and washed in phosphate buffer to remove residual material. The cells were converted to spheroplasts by osmotic shock

(Hancock and Poxton, 1988), then disrupted by sonication. The membranes were then concentrated by centrifugation following DNase and RNase digestion. The inner and outer membranes were separated by sucrose density centrifugation (de Smet et al., 1983; Matsushita et al., 1978; Nieboer et al., 1993). Purity of each fraction was assayed by the presence of KDO, and by NADH oxidase activity. KDO is specific for the outer membrane, and its presence in the inner membrane indicates incomplete separation of the inner and outer membranes. NADH oxidase is specific for the inner membrane, and should not be detected in outer membrane preparations. If specific changes in phospholipid or PLFA are localized to either the inner or the outer membrane, this step allows for the detection of this type of localization.

Identification of Phospholipid Class

The polar lipid fraction obtained from silicic acid separation was further separated into phospholipid classes using one dimensional thin layer chromatography (TLC)(Kates, 1975) developed in chloroform:methanol:water:acetic acid (85:22.5:3:15). Phospholipids were visualized under 254 nm UV light following treatment with rhodamine. Individual phospholipids were identified by comparing R_f values of the sample lane with the R_f values of known standards. The structures were verified by direct probe mass spectrometry following elution of the phospholipid from the silica gel (negative

ion scans). The eluted phospholipids were also analyzed for fatty acid content (see below).

Phospholipids were also quantified and separated using HPLC with an evaporative light scattering detector (Becart et al., 1990). A binary gradient was used with the following solvent system: solvent A chloroform:methanol: 30% ammonium hydroxide (80:19.5:0.5); solvent B chloroform:methanol:water: 30% ammonium hydroxide (60:34:5.5:0.5), with a flow rate of 1 ml/min. This allowed quantitative recovery of each phospholipid class without derivatization. However, this is a destructive technique that doesn't allow for further characterization of the phospholipids, and therefore was only used for quantitation purposes.

Fatty Acid Analysis

Phospholipid fatty acid methyl esters (PLFAME) are obtained by through a transesterification reaction (Guckert et al., 1986). The phospholipid fraction is subject to a mild alkaline methanolysis, which cleaves the ester linkage between the glycerol backbone and the fatty acyl side chains of the phospholipid, and subsequently forms fatty acid methyl esters. PLFAME are separated by gas chromatography. Identifications of individual fatty acids are made by comparing the retention times with FAME standards. Individual fatty acid methyl ester peak areas are quantified by comparison to a peak area of known concentration

of internal standard (dinonadecanoic phosphatidylcholine, 25 pmol/ μ l) added to the sample prior to extraction. Verification of fatty acid structure is achieved using mass spectrometry. Double bond position of monounsaturated fatty acids is verified by mass spectral analysis of dimethyl disulfide adducts of the fatty acids (Nichols et al., 1986).

Statistics

Data points shown are an average of three independent experiments, with all error bars on graphs representing one standard deviation. Calculations were performed with GraphPad Prism software (San Diego, CA). All numbers shown on tables are an average of at least three experiments (n=3), and are shown as the mean $\pm$ one standard deviation. T-tests were used to determine significant differences, using $\alpha = 0.01$.

RESULTS

Growth Curves

The growth curves of *P. putida* MW1200 and *P. putida* Idaho are shown in Figure 2-1. The growth conditions were the same for each strain. The conditions shown are growth in 10 mM succinate minimal salts medium, 10 mM succinate

minimal salts medium + 75 ppm *o*-xylene, and 10 mM succinate minimal salts medium + 200 ppm *o*-xylene (saturated).

When grown in the absence of xylene, both strains exhibit similar patterns of lag and exponential growth. Each strain showed a lag phase of approximately 4.5 - 5.0 hours. However, the Idaho strain shows a rapid loss of viability following a short stationary phase. The MW1200 strain does not exhibit this behavior. In the presence of solvent, the MW1200 strain shows no lag period in the presence of 75 ppm xylene. No growth was observed for this strain in the presence of 200 ppm *o*-xylene.

The Idaho strain exhibits a lag phase in the presence of solvent that exceeds that of cells grown in the absence of solvent. When exposed to 75 ppm *o*-xylene, the lag was 7 hours, and increased to approximately 11 hours in the presence of 200 ppm *o*-xylene. When grown in batch culture, the ability to reach high optical densities in the presence of xylene was decreased compared to cells grown in the absence of xylene. This decrease appeared to be proportional to the amount of xylene added (Figure 2-1). Although a decrease in optical density was observed, it did not appear to be as great as for the cells grown in the absence of xylene. The decrease in optical density was accompanied by a decrease in viable cell number (Figure 2-2).

Doubling times

The doubling time for each strain was established by viable counts (Figure 2-2) taken during exponential phase along with OD readings. The doubling time for *P. putida* MW1200 was 1.36 hours, with no significant difference in doubling time seen for the cells grown in the presence of 75 ppm *o*-xylene. *P. putida* Idaho had a doubling time of 1.41 hours in the absence of solvent. In the presence of solvent the average doubling time increased to 1.54 hours, but was not statistically different ($P = 0.062$).

Based on the 1.41 hour doubling time, a flow rate of 4.1 ml/min. (reactor volume 500 ml) was calculated for continuous culture experiments. In continuous culture, it was possible to maintain *P. putida* Idaho at high cell densities in the presence and absence of xylene for periods of two weeks or more.

Xylene Kill Curves for P. putida MW1200

P. putida MW1200 was challenged with various concentrations of xylene to establish its threshold tolerance for xylene. The threshold tolerance is the amount of xylene which it can tolerate without a loss of viability. The kill curve is shown in Figure 2-2. It was established that 75 ppm was the threshold tolerance for *P. putida* MW1200. At 85 ppm xylene, the cells lost the ability to grow, but could still be cultured after 24 hours. At 100 ppm, viable cells could

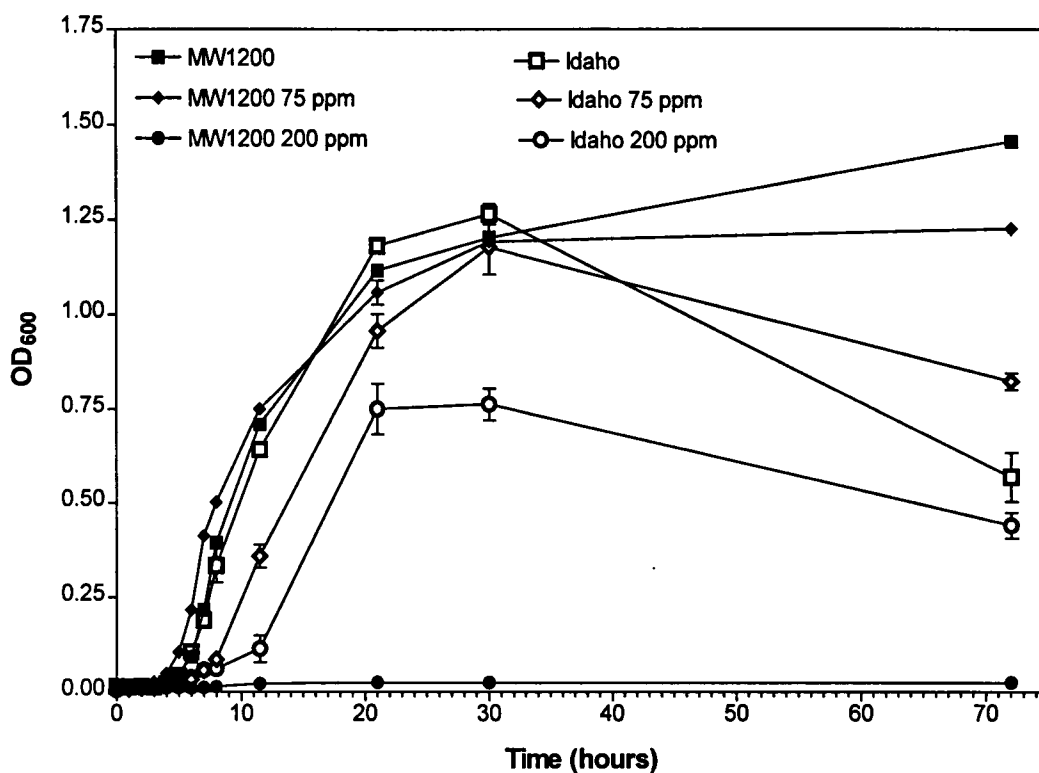


Figure 2-1. Growth curves of *P. putida* MW1200 and *P. putida* Idaho in the absence of *o*-xylene, and in the presence of 75 ppm and 200 ppm *o*-xylene as determined by optical density (wavelength = 600 nm). Error bars represent 1 standard deviation, $n = 3$.

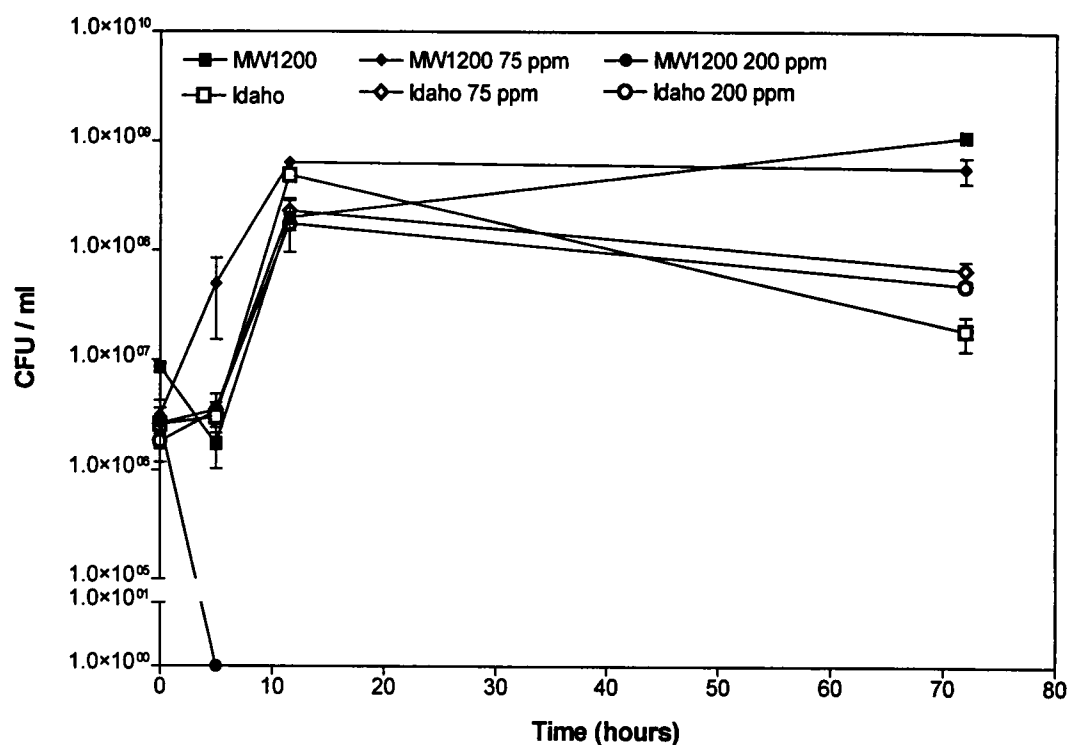


Figure 2-2. Growth curves of *P. putida* MW1200 and *P. putida* Idaho in the absence of *o*-xylene, and in the presence of 75 ppm and 200 ppm *o*-xylene as determined by viable counts (CFU, colony forming unit). Error bars represent 1 standard deviation, $n = 3$.

be cultured at 10 hours, but not after 24 hours. At concentrations exceeding 100 ppm, no viable cells could be cultured after 5 hours. In contrast, *P. putida* Idaho withstands saturated solutions of xylene without loss of viability (Figure 2-2).

Phospholipid Composition

The phospholipid composition of each strain is shown in Table 2-2 . Initial characterization of the phospholipid composition showed the major classes of phospholipid of both strains are phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (diphosphatidylglycerol). These are typical phospholipid classes for fluorescent *Pseudomonas* species (Wilkinson, 1988). HPLC analysis showed the Idaho strain to have a greater percentage of phosphatidylethanolamine than *P. putida* MW1200 when grown with succinate. Phosphatidylserine and phosphatidic acid were detected in trace amounts in both strains (Figure 2-4). Following exposure to xylene, changes were seen in both strains with regards to phospholipid content. The Idaho strain showed an overall increase in phospholipid following exposure to 75 ppm and 200 ppm *o*-xylene. The MW1200 strain showed a decrease in the amount of phospholipid following exposure to its maximum tolerable limit of xylene (Figure 2-4). Loss of phospholipid was expected, as one of the consequences of exposure of bacterial cells to hydrocarbons is the loss of lipid material to the surrounding medium.

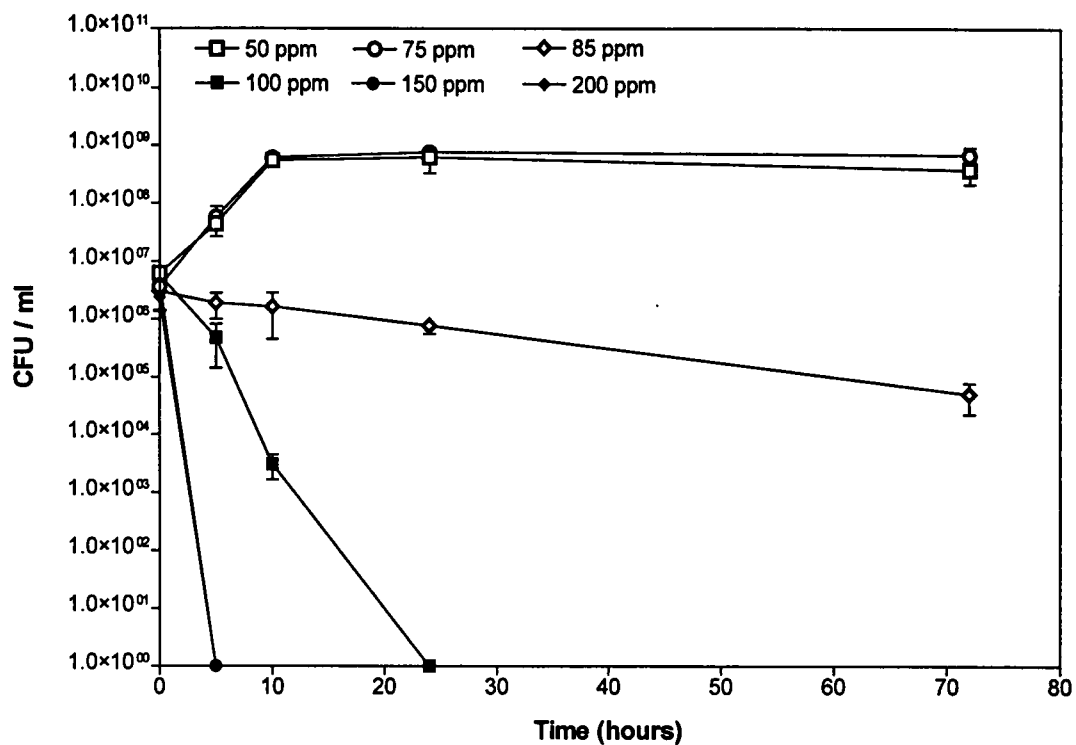


Figure 2-3. Kill curve for *P. putida* MW1200 over various concentrations (in ppm) of *o*-xylene as determined by viable counts (CFU, colony forming unit). Error bars represent 1 standard deviation, n = 3.

Table 2-2. Mole percentages of each class phospholipids and total phospholipid obtained from *P. putida* MW1200 and *P. putida* Idaho in the presence and absence of *o*-xylene.

PL Class ^a	MW1200		Idaho		
	0 ppm	75 ppm	0 ppm	75 ppm	200 ppm
PE	55.97	66.14	73.93	77.07	77.40
PG	28.79	30.74	14.21	19.53	20.43
CL	11.24	6.60	8.88	1.81	0.96
PA	2.40	1.12	1.88	0.93	1.36
PS	1.56	0.78	1.07	0.63	0.55
Total PL ^b	77.0 ± 13.2	38.4 ± 10.7	84.5 ± 12.9	115.2 ± 9.81	166.4 ± 10.3

a. PL, phospholipid; PE, phosphatidylethanolamine; PG phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; PS, phosphatidylserine.

b. Total represents nanomoles phospholipid per milligram cell dry weight ± 1 standard deviation.

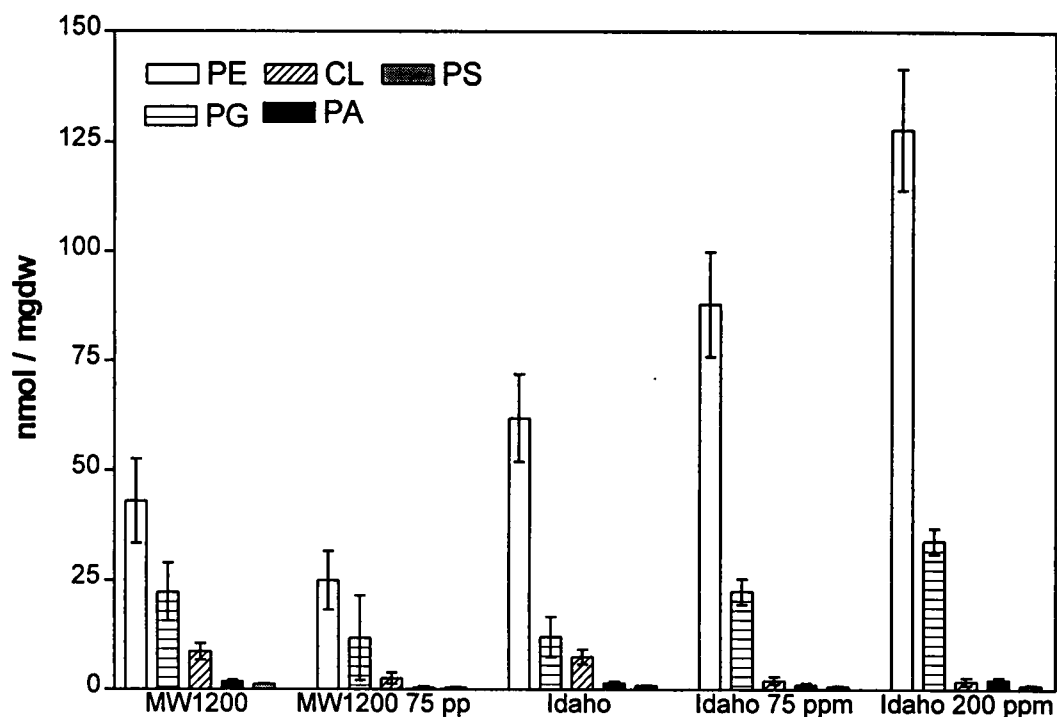


Figure 2-4. Phospholipid composition of *P. putida* MW1200 and *P. putida*

Idaho grown in the presence and absence of *o*-xylene. PE,

phosphatidylethanolamine; PG phosphatidylglycerol; CL, cardiolipin; PA,

phosphatidic acid; PS, phosphatidylserine. Units are expressed as nanomoles of

phospholipid per milligram cell dry weight. Error bars represent 1 standard

deviation, $n = 3$.

Fatty Acid Content and Composition

The fatty acid composition of both *Pseudomonas putida* strains was altered following exposure to xylene (Table 2-3). In the Idaho strain an increase of saturated fatty acids was noted. This increase occurred in palmitic acid (16:0) and stearic acid (18:0). Of the remaining unsaturated fatty acids, primarily *cis*-hexadecenoic acid (16:1 ω 7*cis*) and *cis*-octadecenoic acid (18:1 ω 7*cis*), a conversion from the *cis* isomer to the *trans* isomer was observed. In the Idaho strain this conversion was noted at both 75 ppm *o*-xylene and 200 ppm *o*-xylene, with a greater degree of isomerization as the amount of xylene increased. In the MW1200 strain, the increase in saturation seen in the Idaho strain following exposure to *o*-xylene was not observed. A loss of fatty acid was observed following exposure to 75 ppm *o*-xylene. The phospholipid fatty acid content of *P. putida* Idaho increased with exposure to xylene. The fatty acid content of the MW1200 strain decreased with xylene exposure.

However, the MW1200 strain did have the ability to make *trans*-unsaturated fatty acids. The MW1200 strain made more *trans* fatty acid following exposure to xylene than the Idaho strain at 75 ppm. Small increases in cyclopropyl fatty acids were observed in both strains following exposure to *o*-xylene. No other "specialized" or unusual fatty acids were detected in either strain following exposure to *o*-xylene.

Table 2-3 . Molar percentages and concentrations (± 1 standard deviation, $n = 5$) of phospholipid fatty acids from *P. putida* MW1200 and *P. putida* Idaho grown in the presence or absence of *o*-xylene.

Fatty Acid Type	MW1200		Idaho		
	0 ppm	75 ppm	0 ppm	75 ppm	200 ppm
Saturated					
12:0	0.35 $\pm$ 0.19	1.00 $\pm$ 0.90	0.33 $\pm$ 0.04	0.27 $\pm$ 0.06	0.52 $\pm$ 0.47
14:0	0.23 $\pm$ 0.11	0.26 $\pm$ 0.13	0.23 $\pm$ 0.11	0.27 $\pm$ 0.10	0.35 $\pm$ 0.11
15:0	0.08 $\pm$ 0.06	0.04 $\pm$ 0.09	0.13 $\pm$ 0.13	0.13 $\pm$ 0.10	0.66 $\pm$ 0.08
16:0	32.12 $\pm$ 5.80	29.74 $\pm$ 3.48	28.90 $\pm$ 1.77	28.87 $\pm$ 1.68	30.54 $\pm$ 9.23
18:0	1.26 $\pm$ 0.63	1.64 $\pm$ 0.77	1.06 $\pm$ 0.30	1.51 $\pm$ 0.07	2.50 $\pm$ 0.33
Unsaturated					
16:1 ω 7 <i>cis</i>	31.37 $\pm$ 0.58	27.01 $\pm$ 2.09	27.89 $\pm$ 0.23	25.54 $\pm$ 0.88	16.77 $\pm$ 1.50
16:1 ω 7 <i>trans</i>	2.12 $\pm$ 0.72	5.57 $\pm$ 0.88	1.14 $\pm$ 0.20	2.46 $\pm$ 0.58	13.29 $\pm$ 1.83
18:1 ω 7 <i>cis</i>	22.64 $\pm$ 0.78	21.63 $\pm$ 0.92	26.82 $\pm$ 2.12	26.86 $\pm$ 1.74	22.58 $\pm$ 2.06
18:1 ω 7 <i>trans</i>	1.11 $\pm$ 0.50	1.68 $\pm$ 0.61	0.47 $\pm$ 0.14	0.32 $\pm$ 0.13	4.57 $\pm$ 1.67
cy 17	1.62 $\pm$ 1.03	1.31 $\pm$ 0.80	0.71 $\pm$ 0.12	0.60 $\pm$ 0.03	0.89 $\pm$ 0.24
cy 19	0.10 $\pm$ 0.07	0.74 $\pm$ 0.35	0.06 $\pm$ 0.02	0.25 $\pm$ 0.14	0.36 $\pm$ 0.19
Total PLFA	93.24 $\pm$ 15.40	26.88 $\pm$ 5.26	72.74 $\pm$ 7.77	128.1 $\pm$ 17.10	139.5 $\pm$ 16.50

* Total fatty acid (FA) is expressed in nanomoles per milligram dry weight.

Table 2-4. Changes in fatty acid composition of individual phospholipids of *P. putida* Idaho following exposure to *o*-xylene. Numbers represent the molar percentage of each fatty acid type associated with each phospholipid (mean $\pm$ 1 standard deviation, n=3).

FA Type	PE ^b		PG		CL		PA		PS	
	- xyl	+ xyl	- xyl	+ xyl	- xyl	+ xyl	- xyl	+ xyl	- xyl	+ xyl
Sat. FA	59 $\pm$ 12	60 $\pm$ 12	54 $\pm$ 9	88 $\pm$ 13	97 $\pm$ 8	95 $\pm$ 9	69 $\pm$ 7	86 $\pm$ 8	56 $\pm$ 9	59 $\pm$ 10
<i>Cis</i> FA	35 $\pm$ 6	17 $\pm$ 4	46 $\pm$ 7	9 $\pm$ 3	3 $\pm$ 0.6	5 $\pm$ 1	31 $\pm$ 6	10 $\pm$ 2	40 $\pm$ 7	13 $\pm$ 3
<i>Trans</i> FA	6 $\pm$ 0.7	23 $\pm$ 5	0	3 $\pm$ 1	0	0	0	7 $\pm$ 2	4 $\pm$ 2	28 $\pm$ 4

a. Sat. FA, saturated fatty acids, *Cis* FA, *cis*-unsaturated fatty acids, *Trans* FA, *trans*-unsaturated fatty acids.

b. PE, phosphatidylethanolamine; PG phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; PS, phosphatidylserine.

Analysis of the fatty acid composition of each phospholipid for each strain showed that specific changes in the fatty acid composition were localized to specific classes of phospholipid. For both strains, the *cis-trans* isomerization occurred mainly in phosphatidylethanolamine (Table 2-4). The increase in saturation in the Idaho strain occurred mainly in phosphatidylglycerol (Table 2-4). The MW1200 strain also showed a similar but smaller increase in saturation associated with phosphatidylglycerol (not shown). Phosphatidic acid showed small changes in both saturation and levels of *cis* unsaturated fatty acids. Cardiolipin did not show much change in its fatty acid composition when it was grown under conditions of xylene saturation.

Kinetics of Phospholipid Fatty Acid Change

These experiments were also run in continuous culture. Changes in phospholipid content over time were followed in experiments which were initiated in succinate-minimal salts medium, then amended with 75 and 200 ppm *o*-xylene following the 'time 0' time point. To accomplish this, the influent lines of the continuous cultures were clamped off, and xylene added directly to the growth chamber. The succinate-minimal salts medium was then replaced with succinate-minimal salts medium amended with either 75 ppm *o*-xylene or 200 ppm *o*-xylene, and the influent clamp removed.

The *P. putida* MW1200 strain showed an increase in phospholipid content at 120 hours in the absence of xylene (Figure 2-5). A similar increase was seen in the presence of 75 ppm *o*-xylene, but was significantly less than the amount produced in the absence of xylene (2 standard deviations). *P. putida* MW1200 showed a decrease in phospholipid content following exposure to 200 ppm. Diglycerides were detected in the supernatant of the MW1200 strain following exposure to 200 ppm *o*-xylene at the 400 minute time point, indicating cell death and hydrolysis of phospholipid. The high variation in phospholipid content (high standard deviation) in both strains at the initial time points is probably due to the perturbation caused by the stop and resumption of flow in the continuous culture.

The *P. putida* Idaho strain showed increases in *trans*-unsaturated fatty acids at 5 minutes, with maximal amounts detected at 30 minutes following exposure to xylene. The increase in fatty acid saturation was noted following 15 minutes of exposure, with the maximum amount of saturation seen at two hours following xylene exposure. The increase in total phospholipid is observed as early as 20 minutes following exposure to xylene (Figure 2-6), with subsequent increases between 2 and 6 hours. The same response was seen for *P. putida* MW1200 following exposure to xylene with regards to the synthesis of *trans*-unsaturated fatty acids. However, there was only a small increase in saturated fatty acids over time. Following exposure to 75 ppm xylene, the amount of phospholipid synthesized was less than that observed for growth in the absence

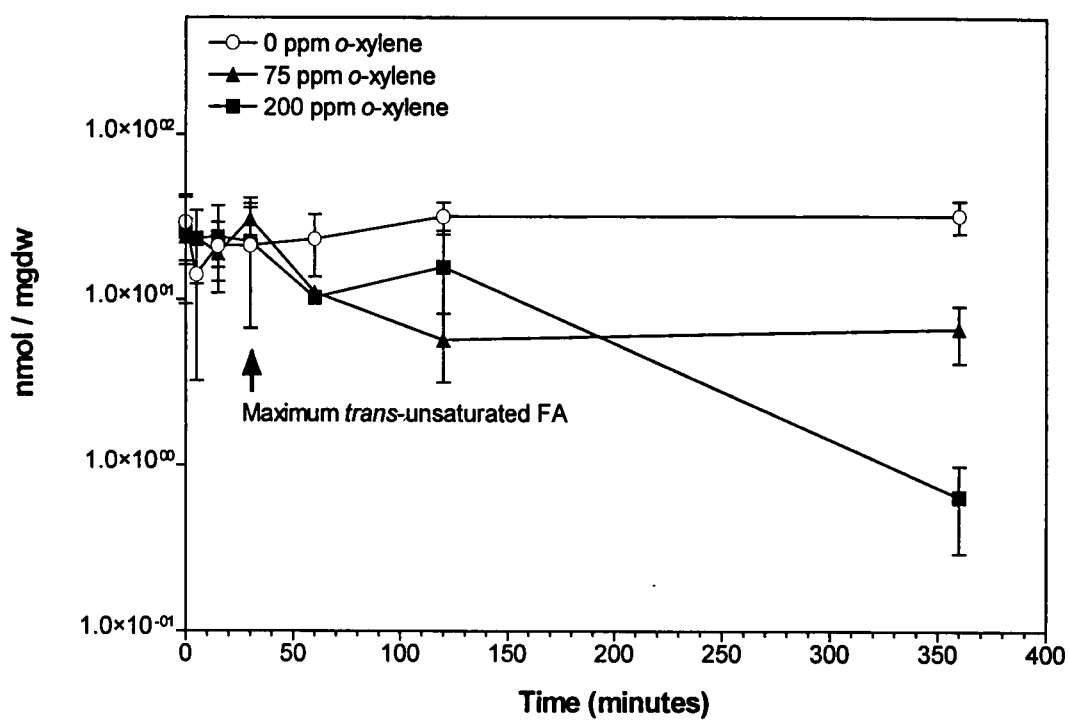


Figure 2-5. Change in total phospholipid concentration in *P. putida* MW1200 over time following exposure to *o*-xylene. Phospholipid concentrations are expressed in nanomoles per milligram cell dry weight. Error bars represent 1 standard deviation, $n = 3$.

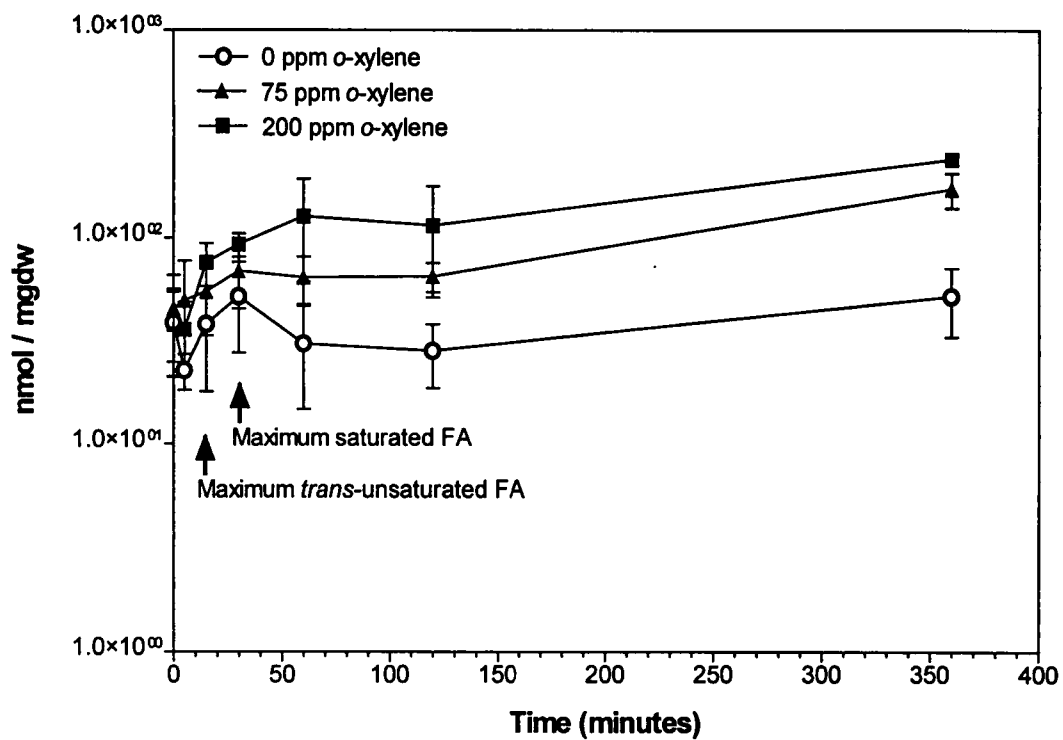


Figure 2-6. Increase in phospholipid in *P. putida* Idaho over time in the presence and absence of *o*-xylene. Phospholipid concentrations are expressed in nanomoles per milligram cell dry weight. Error bars represent 1 standard deviation, $n = 3$.

of the solvent. In the presence of 200 ppm xylene, loss of phospholipid was noted over time, indicating cell death. This loss was small in the initial two hours of exposure, but accelerated soon after the two hour time point.

Location of Phospholipids

Experiments in which the inner and outer membranes were separated by sucrose density centrifugation showed fairly equal proportions of each phospholipid type in each membrane for both strains. Each membrane fraction was pure, with no KDO detected in the inner membrane fraction, and no NADH oxidase activity detected in the outer membrane fraction.

For *P. putida* Idaho, the concentrations of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin were 75%, 22% and 3% for the outer membrane, and 90%, 9% and 1% for the cytoplasmic membrane in the absence of xylene (Table 2-5) lipid material via. In the presence of xylene, the proportions of the phospholipid in both membranes did not change significantly, although 20% more inner membrane phospholipid was recovered from the xylene-grown cells. The proportions of *P. putida* MW1200 phospholipid for phosphatidylethanolamine, phosphatidylglycerol and cardiolipin were 67%, 21%, and 22% for the outer membrane fraction, and 75%, 19% and 6%, respectively for the inner membrane. For both strains, the precursor phospholipids phosphatidic acid and phosphatidylserine were only detected in

Table 2-5. Molar percentages of phospholipid and phospholipid fatty acids associated with the inner membrane (IM) and outer membrane (OM) of each *Pseudomonas putida* strain in the absence and presence of *o*-xylene.

Lipid Class ^a	MW1200		MW1200 75 ppm		Idaho		Idaho 200 ppm	
	IM	OM	IM	OM	IM	OM	IM	OM
PE	75%	65%	78%	67%	88%	75%	89%	76%
PG	19%	22%	16%	21%	9%	22%	11%	23%
CL	6%	23%	6%	22%	3%	3%	0%	1%
PA	trace	-	trace	-	trace	-	trace	-
PS	trace	-	trace	-	trace	-	trace	-
Sat. FA ^b	++	+++	++	+++	++	+++	+++	+++
Trans FA	+	nd	++	+	+	nd	++	++

a. PE, phosphatidylethanolamine; PG phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; PS, phosphatidylserine.

b. Sat. FA, saturated fatty acids, *Trans* FA, *trans*-unsaturated fatty acids. +, <10% of the total fatty acid; ++, 10-50% of the total fatty acid; +++, >50% of the total fatty acid; nd, none detected.

the cytoplasmic membrane fraction, and only in trace amounts (less than 0.1%). For both strains, *trans* fatty acids were found in both inner and outer membrane fractions. Accurate quantitation was difficult, however, because of hydrocarbon contamination from the sucrose density gradient centrifugation. Hexane washes of the silicic acid prior to elution of the phospholipid did little to alleviate the problem.

DISCUSSION

Growth curves for each strain showed similarities in lag, doubling times and exponential phase behavior. For growth in the absence of xylene, there was no significant difference in doubling time between the Idaho strain and the MW1200 strain ($p=0.134$, $\alpha=0.01$). Both strains exhibit a lag phase of about 4.5 - 5.0 hours before entering exponential phase. Following a short period in stationary phase, the Idaho strain showed a rapid loss of viability and decreased optical density. This behavior was independent of exposure to xylene, and did not affect exponential phase growth. By decreasing flow rates slightly, it was possible to cultivate the Idaho strain at cell densities of $>10^9$ cells/ml in continuous culture for two weeks or more. The MW1200 strain demonstrated normal stationary phase behavior. It is possible that the insertion of the TOL genes into the chromosome in the Idaho strain is responsible for the limited stationary phase. Although the location of the genes have not been mapped in

this strain, it is possible that they may reside in an area that controls starvation response, resulting in the loss of viability at that growth stage. Another explanation may be that the differences in fatty acid composition may be responsible for the short stationary phase. Research by Massa et al. (1988) showed that *E. coli* AK7 showed sensitivity to conditions of nutrient depletion that was dependent on microviscosity of the cell envelope. Following manipulation of the fatty acid content of the *E. coli* cells it was found that growth under non-starvation conditions was normal, but upon carbon starvation conditions, the ability to form colonies was lost in cells containing high concentrations of linolenic acid. Those containing higher amounts of oleic acid did not show loss of viability in response to carbon starvation.

The MW1200 strain does not exhibit a lag when exposed to 75 ppm *o*-xylene. The MW1200 strain demonstrated a maximum tolerable limit of 75 ppm *o*-xylene. Concentrations above 75 ppm caused cessation of growth (85 ppm), and loss of viability (100 ppm).

When exposed to 75 ppm *o*-xylene, the Idaho strain exhibits lag phase of about 7 hours before entering exponential phase. This lag is extended to 11 hours in the presence of 200 ppm (saturated) *o*-xylene. This suggests that tolerance to solvents is an inducible event. Exponential growth of the Idaho strain was not affected even when cultivated in succinate minimal salts medium amended with 3000 ppm Triton X-100 and up to 10,000 ppm *o*-xylene (Dr. James Wolfram, personal communication).

The phospholipid composition of both strains was similar, with both strains harboring phosphatidylethanolamine, phosphatidylglycerol and cardiolipin as the major phospholipids, with phosphatidic acid and phosphatidylserine as minor constituents. These are typical phospholipids of fluorescent pseudomonads (Wilkinson and Galbraith, 1973; Wilkinson, 1988).

Following exposure to xylene, the MW1200 strain showed a decrease in all phospholipid classes. The Idaho strain consistently showed greater amounts of phosphatidylethanolamine than the MW1200 strain, in both the presence and absence of xylene. The amount of phosphatidylethanolamine was increased by 15% following exposure to xylene, with a concurrent drop in the percentage of cardiolipin. Phosphatidylglycerol and the minor phospholipids were not proportionately altered. Phosphatidylethanolamine has a higher melting point than phosphatidylglycerol, so increased proportions may help counter increased membrane fluidity in the xylene-exposed cells (Melchior, 1982). Increases in phosphatidylethanolamine to counter the effects of solvents is not a common response among bacteria, however (Table 2-6). Following exposure to organic solvents, a drop in the proportion of phosphatidylethanolamine was observed in *E. coli* (Ingram, 1977, 1986), *Mycobacterium smegmatis* (Taneja and Khuller, 1980) *P. putida* S12 (Heipieper et al., 1992; Weber et al., 1994) *P. putida* MW1200 (this study), and *Zymomonas mobilis* (Carey and Ingram, 1983; Barrow et al., 1983). *P. putida* Idaho and *Microsporon* sp. are the only bacteria that exhibit increased levels of phosphatidylethanolamine (this study, Bansal and

Khuller, 1981). The increase in phosphatidylglycerol was a more common response, exhibited by *P. putida* S12, a solvent-tolerant organism, *P. putida* Idaho, *E. coli*, and the archaebacterium *Acholeplasma laidlawii* (Wieslander et al., 1986). Most of these organisms also showed an increase in cardiolipin (diphosphatidylglycerol), a response not shared by *P. putida* Idaho.

The total phospholipid content of the MW1200 strain dropped by 70% following exposure to xylene. This result was not unexpected. Because of the mechanism of action of xylene, it is likely that the loss resulted from extraction of the membrane components to the surrounding medium. *Mycobacterium smegmatis* exposed to ethanol is reported to show a drop in total phospholipid. *E. coli* exposed to ethanol and phenols is also reported to exhibit a drop in total phospholipid. The *P. putida* Idaho strain showed a quite different response to solvent exposure. The amount of total phospholipid increased by 100%. This suggests a membrane repair mechanism may be present in Idaho, which allows for replacement of membrane components damaged by organic solvents.

Both strains exhibited an ability to alter fatty acid composition following exposure to *o*-xylene. Each strain showed an increase in *trans*-unsaturated fatty acids. This phenomenon has been proposed as a solvent tolerance mechanism by some groups. It has been hypothesized that *trans*-unsaturated fatty acids play a crucial role in solvent tolerance by increasing the phase transition temperature of the phospholipid membrane (Heipieper et al., 1992; Weber et al., 1994) In *Pseudomonas* and *Vibrio* species, the conversion of unsaturated fatty acids from

cis to *trans* has been linked to combating membrane damage due to environmental stresses such as temperature shifts (Guckert et al., 1987; Henderson et al., 1993; Morita et al., 1993) and the presence of phenol and toluene (Heipieper et al., 1991; Heipieper et al., 1992; Weber et al., 1994). The *trans* isomer of the unsaturated fatty acid is thermodynamically more stable than the *cis* isomer, which could decrease membrane fluidity, and has been suggested as a mechanism for avoiding substrate toxicity. However, the results of this study show that both solvent-tolerant and solvent sensitive strains have the ability to make *trans*-unsaturated fatty acids following exposure to organic solvents. These results imply that *trans*-unsaturated fatty acids are probably synthesized as a general response to environmental stress, and is not a specific mechanism of solvent resistance. This is supported by the starvation response shown by *Vibrio* and *P. atlantica* (Guckert et al., 1987; Henderson et al., 1993; Morita et al., 1993). *Shewanella species* (Nichols et al., 1994) and *Sarcina ventriculi* (Jung et al., 1993) also have the ability to make *trans*-unsaturated fatty acids, but have not been reported as being solvent resistant. *Trans*-unsaturated fatty acids are most likely a post-synthetic modification of *cis*-unsaturated fatty acids (Heipieper et al., 1992; Heipieper and de Bont, 1994) because they appear so quickly following exposure to solvent, although evidence also exists that supports their *de novo* production (Guckert et al., 1986).

The Idaho strain showed an increase in saturated fatty acids following exposure to xylene. This increase was seen mainly in hexadecanoic acid (16:0)

and in octadecanoic acid (18:0), with an overall relative increase in saturated fatty acids seen in cells exposed to 200 ppm xylene. A similar response has been seen in other genera, where exposure to alcohols resulted in an increase in the relative percentage of saturated fatty acids to compensate for solvent-induced increased membrane fluidity in *E. coli* (Ingram, 1977; Keweloh et al., 1991), some *Clostridium* species (Lepage et al., 1987; Lovitt et al., 1984), *Microsporium* (Bansal and Khuller, 1981), *Mycobacterium*, and *Rhizobium* species. This isn't always the response seen in response to organic solvents, however. Several bacterial species respond to organic solvents by decreasing the ratio of saturated to unsaturated fatty acids. The bacteria eliciting this response include *Clostridium thermocellum* (Herrero et al., 1982), and *Lactobacillus* species (Uchida, 1975) (Table 2-6). Although the reason for the increased unsaturation is not known, it has been postulated that the presence of solvent inhibits the enzymes responsible for production of saturated fatty acids (Heipieper et al., 1994).

The combination of increased saturation and the isomerization of unsaturated fatty acids from *cis* to *trans* result in a phospholipid membrane that is more rigid and organized, and help compensates for the fluidizing action of *o*-xylene. Acidophilic bacteria have been shown to contain high concentrations of cyclopropyl and cyclohexyl fatty acids (Langworthy et al., 1982). These are also post-synthetic modifications, and have been suggested as an advantageous adaptation for maintaining membrane integrity in acidic environments.

Table 2-6. Membrane adaptations reported for various bacteria in response to exposure to organic solvents.

Organism	Solvent	FA Comp. ¹	PL Headgroup ²	Others
<i>Bacillus cereus</i>	Ethanol Propanol	Branched FA↑		
<i>B. subtilis</i>	Ethanol	Branched FA↓		
<i>Clostridium acetobutylicum</i>	C2-C8 Alcohols	Unsat:Sat↓		
<i>C. thermocellum</i>	Ethanol	Unsat:Sat↑ Chain length↓ Anteiso↑		
<i>Escherichia coli</i>	Ethanol	Unsat:Sat↑ Chain length↑	PE↓ CL↑ PG↑	Lipid:protein↓
	C6-C10 Alcohols	Unsat:Sat↓		
	Phenol	Unsat:Sat↓		Lipid:protein↓
<i>Lactobacillus heterohiochii</i>	Ethanol	Unsat:Sat↑		
<i>L. homohiochii</i>	C1-C7 Alcohols	Unsat:Sat↑		
<i>Microsporum</i> sp.	Ethanol	Unsat:Sat↓	PE↑ PS↓	
<i>Mycobacterium smegmatis</i>	Ethanol 27C	Unsat:Sat↑		Total PL↓
	Ethanol 37C	Unsat:Sat↓	PE↓ PI↑	Total PL↓
<i>Pseudomonas putida</i> S12	Ethanol	Unsat:Sat↑ <i>Trans:cis</i> ↑		
	Phenol	Unsat:Sat↓ <i>Trans:cis</i> ↑		
	Toluene	<i>Trans:cis</i> ↑	PE↓ PG↑ CL↑	
<i>P. putida</i> Idaho	<i>O</i> -Xylene	Unsat:Sat↓ <i>Trans:cis</i> ↑	PE↑ PG↑ CL↓	Total PL↑
<i>P. putida</i> MW1200	<i>O</i> -Xylene	<i>Trans:cis</i> ↑	PE↓ PG↑ CL↓	Total PL↓
<i>Rhizobium</i> sp.	2,4-D	Unsat:Sat↓		
<i>Sarcina ventriculi</i>	Ethanol Butanol	Long-chain di-carboxylic FA↑		
<i>Zymomonas mobilis</i>	Ethanol	Chain length↑	PE↓ CL↑	Lipid:protein↓ Hopanoids↑

1. FA, fatty acid; Unsat:Sat, ratio of unsaturated fatty acids to saturated fatty acids; *Trans:cis*, ratio of *trans*-unsaturated fatty acids to *cis*-unsaturated fatty acids.

2. PL, phospholipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PI, phosphatidylinositol; PS, phosphatidylserine.

The changes in fatty acid composition were found to be localized to specific phospholipids. The *cis-trans* isomerization was found to be mainly in phosphatidylethanolamine, while the increase in saturation was localized to phosphatidylglycerol, with a small amount present in phosphatidylethanolamine. Cardiolipin also showed few changes in saturation. The precursor phospholipid phosphatidylserine showed changes both in *cis-trans* isomerization and increased saturation. Phosphatidic acid showed increased saturation following exposure to xylene. The increase in *trans*-unsaturated fatty acid concentration happened quickly, first appearing at five minutes following xylene exposure, reaching a maximal concentration at 30 minutes. This response allows a quick adjustment to increases in membrane fluidity. The increase in saturation was observed initially at twenty minutes following xylene exposure, with maximum saturation observed at 2 hours.

Phosphatidic acid is a precursor for phosphatidylglycerol (Raetz and Dowhan, 1990). Therefore it is logical that the increase in saturation is seen there, and later in phosphatidylglycerol. *Trans* fatty acid was observed in both phosphatidylserine and phosphatidylethanolamine. This indicates that production of phosphatidylethanolamine coincides with the production of *trans*-unsaturated fatty acids. It is unclear at this time whether the formation of *trans* fatty acid was de novo, or was a direct isomerization of phosphatidylserine. It is clear that the precursor of phosphatidylethanolamine, phosphatidylserine,

contained the *trans* fatty acids prior to the decarboxylation of phosphatidylserine to form phosphatidylethanolamine. It is possible that the production of *trans*-unsaturated fatty acid is regulated at some level, and is specifically directed toward incorporation into phosphatidylethanolamine. Since a certain proportion of unsaturated fatty acids is required for proper membrane function, it is logical that the unsaturated fatty acids be directed towards a phospholipid with a higher phase transition temperature. This would explain the increase in phosphatidylethanolamine and the high percentage of *trans*-fatty acid seen when *P. putida* Idaho is exposed to 200 ppm. The increase in saturation seen in the phosphatidylglycerol may compensate for its lower melting point, thereby increasing the overall phase transition state of the membrane.

In summary, both strains of *P. putida* showed membrane alterations following exposure to solvent. Each strain demonstrated the ability to make *trans*-unsaturated fatty acids, a modification which creates a higher phase transition temperature in lipids. The solvent tolerant strain showed the additional ability to increase the total amount of cellular phospholipid following xylene exposure, as well as an increase in saturated fatty acids. These modifications also serve to increase membrane phase transition temperature, and indicate increased membrane synthesis following xylene exposure.

PART III.

PHOSPHOLIPID BIOSYNTHETIC RATE AND TURNOVER RATE IN SOLVENT-TOLERANT AND SOLVENT- SENSITIVE *PSEUDOMONAS PUTIDA* STRAINS

INTRODUCTION

In the previous section, it was observed that *P. putida* Idaho showed an increase in the amount of phospholipid produced following exposure to xylene. One possible explanation for this is that the Idaho strain has an increased rate of membrane biosynthesis in response to exposure to xylene. An increased rate of membrane biosynthesis would be beneficial to this strain in that it would allow rapid replacement of membrane damaged by exposure to xylene. An increased rate of membrane biosynthesis might also provide an explanation for truncated stationary phase exhibited by this strain. If this strain kept up the biosynthesis of phospholipid in stationary phase, it would rapidly run out of substrates and energy required for stationary phase maintenance. The results from this experiment can be used to determine the rate of turnover of phospholipid, and be used to assess whether the Idaho strain has an increased rate of biosynthesis of phospholipid, or decreased rate of turnover, either of which could explain the increased amount of phospholipid found in the Idaho strain (Figure 2-4). Increased biosynthetic rate of phospholipid in the Idaho strain indicates that this strain can replace damaged membrane more quickly than the MW1200 strain.

While increased rate of membrane biosynthesis in response to cell damage has not been reported as of yet, repair of damaged cell components as a resistance mechanism is not an unusual phenomenon. *Deinococcus radiodurans*, for example, has a DNA polymerase that is 100 - 150 times as efficient as that

found in *E. coli*. It is this attribute that allows the species to survive high doses of ionizing radiation because it is extremely efficient at repairing damaged DNA (Smith et al., 1992). Resistance to the antibiotic trimethoprim has been acquired by some bacterial strains by overproducing the target molecule of the antibiotic, dihydrofolate reductase, by 600 times that of the normal level, thus negating the effect of trimethoprim. In *Staphylococcus aureus*, resistance to methicillin is acquired through overproduction of PBP2a (Franklin and Snow, 1989). In *E. coli*, a mutation in cardiolipin synthesis conferred resistance to 3,4-dihydroxybutyl 1-phosphonate (Hwang et al., 1984).

In this study, the rates of membrane biosynthesis and phospholipid turnover will be examined in both solvent-tolerant and solvent-sensitive *P. putida* strains.

MATERIALS AND METHODS

Pulse-chase Protocol

Pulse-chase methodology was utilized in this experiment (Joseleu-Petit, 1987; Pierucci, 1979; Russell et al., 1985). Each strain was grown in continuous culture with succinate mineral salts medium prior to this experiment. Plate counts were performed from a 'cold' culture treated identically to the experimental cultures at the start and end of this experiment to estimate cell

number. Two controls were utilized in this experiment. One is an abiotic control, to which no cells are added. The other control will use azide-killed cells, to account for any labeled phosphate that was associated with the cell surface. Cells (100 ml) were removed from the continuous cultures, and filtered (Supor filters, 0.2 micron) to remove the cells from the culture. The cells retained on the filter were washed three times in phosphate-free buffer. Washed cells were transferred to culture flasks containing phosphate-limited minimal salts minimal supplemented with 10 mM succinate, or phosphate-limited minimal salts-succinate media supplemented with 75 ppm or 200 ppm *o*-xylene. The controls contained only the minimal salts-succinate medium. [^{32}P]- orthophosphoric acid (50 $\mu\text{Ci}/100\text{ ml}$) was added to each flask. The cells were exposed to the [^{32}P]- orthophosphoric acid for 60 minutes, harvested by filtration, washed in phosphate-free buffer, then resuspended in unlabeled media. The cold chase was allowed to proceed for 2.0 hours.

During the initial pulse, 2 ml samples were removed every ten minutes for the first hour, then every thirty minutes until the end of the cold chase. The phospholipids were extracted in the same manner as described previously. One hundred microliter aliquots were removed from the aqueous and organic fractions of the Bligh-Dyer extraction and the radioactivity assessed by scintillation counting. The organic fraction was then applied to a thin layer plate (TLC) to isolate the individual phospholipids (Kates, 1986). Radioactivity was

measured directly from the TLC plate using the Bioscan Imaging Scanner System (Baird et al., 1979). Following data acquisition from the TLC plate, all radioactive spots were removed from the TLC plates, and analyzed for total lipid content. Specific activity was then calculated from this data. This experiment generated several types of data, rate of incorporation of radiolabel into phospholipid, and rate of loss from phospholipid, which was obtained by analysis of total phospholipid (total organic fraction), and also gave incorporation values and turnover data for individual phospholipids.

Statistics

Total phospholipid biosynthesis and turnover rates were calculated using linear regression analysis (GraphPad Prism software, San Diego, CA). Rates of biosynthesis and turnover are the result of three independent experiments. Each data point represents the mean of three experiments. Error bars on the graphs represent one standard deviation. T-tests were used to determine significant differences between the biosynthesis and turnover rates of each strain under the different growth conditions ($\alpha = 0.01$).

RESULTS

Cell numbers at the start of the experiment were $3.1 (\pm 0.8) \times 10^7$ CFU/ml for the *P. putida* MW1200 strain, and $4.1 (\pm 1.2) \times 10^7$ CFU/ml for the *P. putida* Idaho strain. Ending cell numbers were $6.3 (\pm 1.1) \times 10^7$ CFU/ml and $5.9 (\pm 1.4) \times 10^7$ CFU/ml, respectively, indicating a small increase in population over the course of the experiment. Lipid data was normalized to the increase in cell number over time. The azide-killed cells showed minimal uptake of radiolabel, with incorporation of an average of $0.12 \text{ cpm nmol}^{-1} \text{ min}^{-1}$. This value was subtracted from rates of calculated for each experimental strain. Incorporation of label into phospholipid was followed over time (average of three experiments), and is expressed as specific activity (cpm/nanomole phospholipid), and is shown in Figure 3-1. The MW1200 strain showed incorporation of ^{32}P into phospholipid at an average rate of $207.3 (\pm 21.04) \text{ cpm nmol}^{-1} \text{ min}^{-1}$ ($r^2=0.907$). In the presence of 75 ppm *o*-xylene, this rate increased to $273.6 (\pm 17.00) \text{ cpm nmol}^{-1} \text{ min}^{-1}$ ($r^2=0.963$). The Idaho showed a higher rate of incorporation. In the absence of solvent, the Idaho strain exhibited an average incorporation rate of $532.7 (\pm 23.16) \text{ cpm nmol}^{-1} \text{ min}^{-1}$ ($r^2=0.981$). In the presence of 75 ppm *o*-xylene, this rate increased to $852.0 (\pm 31.47) \text{ cpm nmol}^{-1} \text{ min}^{-1}$ ($r^2=0.987$). The incorporation rate for *P. putida* Idaho in the presence of 200 ppm xylene was to $882.6 (\pm 29.23) \text{ cpm nmol}^{-1} \text{ min}^{-1}$ ($r^2=0.989$). All incorporation rates were significantly linear.

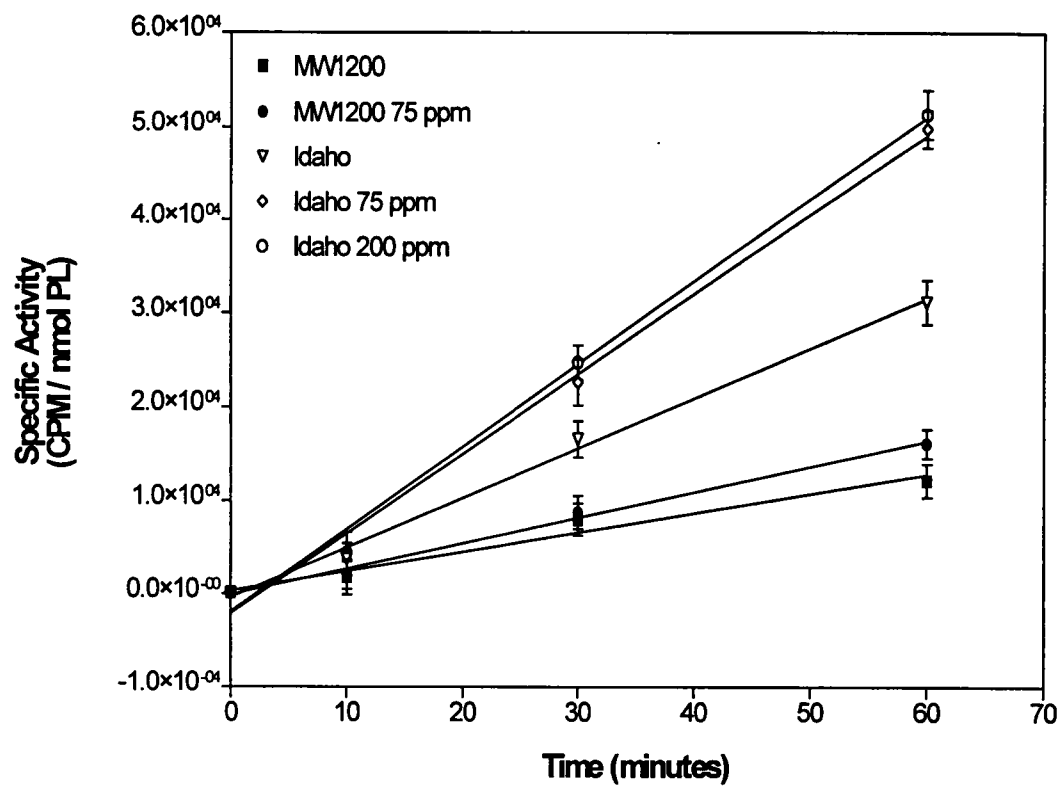


Figure 3-1. Rates of ^{32}P -orthophosphate incorporation into phospholipid for *P. putida* MW1200 and *P. putida* Idaho in the absence and presence of *o*-xylene. Incorporation rates are expressed as $\text{cpm nmol}^{-1} \text{ min}^{-1}$.

Table 3-1. T-tests for phospholipid biosynthetic rate data ($\alpha = 0.01$).

Rate Comparison	P-value	Significance
MW1200 _s vs. MW1200 _{75x}	0.0512	Not Significant
Idaho _s vs. Idaho _{75x}	0.0006	Significant
Idaho _s vs. Idaho _{200x}	0.0004	Significant
Idaho _{75x} vs. Idaho _{200x}	0.4596	Not Significant
MW1200 _s vs. Idaho _s	<0.0001	Significant
MW1200 _{75x} vs. Idaho _{75x}	<0.0001	Significant
MW1200 _{75x} vs. Idaho _{200x}	<0.0001	Significant

s = growth in succinate minimal salts medium

75x = growth in succinate minimal salts medium + 75 ppm *o*-xylene

200x = growth in succinate minimal salts medium + 200 ppm *o*-xylene

T-tests (Table 3-1) run on the above data showed the following results: 1) For the MW1200 strain, there was no significant difference between the rate of incorporation of radiolabel into phospholipid in cells grown in the presence and absence of xylene, $p=0.0512$; 2) there is no significant difference between the rates of incorporation of *P. putida* Idaho grown in the presence of 75 ppm *o*-xylene vs. 200 ppm *o*-xylene, $p=0.4596$; 3) there is a significant difference between the rates of incorporation of radiolabel into phospholipid in *P. putida* Idaho cells grown in the presence and absence of xylene, $p = 0.0006$; 4) there is a significant difference between the rates of incorporation of radiolabel into phospholipid of *P. putida* MW1200 and *P. putida* Idaho grown in the absence of *o*-xylene, $p<0.0001$; 5) there is a significant difference between the rates of incorporation of radiolabel into phospholipid of *P. putida* MW1200 and *P. putida* Idaho grown in the presence of *o*-xylene, $p<0.0001$.

Data on the loss of label (turnover) from the phospholipid is shown in Figure 3-2. The rate of loss of label from the MW1200 strain in the absence of *o*-xylene was $24.89 (\pm 9.24) \text{ cpm nmol}^{-1} \text{ min}^{-1}$ ($r^2 = 0.358$). The rate of loss of label from the MW1200 strain in the presence of *o*-xylene was $42.89 (\pm 11.24) \text{ cpm nmol}^{-1} \text{ min}^{-1}$ ($r^2 = 0.5284$). The rate of loss of label from the Idaho strain in the absence of *o*-xylene was $56.44 (\pm 16.32) \text{ cpm nmol}^{-1} \text{ min}^{-1}$ ($r^2 = 0.4793$). The rate of loss of label from the Idaho strain in the presence of 75 ppm *o*-xylene was $131.7 (\pm 12.38) \text{ cpm nmol}^{-1} \text{ min}^{-1}$ ($r^2 = 0.897$). The rate of loss of label from the Idaho strain in the presence of 200 ppm *o*-xylene was $178.3 (\pm 18.22) \text{ cpm}$

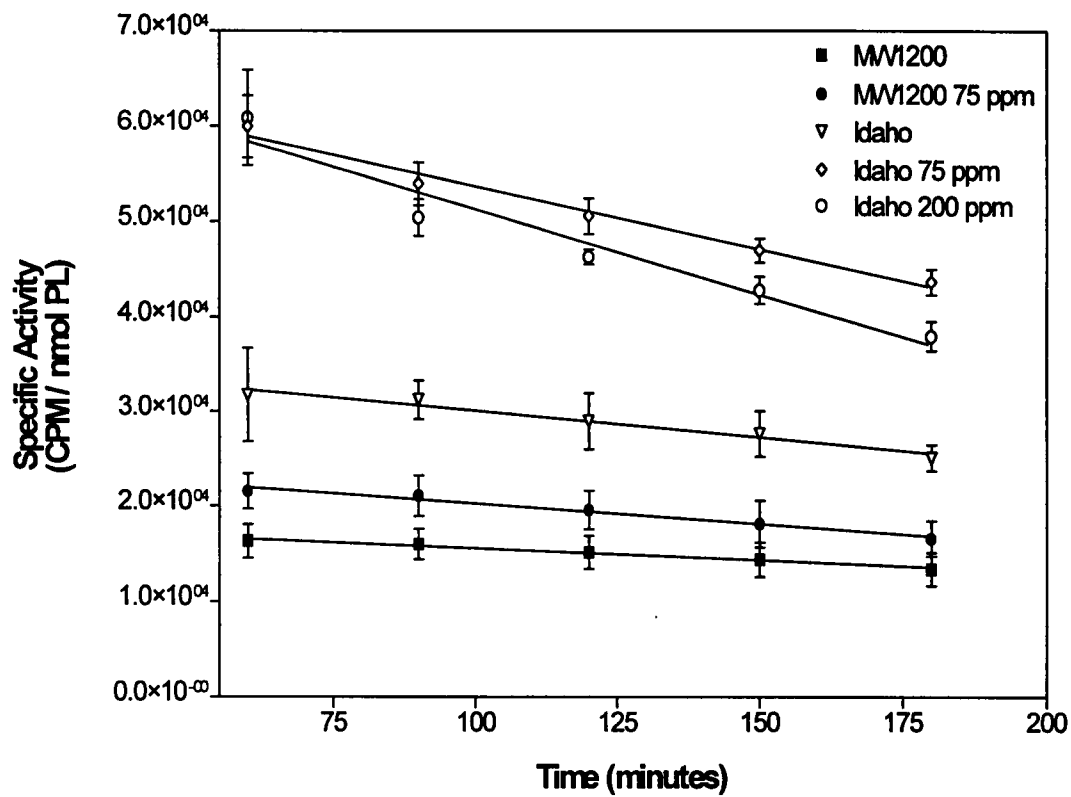


Figure 3-2. Rates of ³²P-orthophosphate loss from phospholipid for *P. putida* MW1200 and *P. putida* Idaho in the absence and presence of *o*-xylene.

Table 3-2. T-tests for rate of loss (phospholipid turnover) data ($\alpha = 0.01$)

Rate Comparison	P-value	Significance
MW1200 _s vs. MW1200 _{75x}	0.0242	Not Significant
Idaho _s vs. Idaho _{75x}	0.0014	Significant
Idaho _s vs. Idaho _{200x}	0.0002	Significant
Idaho _{75x} vs. Idaho _{200x}	0.8452	Not Significant
MW1200 _s vs. Idaho _s	0.0186	Not Significant
MW1200 _{75x} vs. Idaho _{75x}	0.0014	Significant
MW1200 _{75x} vs. Idaho _{200x}	0.0002	Significant

s = growth in succinate minimal salts medium

75x = growth in succinate minimal salts medium + 75 ppm *o*-xylene

200x = growth in succinate minimal salts medium + 200 ppm *o*-xylene

$\text{nmol}^{-1} \text{ min}^{-1}$ ($r^2 = 0.881$). The rates of loss from both strains in the absence of *o*-xylene was not significantly different from zero. The rates of loss for both strains following exposure to xylene were significantly different from zero.

T-tests (Table 3-2) run on the total phospholipid turnover data showed the following results: 1) For the MW1200 strain, there was no significant difference between the rate of loss of radiolabel into phospholipid in cells grown in the presence and absence of xylene, $p=0.0242$; 2) there is no significant difference between the rates of loss of between *P. putida* Idaho and *P. putida* MW1200 grown in the absence of *o*-xylene, $p=0.186$; 3) there is no significant difference between the rates of loss of between *P. putida* Idaho grown in the presence of 75 ppm *o*-xylene vs. 200 ppm *o*-xylene, $p=0.8452$; 4) there is a significant difference between the rates of incorporation of radiolabel into phospholipid in *P. putida* Idaho cells grown in the presence and absence of xylene, $p \leq 0.0014$; 5) there is a significant difference between the rates of incorporation of radiolabel into phospholipid of *P. putida* MW1200 and *P. putida* Idaho grown in the presence of *o*-xylene, $p \leq 0.0014$.

In the absence of solvent, the MW1200 strain showed greatest incorporation of label into phosphatidylglycerol, followed by cardiolipin and phosphatidylethanolamine. Cardiolipin showed the most rapid turnover, followed by phosphatidylglycerol and phosphatidylethanolamine in *P. putida* MW1200 (Figure 3-3). Phosphatidic acid was detected only at very low levels, and phosphatidylserine was not detected at all. In the presence of *o*-xylene,

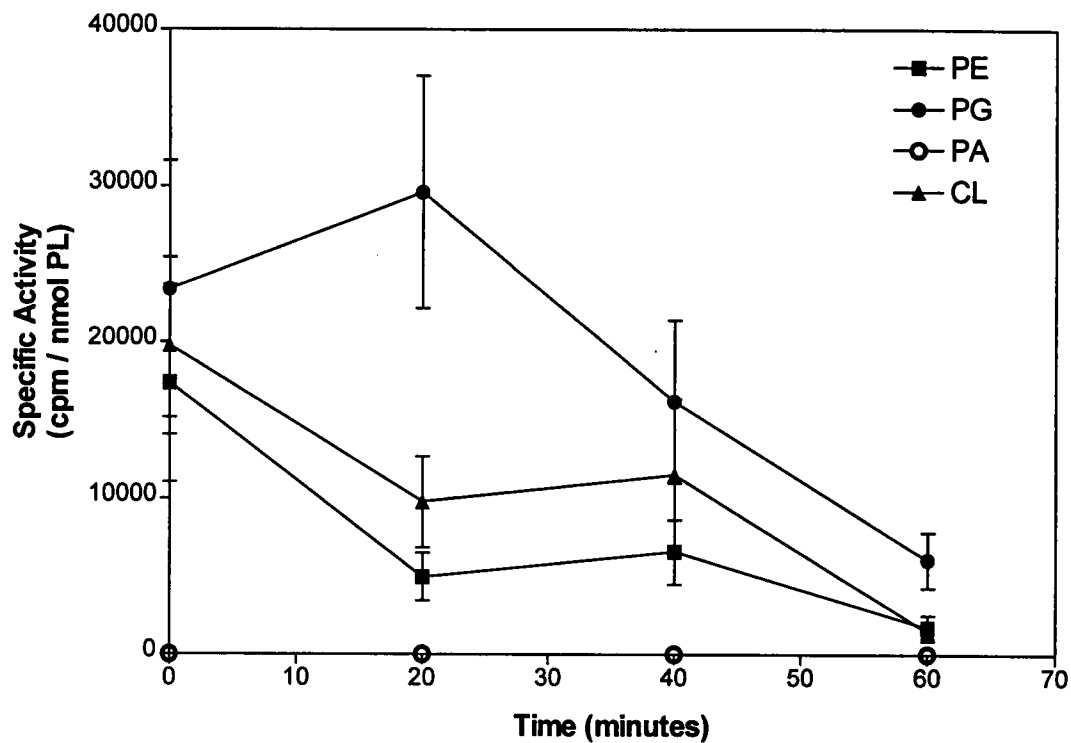


Figure 3-3. Turnover of individual phospholipids of *P. putida* MW1200 in the absence of *o*-xylene. PE, phosphatidylethanolamine, PG, phosphatidylglycerol, PA, phosphatidic acid, CL, cardiolipin. Error bars represent 1 standard deviation, $n = 3$.

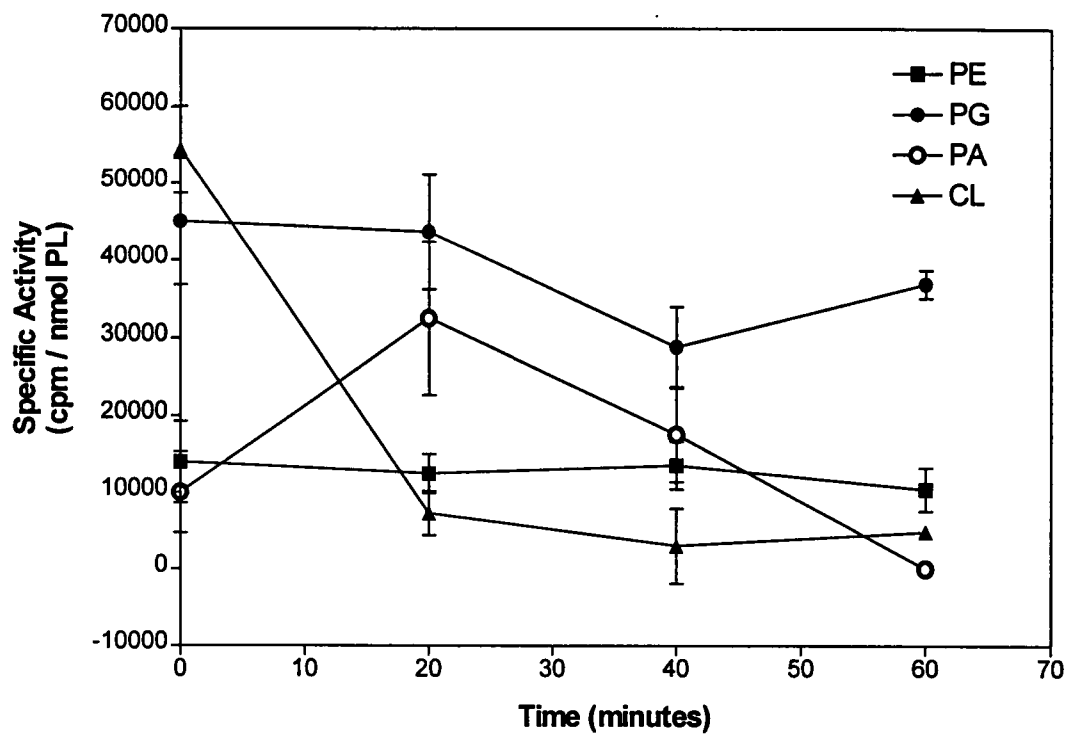


Figure 3-4. Turnover of individual phospholipids of *P. putida* MW1200 in the presence of 75 ppm *o*-xylene. PE, phosphatidylethanolamine, PG, phosphatidylglycerol, PA, phosphatidic acid, CL, cardiolipin. Error bars represent 1 standard deviation, $n = 3$.

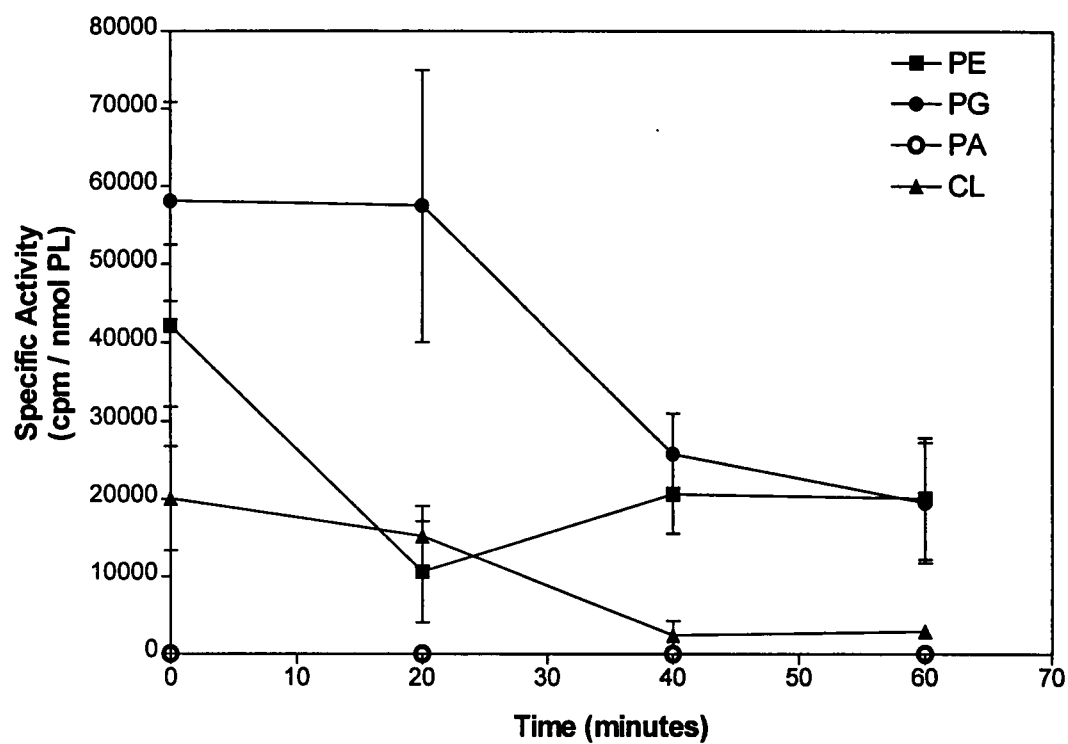


Figure 3-5. Turnover of individual phospholipids of *P. putida* Idaho in the absence of *o*-xylene. PE, phosphatidylethanolamine, PG, phosphatidylglycerol, PA, phosphatidic acid, CL, cardiolipin. Error bars represent 1 standard deviation.

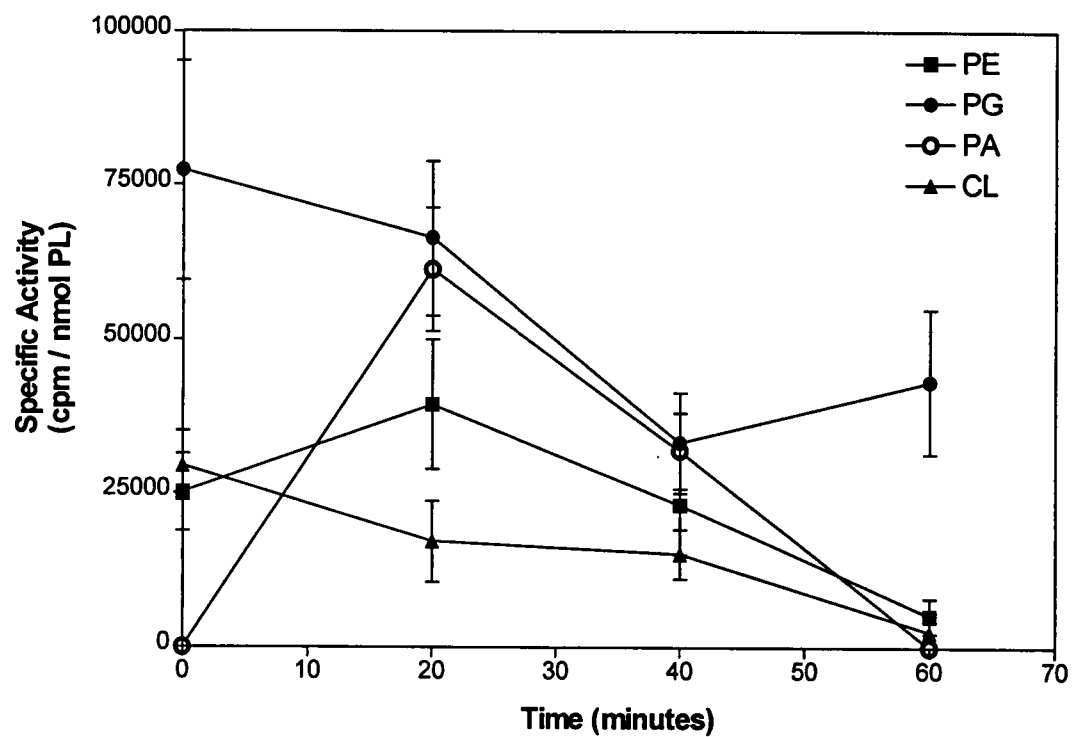


Figure 3-6. Turnover of individual phospholipids of *P. putida* Idaho in the presence of 75 ppm *o*-xylene. PE, phosphatidylethanolamine, PG, phosphatidylglycerol, PA, phosphatidic acid, CL, cardiolipin. Error bars represent 1 standard deviation, $n = 3$.

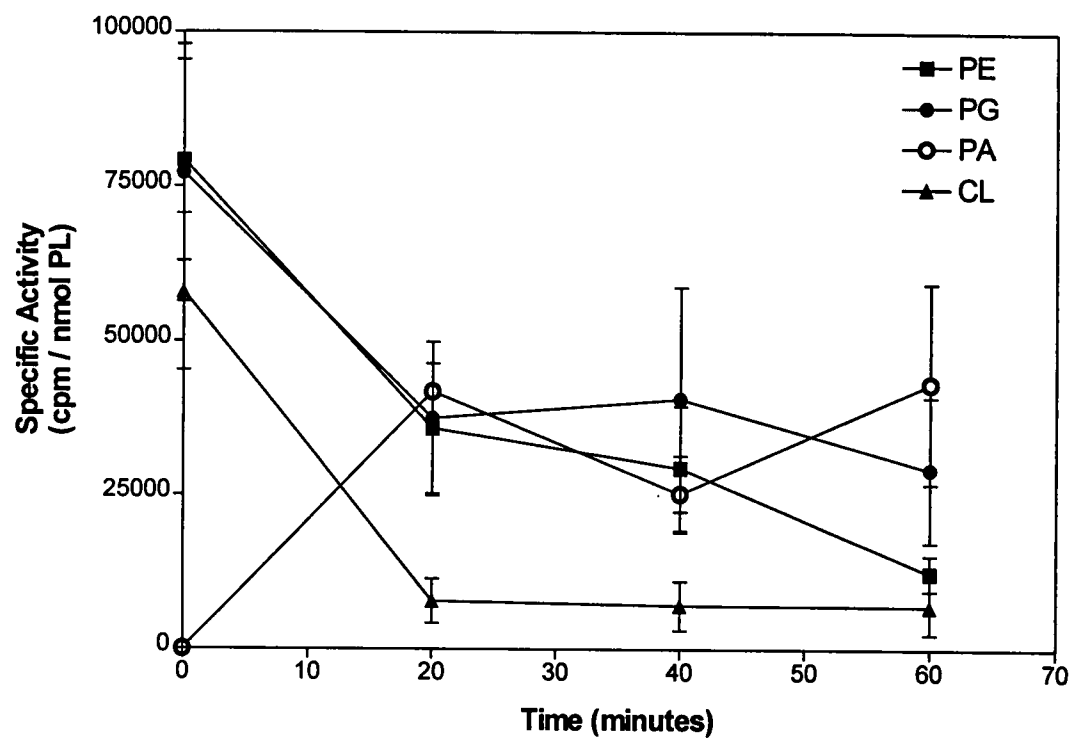


Figure 3-7. Turnover of individual phospholipids of *P. putida* Idaho in the presence of 200 ppm *o*-xylene. PE, phosphatidylethanolamine, PG, phosphatidylglycerol, PA, phosphatidic acid, CL, cardiolipin. Error bars represent 1 standard deviation, $n = 3$.

cardiolipin showed the greatest incorporation of label, followed by phosphatidylglycerol, phosphatidic acid, and phosphatidylethanolamine (Figure 3-4). Cardiolipin again showed the most rapid turnover, followed by phosphatidic acid, a phosphatidylglycerol precursor. Phosphatidylethanolamine and phosphatidylglycerol showed relatively little turnover following exposure to *o*-xylene.

In the absence of *o*-xylene, *P. putida* Idaho exhibited the greatest incorporation of label into phosphatidylglycerol, followed by phosphatidylethanolamine and cardiolipin (Figure 3-5). Phosphatidylglycerol was the most rapidly turned over phospholipid, followed by phosphatidylethanolamine and cardiolipin in *P. putida* Idaho. Phosphatidic acid was detected only at low levels, with no phosphatidylserine detected. In the presence of 75 ppm *o*-xylene, phosphatidylglycerol showed the greatest incorporation of label, followed by phosphatidic acid, phosphatidylethanolamine and cardiolipin. Phosphatidic acid showed the greatest turnover, followed by phosphatidylglycerol (Figure 3-6). Phosphatidylethanolamine and cardiolipin both showed turnover, but at a much lower rate. At 200 ppm *o*-xylene exposure, phosphatidylethanolamine showed the greatest incorporation of label, followed closely by phosphatidylglycerol (Figure 3-7). Cardiolipin and phosphatidic acid also showed incorporation of label. Interestingly, at this concentration, phosphatidylethanolamine showed the greatest turnover, followed by cardiolipin, phosphatidylglycerol, and phosphatidic acid.

DISCUSSION

In the previous section, it was noted that the *P. putida* Idaho strain increased its total phospholipid following exposure to *o*-xylene. This is an unusual response, with all documented microorganisms to date showing loss of phospholipid following exposure to solvents (see Table 2-5). One possible explanation for the increase in total phospholipid is that the Idaho strain has an increased rate of phospholipid biosynthesis in response to exposure to organic solvents. Analysis of phospholipid biosynthetic rates showed several interesting aspects of both the solvent tolerant and sensitive strains.

The *P. putida* MW1200 strain showed an increase in the mean phospholipid biosynthetic rate following exposure to *o*-xylene, compared to the rate shown by cells not exposed to *o*-xylene. Although this increase was not considered significant, it showed an ability to increase phospholipid biosynthesis in response to exposure to *o*-xylene. Although it showed this ability, this increased rate of biosynthesis did not result in increased phospholipid following exposure to xylene. On the contrary, it showed a loss of phospholipid following exposure to *o*-xylene (Table 2-3, Figure 2-4). Therefore it seems that a small increase in biosynthetic rate of phospholipid is not sufficient to afford protection against exposure to solvents.

The *P. putida* Idaho strain showed a basal rate of phospholipid biosynthesis that was significantly greater than that of the *P. putida* MW1200

strain. This is interesting because it did not result in significantly increased total phospholipid in the absence of *o*-xylene over the solvent-sensitive strain ($p = 0.2981$). In the presence of *o*-xylene, the rate of phospholipid biosynthesis increased significantly. The increase in biosynthetic rate was independent of *o*-xylene concentration, as the increase was essentially the same for exposure to both 75 ppm and 200 ppm *o*-xylene. The fact that the biosynthetic rate was significantly greater following exposure to *o*-xylene than that observed in the absence of xylene indicates that the increased rate is an inducible event. In contrast to the slight increase observed for *P. putida* MW1200, the increase in biosynthetic rate following solvent exposure, the increase in biosynthetic rate in *P. putida* Idaho resulted in an increase in total phospholipid. Although this response has not yet been reported in the literature as a response to solvents, increased phospholipid biosynthesis has been reported for other environmental stresses. For example, Russell et al. (1985) reported an increase in the rate of phospholipid (phosphatidylglycerol and phosphatidylethanolamine) synthesis after shifting a *Vibrio costicola* culture to a 3M NaCl from a 1M NaCl culture. A much greater increase in phosphatidylglycerol was noted for this strain. This was thought to occur because phosphatidylglycerol is a negatively charged phospholipid, while phosphatidylethanolamine is a neutral lipid. This increase in charged phospholipids was thought to be important in maintaining charge balance at the membrane level.

The phospholipid turnover rate (rate of loss of labeled phospholipid) also yielded interesting results. The rate of turnover in both strains was higher in xylene-exposed cells than in cells not exposed to xylene. This was an expected result, because membrane phospholipids are damaged by xylene and must be replaced to maintain membrane integrity. Cells not exposed to xylene showed no significant turnover of phospholipid (rates were not significantly different from zero). This was also an expected result, as the membranes of cells grown in the absence of solvent would not be damaged, therefore no turnover is necessary and would be a waste of cellular energy. The greater rate of turnover in the Idaho strain indicates that this strain may be more efficient at scavenging damaged membrane components for use in phospholipid synthesis.

Analysis of individual phospholipid turnover showed several unexpected results. From the section II data it was observed that phosphatidylethanolamine was the most abundant phospholipid in both strains. Therefore it would seem likely that the most incorporation of labeled phosphate would be in phosphatidylethanolamine. However, for the most part this was not the case. In the MW1200 strain it showed the lowest incorporation in both the presence and absence of *o*-xylene. It also showed the least turnover in both the presence and absence of *o*-xylene. In the Idaho strain, phosphatidylethanolamine also showed less incorporation and less turnover in the absence of *o*-xylene, and in the presence of 75 ppm *o*-xylene. Only at 200 ppm did phosphatidylethanolamine show significant turnover. It is possible that phosphatidylethanolamine does not

incur as much damage as other phospholipids, and therefore does not need to be synthesized at a high a level as the other phospholipids. This may explain why incorporation was only high in the Idaho strain at 200 ppm and not at the lower 75 ppm concentration.

Surprisingly, cardiolipin showed significant turnover in both strains grown in the presence of xylene. This was surprising because cardiolipin constitutes a very low proportion of the total phospholipid in these *P. putida* strains. It is interesting to note that increases in phosphatidic acid and phosphatidylglycerol follow decreases in cardiolipin specific activity. This indicates that phosphatidylglycerol is being synthesized at the expense of cardiolipin. The fact that the amount of cardiolipin proportionally decreases in xylene exposed cells (both strains) supports this conclusion.

In both strains, phosphatidylglycerol generally shows the greatest incorporation of label. It also shows a higher rate of turnover than phosphatidylethanolamine in all cases except *P. putida* Idaho in the presence of 200 ppm o-xylene. This was unexpected because although this is the second most abundant phospholipid in each strain, it accounts for 25% of the total phospholipid (at most), and then only in the MW1200 strain. It generally accounts for 20% or less in the Idaho strain. Because it shows high incorporation and significant turnover, it is likely that this phospholipid is more readily damaged by xylene than phosphatidylethanolamine, and needs to be replaced more rapidly. This could also account for the rapid turnover and proportional

decrease in cardiolipin. The turnover of cardiolipin, which contains 2 molecules of phosphatidylglycerol, is an efficient method to replenish phosphatidylglycerol pools.

It is apparent by analysis of the biosynthetic rates of solvent-tolerant and solvent-sensitive strains that the solvent-tolerant strain has a statistically significant increased biosynthetic rate following exposure to *o*-xylene. This indicates that an inducible membrane repair mechanism exists in *P. putida* Idaho which allows rapid repair of damaged membrane components. Investigators of solvent-tolerant organisms have noted an increased requirement for magnesium for these strains. Ramos et al.(1995) showed that the addition of excess magnesium ions aided in decreasing the lag phase for cells grown in high concentrations of toluene. However, it is not clear from their study whether the magnesium ions were required for solvent tolerance or for toluene degradation, since toluene was also the carbon source in that experiment. If rapid membrane repair is occurring in the solvent-tolerant strains, then a requirement for magnesium ions would exist because magnesium is known to be one of the important cations in phospholipid and LPS stabilization (Labischinski et al., 1985). If extra magnesium were not added to the medium, cells would have to recycle their own magnesium during lipid turnover, which may account for the observed lag phase in *P. putida* Idaho following exposure to *o*-xylene. Magnesium deficiency could also contribute to the stationary phase sensitivity observed in *P. putida* Idaho.

Although many investigators have examined fatty acid content and phospholipid content of various bacterial strains in response to organic solvents (see previous section), to date no one has examined phospholipid biosynthetic rate following exposure to organic solvents. In fact, phospholipid biosynthesis has rarely been studied at all in bacteria, with the exception of *E. coli* and a few related enteric bacteria. It is difficult to study other aspects of increased activity such as increased mRNA levels because these genes have not been identified in *P. putida*. Because of the statistically significant increase phospholipid biosynthesis seen in *P. putida* Idaho, it is reasonable to assume that this is an inducible event, but it is difficult to be absolutely certain until it is possible to study mRNA, or increased activity of proteins involved in phospholipid biosynthesis. These proteins have not as yet been characterized for *P. putida*.

PART IV.

OUTER MEMBRANE RESPONSES OF SOLVENT- TOLERANT AND SOLVENT-SENSITIVE *PSEUDOMONAS* *PUTIDA* STRAINS TO *O*-XYLENE

INTRODUCTION

One mechanism of preventing damage to the cytoplasmic membrane may be to limit permeabilizing concentrations of the solvent from reaching the inner membrane. This would have to be accomplished by the outer membrane. It has been demonstrated that changes in the outer membrane structure are responsible for resistance to antibiotics in *Pseudomonas aeruginosa* (Arnoff, 1988; Legakis et al., 1989; Leying et al., 1992; Michea-Hamzehpour, 1991; Piddock et al., 1992). By altering the amount of LPS and surface protein in the outer membrane, these strains became antibiotic resistant. It has been found that by increasing cell surface hydrophobicity, resistance to quinolones was increased. Lack of O-antigen chains provides increased surface hydrophobicity in *Shewanella* oil field isolates (Pickard et al., 1993) which allows for greater interaction with oil droplets. Conversely, it is possible for bacterial cells to decrease their surface hydrophobicity by altering the composition and chain length of O-antigen. Changes in the structure of the core region could also decrease surface hydrophobicity. It was shown in *P. putida* S12 that exposure to toluene resulted in a decreased water contact angle when compared to cells grown in glucose (Weber, 1995). It is possible that this type of alteration in the outer membrane of the Idaho strain renders the cell less permeable to *o*-xylene by decreasing its surface hydrophobicity.

Alterations in membrane permeability could be a significant factor in the solvent tolerance of *Pseudomonas putida* Idaho. Changes in the membrane ultrastructure could result in decreased diffusion of substrates such as xylene and toluene. The differences in surface structure were analyzed by scanning electron microscopy, and by chemical analysis of LPS. Surface hydrophobicity was assayed by the bacterial adhesion to hydrocarbons (BATH) assay. Differences in the permeability between *Pseudomonas putida* Idaho and control strain MW1200 to hydrophobic compounds was determined by two methods, by hydrophobic dye uptake (Tsuchido et al., 1989) and by comparison of sensitivity to difloxacin, a hydrophobic antibiotic with an octanol-water partition coefficient equal to that of xylene. Decreased permeability to these compounds by the Idaho strain would indicate that permeability to xylene and related compounds is reduced compared to the control strain. The goal of these experiments was to examine the outer membrane of each strain for significant structural changes in the LPS and protein content in response to exposure to solvent, and relate these changes to solvent tolerance in terms of each strain's relative permeability to hydrophobic substrates.

MATERIALS AND METHODS

Growth Conditions

Each strain was grown in continuous culture for SEM and LPS experiments. The continuous cultures contained minimal salts medium with 10 mM sodium succinate as the carbon source. The minimal salts medium was formulated as follows: 0.7 g K_2HPO_4 , 0.7g KH_2PO_4 , 0.5 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4$, and 1 ml Wolfe's Minerals (Balch et al., 1979) per liter of nanopure water (pH 6.8). Cultures were started using 5 mg lyophilized cells, and grown in batch phase until the OD_{600} was 0.4. At this point the continuous culture was started and allowed to run for 24 hours (approximately 17 generations) prior to removing samples for LPS analysis.

P. putida Idaho was cultivated in 10 mM succinate-minimal salts medium, in 10 mM succinate-minimal salts medium supplemented with 75 ppm *o*-xylene, and in 10 mM succinate-minimal salts medium supplemented with 200 ppm *o*-xylene. *O*-xylene was added directly to the aqueous phase of the medium. The concentration of *o*-xylene in the aqueous phase was verified by gas chromatographic analysis. *P. putida* MW1200 was cultivated in 10 mM succinate-minimal salts medium, and in 10 mM succinate-minimal salts medium supplemented with 75 ppm *o*-xylene. A 100 ml sample of this strain previously grown in 75 ppm *o*-xylene was subsequently exposed to 200 ppm *o*-xylene for two hours, then subjected to LPS extraction procedures and SEM analysis.

Scanning Electron Microscopy (SEM)

Each strain was grown in the conditions described above. Fifty milliliter samples were then collected from each continuous culture, centrifuged to concentrate the cells, then washed three times in 0.1 M phosphate buffer. The washed cells were then fixed in a 2.5% glutaraldehyde solution for 15 minutes. The fixed cells were then washed twice in phosphate buffer to remove the residual glutaraldehyde. The fixed cells were dehydrated using ethanol and amyl acetate, then dried using a critical point drying method. The dried cells were then subsequently applied to a silicon wafer. Specimens were examined at 700 V using a high resolution S-4500 scanning electron microscope.

Extraction and Purification of LPS

LPS was quantitatively recovered from each *Pseudomonas* strains using the Darveau and Hancock (1983) technique. This method involves repeated ultracentrifugation in the presence of 0.5 M EDTA , and 2% SDS in a Tris-HCL buffer system. DNA, RNA, and protein are removed using DNase, RNase and proteinase K digestion steps. The purity of the LPS was assessed by detection of the presence of DNA and RNA (UV absorption at 260 nm and 280 nm), and the presence of protein using the Lowry technique. LPS was initially quantified by assaying the amount of KDO in the sample. The recovered LPS was then analyzed using a tricine-sodium dodecyl sulfate- polyacrylamide gel (TSDS-

PAGE) (Lesse et al., 1990). Separating gels were formed with 14% acrylamide with 0.1% SDS, with a 7% acrylamide stacking gel. Samples of approximately 3 µg LPS per lane were loaded per lane. Electrophoresis was performed with a constant voltage of 64V for 15 minutes, then 80V for 100 minutes. LPS bands were visualized using the silver stain method of Tsai and Frasch (1982).

LPS Component Analysis

The O-antigen region of *Pseudomonas* LPS was analyzed by polyacrylamide gel electrophoresis (Laemmli, 1970). This assay gave qualitative information on the type of LPS (smooth or rough) present on the cell.

The fatty acid content of the Lipid A was established by direct extraction of LPS using a sequential alkaline methanolysis/acid hydrolysis developed by Mayberry and Lane (1993). This method allows for quantitative recovery of both ester-linked and amide-linked fatty acids from the disaccharide backbone of Lipid A. The derivatized fatty acids were then separated, quantified and identified using GC/MS.

Carbohydrate composition of LPS was determined by preparing trimethylsilyl methylglycoside derivatives, followed by GC-MS. The LPS was treated with anhydrous methanolic HCl (1M, 80°C, 16 hours), then *N*-acetylated (methanol-pyridine-acetic anhydride) for 6 hours at room temperature. The resultant methylglycosides were then derivatized with trimethylsilane reagent

(Tri-Sil, Pierce Chemical Co.), and analyzed by GC-MS using a 50 meter Quadrex column.

Cell Surface Hydrophobicity

Cell surface hydrophobicity was assayed using the BATH (bacterial adhesion to hydrocarbons) technique (Rosenburg et al., 1980; Lichtenberg et al., 1985). In this assay, each strain was grown in continuous culture in the absence of *o*-xylene, and in the presence of various concentrations of *o*-xylene. The OD₆₀₀ of the continuous culture was 0.4. Ten milliliters of culture was removed, centrifuged and washed three times with 0.1 M phosphate buffer to remove *o*-xylene associated with the cells. The cells were then resuspended in sterile minimal salts medium and readjusted to an OD₆₀₀ of 0.4. Three milliliters of washed cell culture was removed to a new tube which also contained 3 ml of hexadecane. The tube was vortexed for 1 minute, then allowed to separate for 20 minutes. The OD₆₀₀ of the aqueous layer was then assayed again. The measure of hydrophobicity was recorded as the percentage of cells remaining in the aqueous phase. Following this measurement the aqueous phase was checked microscopically for cell lysis, which could interfere with the assay.

Difloxacin Permeability Assay

Each strain was grown in the absence of *o*-xylene, then transferred to medium containing various concentrations of difloxacin. Difloxacin is a hydrophobic antibiotic which has an octanol-water partition coefficient of 3.1, equal to that of xylene. After establishing a minimum inhibitory concentration (MIC) for each strain, the experiment was repeated in the same manner, but with the addition of *o*-xylene in the concentration of 75 ppm to the medium for the MW1200 strain. The Idaho strain was subjected to 75 ppm and 200 ppm *o*-xylene. Following a 3 hour exposure to the solvent, the cells were concentrated by centrifugation (10 min, 8000 x g), then resuspended in fresh medium containing difloxacin and assayed again for minimum inhibitory concentration.

NPN Permeability Assay

Permeability of the cell to hydrophobic substrates can also be assessed by the use of 1-*N*-phenylnaphthylamine (NPN) (Tsuchido et al., 1989). NPN is an uncharged, lipophilic dye that fluoresces weakly in aqueous environments, but fluoresces strongly in hydrophobic environments such as the cell membrane. Analysis of changes in the fluorescence intensity between a solvent tolerant strain and a solvent sensitive strain gives insight as to relative permeability of each strain to hydrophobic substrates.

Each strain was grown under continuous culture conditions with 10 mM succinate as the carbon source, and amended with 75 ppm *o*-xylene or 75 ppm and 200 ppm *o*-xylene for the MW1200 strain and the Idaho strain, respectively. Twenty milliliter aliquots were withdrawn from each culture. The cells were then centrifuged and washed three times with 0.1 M phosphate buffer. The cells were then resuspended in sterile minimal salts medium, and the volumes adjusted to a final OD₆₁₀ of 0.2. From these cultures, a 3.96 ml aliquot was removed to a 10 ml opaque scintillation vial. Forty microliters of NPN (dissolved in acetone) was added to bring the total concentration in the vial to 5μM. The samples were incubated at room temperature with the NPN for 10 minutes, then assayed for fluorescence (excitation wavelength of 348 nm, emission 408 nm). Fluorescence was measured for cells in minimal salts medium without the addition of NPN, and for NPN to sterile minimal salts medium without cells for use as controls.

Statistics

Each experiment was performed at least three times. Data points for each graph are an average of three independent experiments, with error bars representing one standard deviation. Data points shown in tables expressed as the mean $\pm$ one standard deviation. T-tests ($\alpha = 0.01$) were performed to determine significant

differences for values obtained for *P. putida* Idaho and *P. putida* MW1200 grown under the different growth conditions.

RESULTS

SEM

P. putida MW1200 grown in continuous culture in the absence of *o*-xylene has an ordered, slightly wrinkled cell surface. Most of the cells have this appearance, and are normal, rod-shaped cells ranging from 1-2 microns in length (Figure 4-1). *P. putida* MW1200 grown in the presence of 75 ppm *o*-xylene appear similar to those grown in the absence of *o*-xylene, with some minor cell surface blebbing (data not shown). Exposure to 200 ppm *o*-xylene results in cells with cell surface blebbing and a shrunken, crenated appearance. The majority of these cells measured less than 1 micron (Figure 4-2).

P. putida Idaho grown in continuous culture in the absence of xylene has a much less wrinkled cell surface than the MW1200 strain, showing a more smooth appearance. These cells range from 1-1.5 microns in length (Figure 4-3).

P. putida Idaho grown in continuous culture in the presence of 200 ppm *o*-xylene show a different response to xylene. About half the cells appear unaffected, while the rest show distinct invaginations. these cells also range from 1-1.5 microns in length (Figure 4-4).

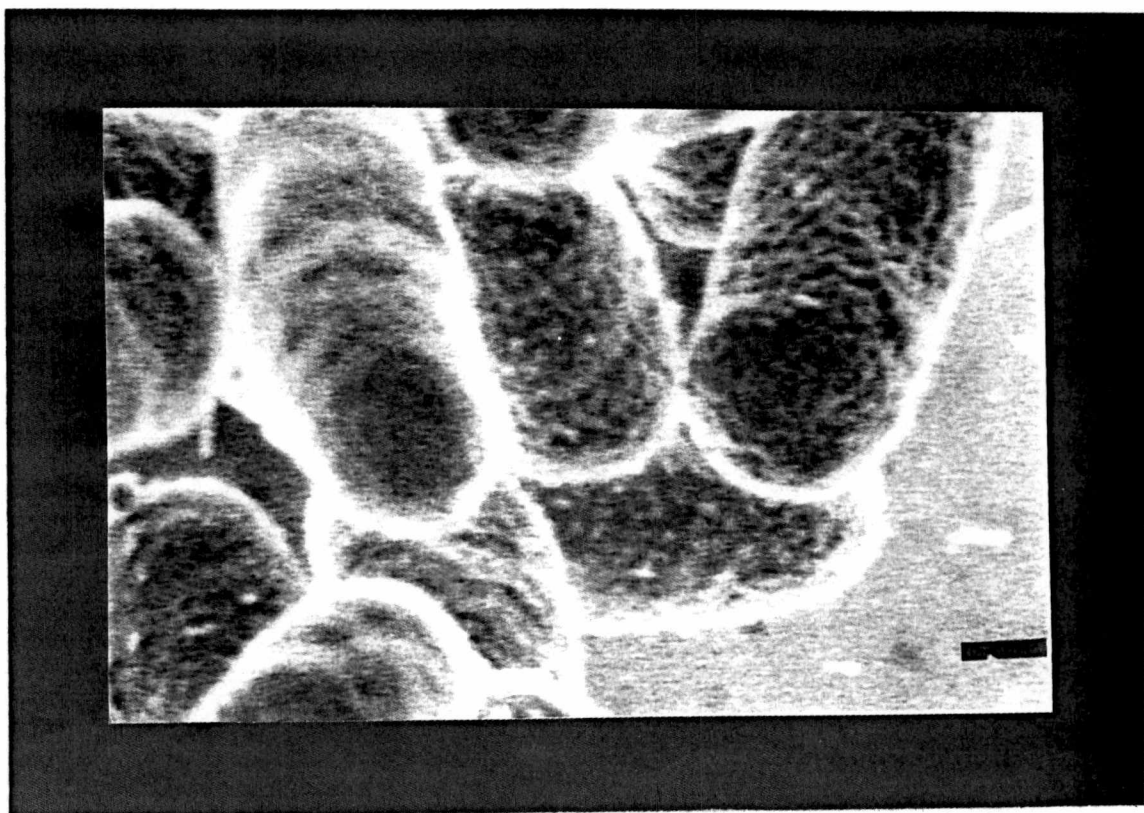


Figure 4-1. SEM micrograph of *Pseudomonas putida* MW1200 grown in the absence of *o*-xylene. Magnification x 50,000. Bar = 200 nm.

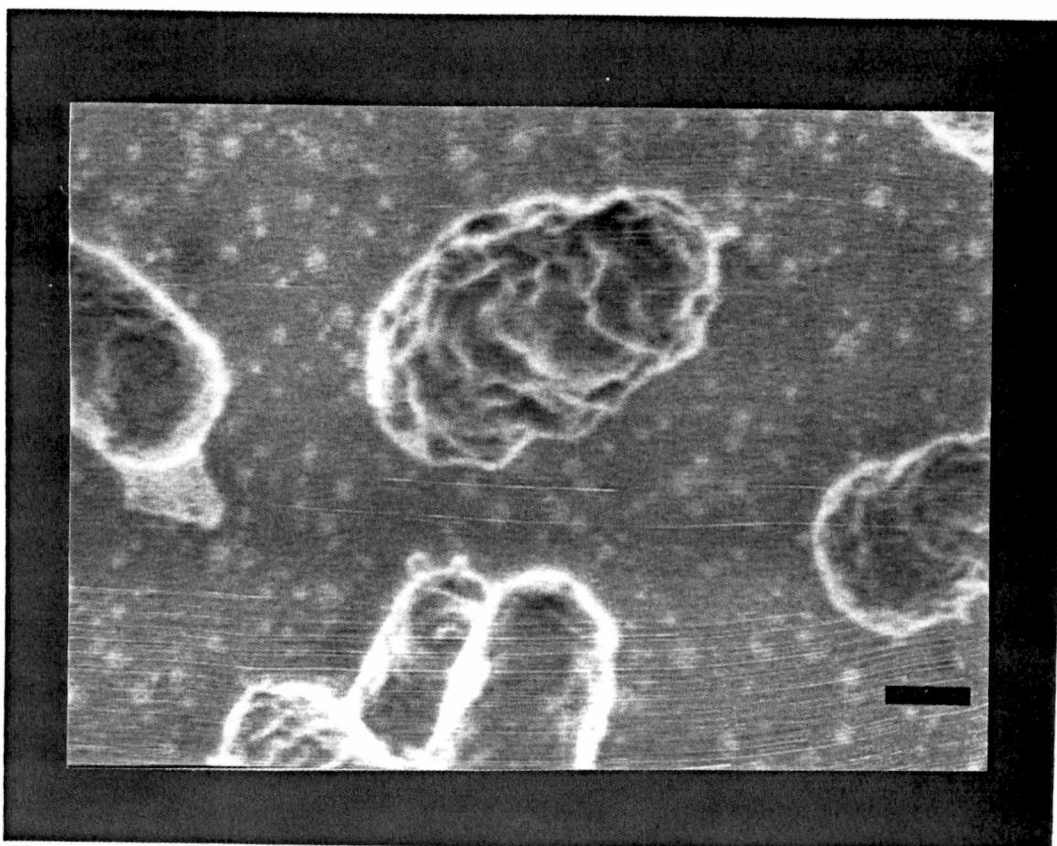


Figure 4-2. SEM micrograph of *Pseudomonas putida* MW1200 exposed to 200 ppm *o*-xylene. Magnification x 50,000. Bar = 325 nm.

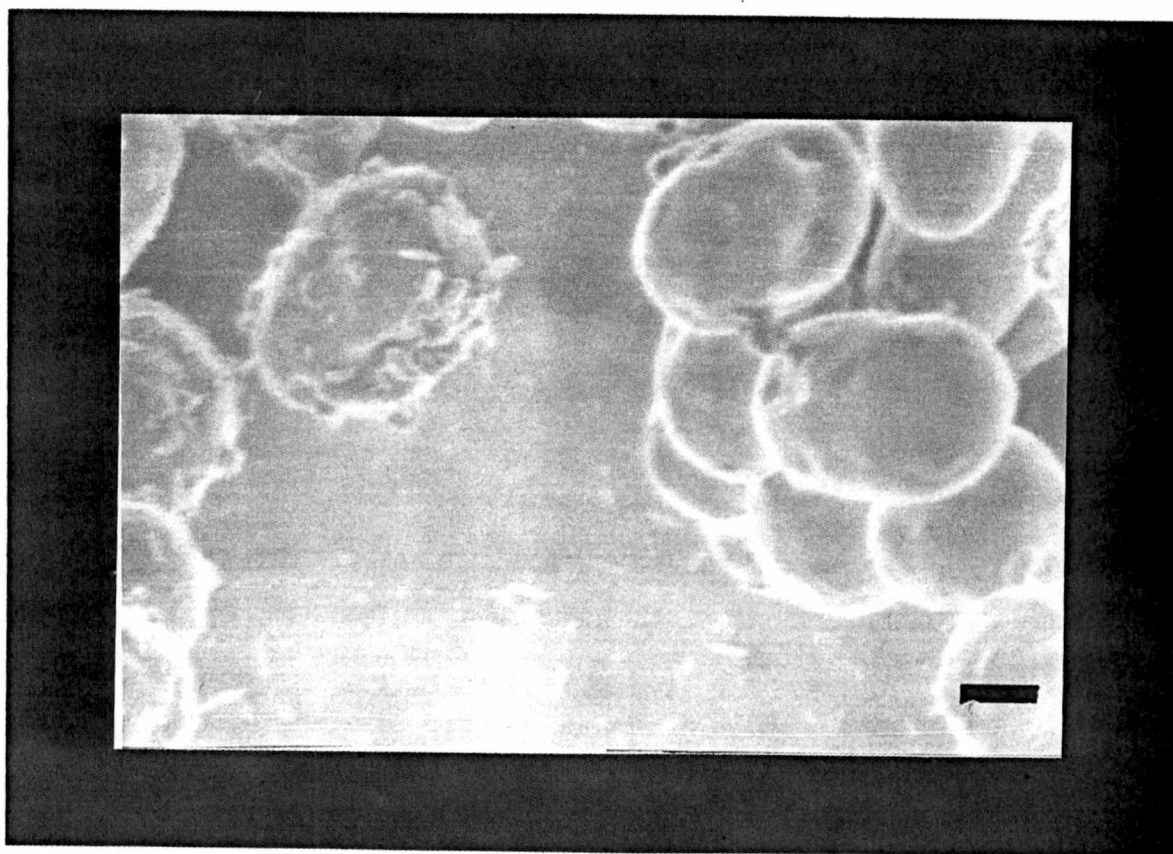


Figure 4-3. SEM micrograph of *Pseudomonas putida* Idaho grown in the absence of *o*-xylene. Magnification x 50,000. Bar = 500 nm.

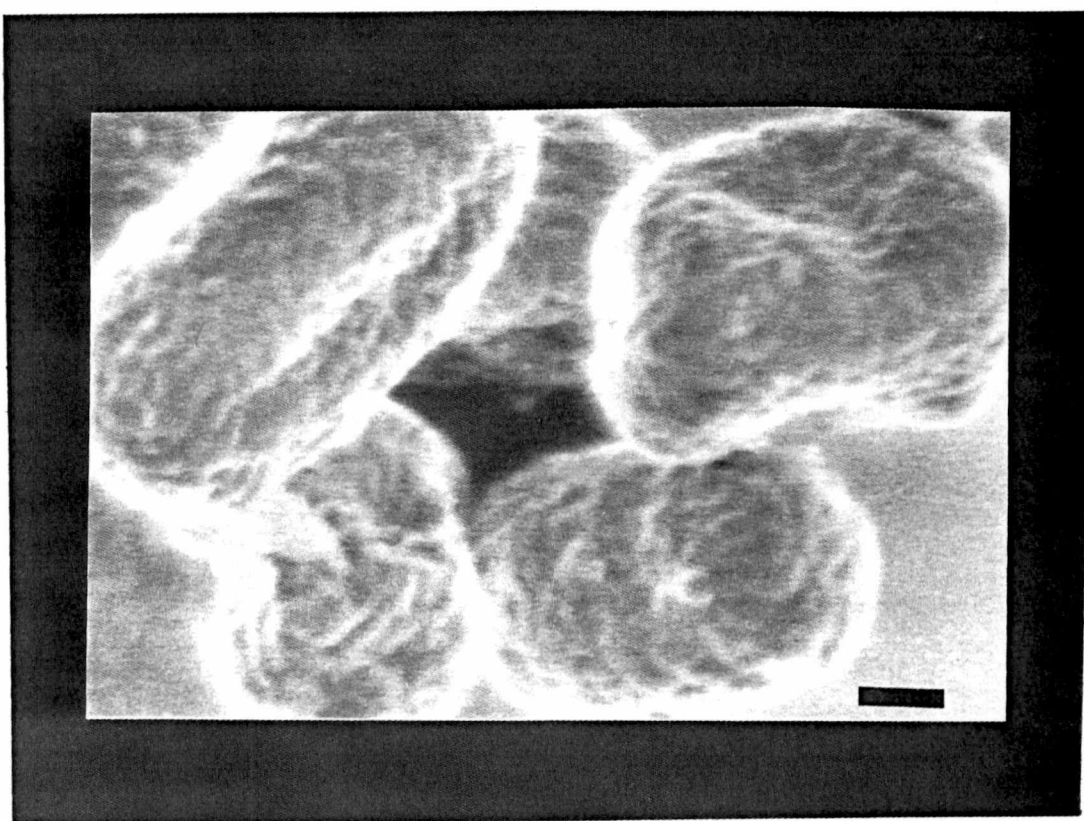


Figure 4-4. SEM micrograph of *Pseudomonas putida* Idaho exposed to 200 ppm *o*-xylene. Magnification x 50,000. Bar = 250 nm.

PAGE Analysis

Purified LPS obtained from both strains exhibited no detectable amounts of protein or nucleic acid. A silver-stained polyacrylamide gel showing LPS of each strain grown in the presence and absence of *o*-xylene is shown in Figure 4-5. The solvent-sensitive MW1200 strain shows a ladder-like banding pattern indicative of smooth-type LPS (lanes 1). No significant change in LPS banding pattern was seen in cells grown in the presence of 75 ppm xylene (lane 2). When this strain is subjected to 200 ppm *o*-xylene and incubated for 2 hours, it is difficult to isolate good quality LPS from these cells (lane 3). This is most likely due cell lysis (verified by light microscopy). *Escherichia coli* LPS is shown in lane 9 as an example of smooth LPS, for comparison purposes.

The Idaho strain shows typical rough-type LPS when grown both in the presence and absence of xylene (lane 4). A higher molecular weight band is seen in the LPS of the Idaho strain grown in the absence of xylene, which disappears from the LPS of the xylene-exposed cells. It appears to be replaced by a lower molecular weight molecule which migrates more quickly through the gel (lanes 5 and 6).

Chemical Composition of LPS

The LPS composition of each strain is showed in Figure 4-6. The *P. putida* MW1200 strain contained 62.8% glucose, 27% ribose, 5.3% KDO, 2.3%

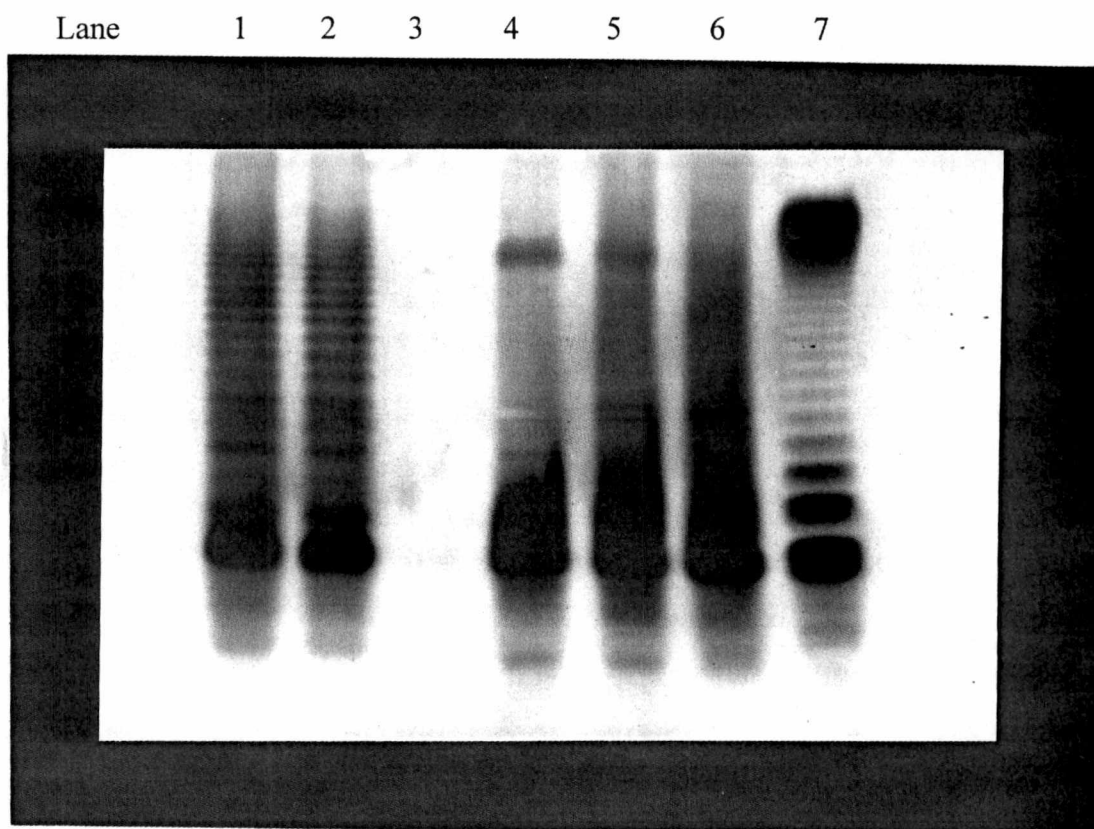


Figure 4-5. Silver stained polyacrylamide gel of LPS from *P. putida* MW1200 and *P. putida* Idaho. Lane 1, *P. putida* MW1200 LPS from cells grown in the absence of *o*-xylene; lane 2, *P. putida* MW1200 LPS from cells grown in the presence of 75 ppm *o*-xylene; lane 3, *P. putida* MW1200 LPS from cells grown in the presence of 200 ppm *o*-xylene. Lane 4, *P. putida* Idaho LPS from cells grown in the absence of *o*-xylene; lane 5, *P. putida* Idaho LPS from cells grown in the presence of 75 ppm *o*-xylene; lane 6, *P. putida* Idaho LPS from cells grown in the presence of 200 ppm *o*-xylene; lane 7, *Escherichia coli* smooth type LPS (for comparison purposes).

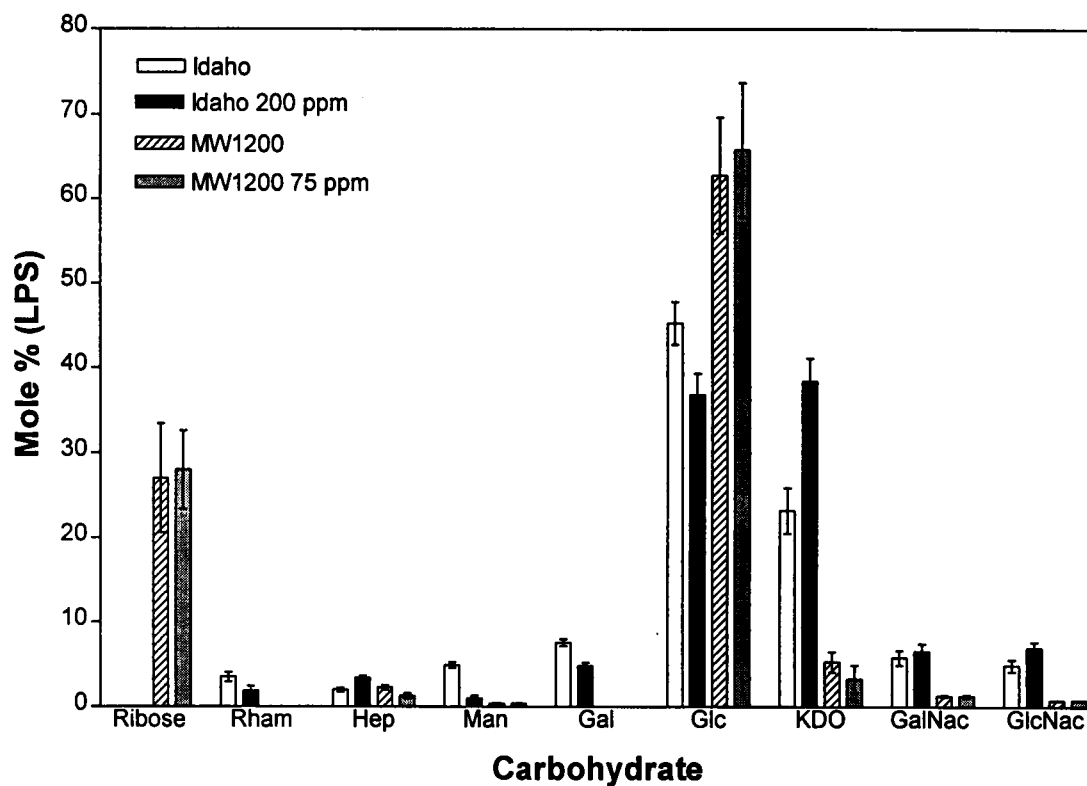


Figure 4-6. Carbohydrate composition of LPS from *P. putida* MW1200 and *P. putida* Idaho in the presence and absence of *o*-xylene. Rham, rhamnose; Hep, heptose; Man, mannose; Gal, galactose; Glc, glucose; KDO, 2-keto-3-deoxyoctulosonate; GalNac, galactosamine, GlcNac, glucosamine. Error bars represent 1 standard deviation, $n = 3$.

Table 4-1. Fatty acid composition (mole percentage) of LPS Lipid A for *P.*

putida MW1200 and *P. putida* Idaho in the presence and absence of *o*-xylene.

Total LPS fatty acid is shown in the last row. Values are ± 1 standard deviation,

n = 5.

Fatty Acid Type	MW1200		Idaho	
	0 ppm	75 ppm	0 ppm	200 ppm
Hydroxy				
3OH10	6.1 $\pm$ 1.22	15.7 $\pm$ 1.99	17.7 $\pm$ 3.14	31.6 $\pm$ 4.23
2OH12	17.6 $\pm$ 2.86	23.6 $\pm$ 3.21	25.4 $\pm$ 2.97	30.9 $\pm$ 5.46
3OH12	17.6 $\pm$ 2.18	20.0 $\pm$ 3.09	21.0 $\pm$ 3.54	22.4 $\pm$ 4.01
Saturated				
15:0	4.7 $\pm$ 1.23	5.3 $\pm$ 0.95	0.2 $\pm$ 0.02	0.1 $\pm$ 0.02
16:0	37.7 $\pm$ 5.11	26.7 $\pm$ 4.65	23.4 $\pm$ 3.64	9.9 $\pm$ 1.82
18:0	9.9 $\pm$ 1.08	2.3 $\pm$ 0.36	11.8 $\pm$ 2.18	5.0 $\pm$ 0.81
Total FA *	11.64 $\pm$ 1.83	3.30 $\pm$ 0.65	8.93 $\pm$ 0.96	17.14 $\pm$ 2.04

*Total is expressed in nanomoles fatty acid per milligram dry weight.

heptose, 1.1% *N*-acetyl galactosamine, and 1% *N*-acetylglucosamine. No rhamnose or galactose was detected. The MW1200 strain showed no compositional changes following exposure to *o*-xylene. However, the total LPS recovered from xylene-exposed cells was 20% less than that recovered from cells grown in the absence of xylene. Carbohydrates accounted for 8.96% of the total LPS for this strain in the absence of *o*-xylene. In the presence of 75 ppm *o*-xylene, carbohydrates accounted for 29.87% of the total LPS. The fatty acid composition of lipid A for *P. putida* MW1200 is shown in Table 4-1. The LPS fatty acid concentration in the absence of *o*-xylene was 11.64 ± 1.8 nanomoles per milligram dry weight. In the presence of *o*-xylene this concentration decreased to 3.28 ± 0.65 nanomoles per milligram dry weight. The percentage of 16:0 (palmitic acid) increased following exposure to *o*-xylene, as did 3OH10.

The *P. putida* Idaho strain grown in the absence of *o*-xylene showed 45.2% glucose, 23.2% KDO, 7.6% galactose, 5.8% *N*-acetyl galactosamine, 4.9% *N*-acetyl glucosamine, 4.9% mannose, 3.5 % rhamnose, and 2.3% heptose. Carbohydrates accounted for 20.39% of the total LPS weight in the absence of *o*-xylene. Following exposure to 200 ppm *o*-xylene, glucose decreased to 36.8%, rhamnose to 1.9%, mannose to 1.0%, and galactose to 4.8%. KDO increased to 38.4%, *N*-acetyl galactosamine to 6.6%, and *N*-acetyl glucosamine to 7.0%, and the heptose to 3.4%. The total LPS recovered from *P. putida* Idaho increased by 30% following exposure to *o*-xylene. Carbohydrates only accounted for 6.67% of the total LPS weight in the presence of *o*-xylene.

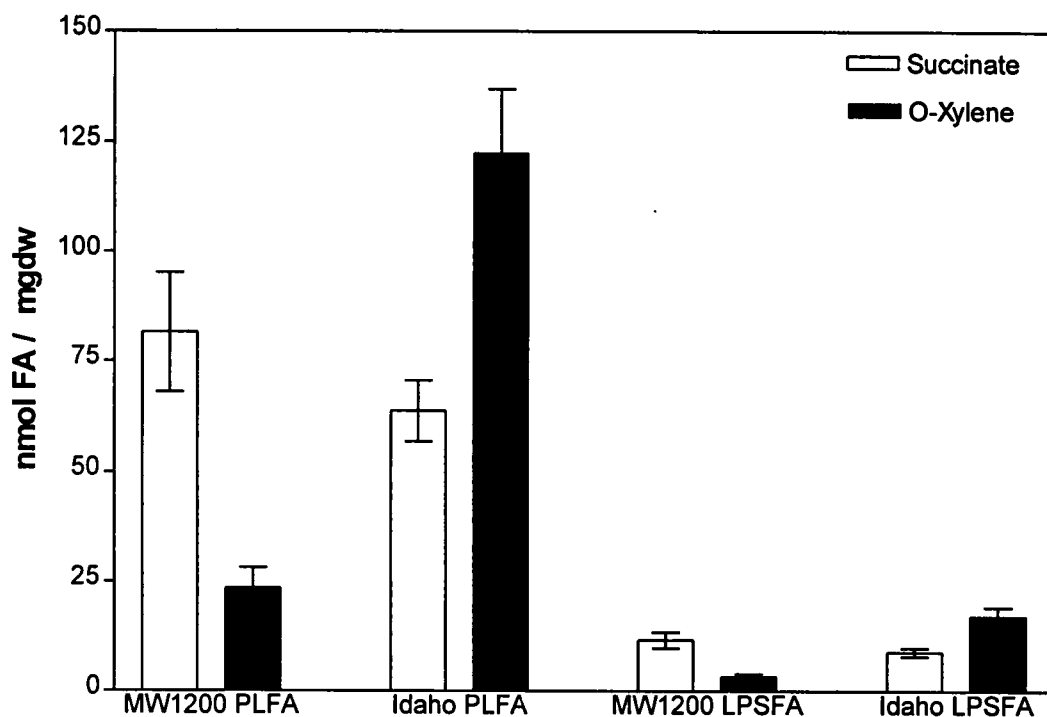


Figure 4-7. A comparison of the fatty acid content of *P. putida* Idaho and *P. putida* MW1200 in the absence and presence of *o*-xylene. Error bars represent 1 standard deviation, n = 5. The *o*-xylene concentration for *P. putida* MW1200 was 75 ppm and was 200 ppm for *P. putida* Idaho. PLFA, phospholipid fatty acid; LPSFA, LPS fatty acid.

The fatty acid composition of lipid A for *P. putida* Idaho is shown in Table 4-1. Following exposure to *o*-xylene, the concentrations of all hydroxy fatty acids showed significant increases, with proportional decreases noted in all non-hydroxy fatty acids. The LPS fatty acid concentration in the absence of *o*-xylene was 8.94 ± 0.97 nanomoles per milligram cell dry weight (Figure 4-7). The LPS fatty acid concentration in the presence of *o*-xylene was 17.1 ± 2.06 nanomoles per milligram cell dry weight.

Surface Hydrophobicity Assay

Results of the BATH assay are shown in Figure 4-8. Throughout the *o*-xylene concentrations tested, both strains showed consistent results. The mean percentage of cells remaining in the aqueous phase for *P. putida* MW1200 was 54.02 ± 1.26 . This value was consistent over the range of *o*-xylene concentrations tested. The mean percentage of cells remaining in the aqueous phase for *P. putida* Idaho was 91.23 ± 2.20 . This value was also consistent over the range of *o*-xylene concentrations tested, with no significant differences noted between concentrations. The difference in cell surface hydrophobicity between the Idaho strain and the MW1200 strain was significant ($p < 0.0001$).

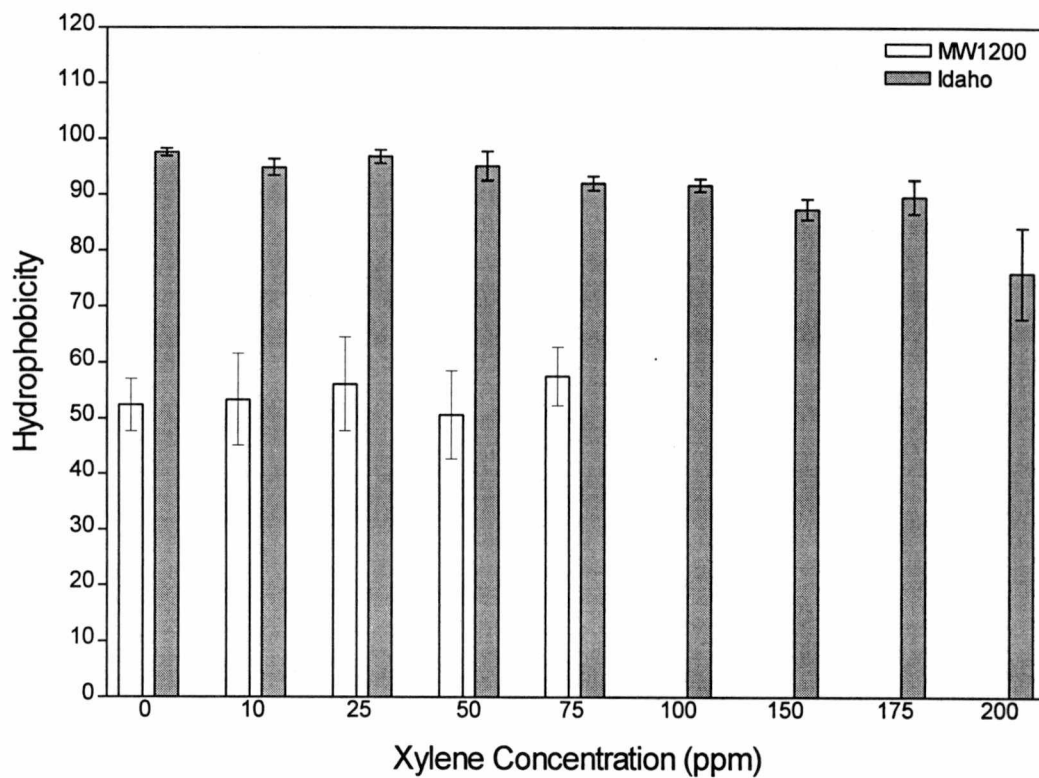


Figure 4-8. Bacterial adhesion to hydrocarbons (BATH) assay performed on *P. putida* MW1200 and *P. putida* Idaho. Hydrophobicity represents the percentage of cells remaining in the aqueous phase following incubation with hexadecane (0 = most hydrophobic, 100 = most hydrophilic). Error bars equal 1 standard deviation, n = 5.

Table 4-2. Difloxacin permeability assay for *P. putida* MW1200 and *P. putida* Idaho as measured by sensitivity to the hydrophobic antibiotic (MIC = minimum inhibitory concentration).

Strain	MIC (µg / ml)
<i>P. putida</i> MW1200	0.1
<i>P. putida</i> MW1200 + 75 ppm <i>o</i> -xylene	<0.05
<i>P. putida</i> Idaho	10
<i>P. putida</i> Idaho + 75 ppm <i>o</i> -xylene	10
<i>P. putida</i> Idaho + 200 ppm <i>o</i> -xylene	10

Difloxacin Permeability Assay

The results of the permeability assay are shown in Table 4-2. *P. putida* MW1200 demonstrated a minimum inhibitory concentration (MIC) of 0.1 µg/ml when grown in the absence of *o*-xylene. The MIC value for *P. putida* MW1200 following exposure to 75 ppm *o*-xylene diminished to less than 0.05 µg/ml, showing increased sensitivity. *P. putida* Idaho demonstrated a MIC of 10 µg/ml in the absence of *o*-xylene. Following exposure to *o*-xylene, the MIC remained the same at 10 µg/ml, demonstrating neither increased nor decreased sensitivity to this hydrophobic antibiotic.

NPN Permeability Assay

The results for the NPN permeability assay are shown in Figure 4.9. Cells in sterile minimal salts medium without the addition of NPN showed no fluorescence over background. The sterile minimal salts medium with NPN addition (no cells) demonstrated an average fluorescence of $8.67 (\pm 0.76) \times 10^5$ (arbitrary units). This value was subsequently subtracted from the values obtained for the cells exposed to NPN.

The MW1200 strain showed a significant increase from the initial value of 6.60×10^5 in the absence of xylene to 2.02×10^6 in the presence of *o*-xylene ($p < 0.0001$). The increase in fluorescence indicates an increase in cellular permeability to hydrophobic substrates. The Idaho strain showed no significant increase between the fluorescence value in the absence of *o*-xylene, 4.16×10^5 ,

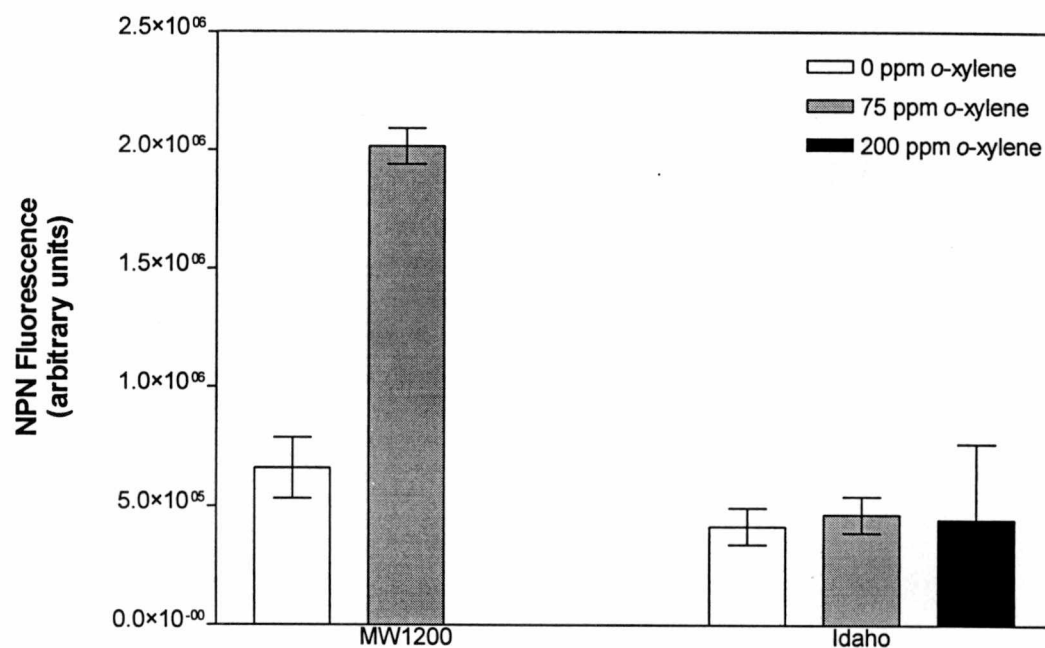


Figure 4-9. NPN permeability assay for *P. putida* MW1200 and *P. putida* Idaho in the absence and presence of *o*-xylene (given in concentrations of ppm). Error bars represent 1 standard deviation, n = 3.

and those values obtained for growth in the presence of 75 ppm *o*-xylene (4.66×10^5) and 200 ppm *o*-xylene (4.460×10^5). T-tests showed values for the difference in permeability Idaho exposed to 75 ppm and no exposure have a p value of 0.4676. The p value for no exposure versus 200 ppm was 0.8812. This indicates that the Idaho strain showed no increase in permeability to hydrophobic substrates following exposure to *o*-xylene.

DISCUSSION

SEM characterization showed that the surface structure of *P. putida* MW1200 and *P. putida* Idaho was significantly different, even in the absence of *o*-xylene. It also showed that the MW1200 strain is in fact damaged on an ultrastructural level following exposure to *o*-xylene. The Idaho strain also showed ultrastructural changes following exposure to *o*-xylene, but in a different manner than the MW1200 strain. No blebbing was observed, but some of the cells appeared to have concave areas, giving the impression that it had collapsed in on itself. It is possible that this is an artifact of the drying process, or may be due to xylene-induced cell damage. It is more likely an artifact of the drying process, since the Idaho strain does not show any of the typical damage such as blebbing and "peeling" observed by other investigators of organic solvent damage to bacterial membranes (de Smet et al., 1978; Woldringh et al., 1974).

The original isolation of LPS and characterization using PAGE showed that the LPS of the MW1200 strain and the Idaho strain were different, and that the LPS of the Idaho strain was modified following exposure to *o*-xylene. Chemical characterization of LPS showed several distinct differences between the LPS of the two strains. The MW1200 strain exhibited an LPS that contained an average of 25% ribose, a sugar not present in *P. putida* Idaho. The MW1200 strain also exhibited a loss of lipid from LPS following exposure to xylene, as indicated by the increase in carbohydrate content of the LPS and the decrease in LPS content. This loss is most likely due to xylene damage, because the carbohydrate composition did not differ significantly between cells in the presence and absence of xylene, as indicated by the chemical composition analysis and the PAGE data.

The Idaho strain shows some interesting changes in LPS following xylene exposure. The amount of total LPS recovered per milligram dry weight increased. This indicates the presence of a more ordered cell surface. A decrease in porin content accompanied by a concurrent increase in LPS has been associated with decreased permeability to antibiotics. Several *Pseudomonas* strains are antibiotic-resistant due to changes in the LPS and protein structure of the cell surface, which change the cell surface hydrophobicity and permeability properties (Arnoff, 1988; Legakis et al., Leying, 1992; 1989; Michea-Hamzehpour et al., 1991; Piddock et al., 1992). The Idaho strain also shows shifts in carbohydrate composition following xylene exposure. The neutral

sugars glucose, galactose, mannose, and rhamnose all decreased in concentration. The charged sugars *N*-acetyl glucosamine and *N*-acetyl galactosamine increased, as did the concentration of KDO. By increasing the proportion of charged sugars in LPS, the cell surface would become more hydrophilic, and less permeable to hydrophobic substrates. Increased KDO corresponds to the increase in fatty acid content displayed by the Idaho strain following exposure to xylene. The increased concentration of KDO and hydroxy fatty acid creates a very ordered lipid interior that is less likely to become disorganized in the presence of xylene. The increase in hydroxy fatty acids would create a moderately charged lipid environment less likely to incorporate xylene, thereby reducing the chance for outer membrane damage.

The Idaho strain did have a more hydrophilic cell surface than the MW1200 strain, as shown by the BATH assay, but did not become more hydrophilic following xylene exposure. It is interesting to note that the changes in *P. putida* Idaho LPS don't appear to make the cell less permeable to hydrophobic substrates. Following exposure to xylene, the Idaho strain does not show an increased MIC towards difloxacin. This would be expected if the cell were less permeable to hydrophobic substrates following the shift in LPS composition. Similar results were seen in the NPN assay, where there was no significant difference between cells exposed to xylene and cells grown in the absence of xylene.

What is unclear is the consequence of the changes observed in the chemical composition of the LPS of the Idaho strain. The LPS composition was significantly altered, yet did not appear to make the cell surface more hydrophilic or less permeable following exposure to xylene than it was prior to xylene exposure. It is possible that the techniques employed were not sensitive enough to detect small changes in permeability and hydrophobicity brought about by alterations in LPS structure. It is also possible that the altered LPS is more stable in the presence of xylene than the original LPS structure.

However, it is clear that the relatively neutral, hydrophobic surface of the MW1200 strain affords little protection against hydrophobic substrates. The MIC for difloxacin in this strain in the absence of *o*-xylene was 100 fold less than that of the Idaho strain. Following exposure to *o*-xylene, the MIC for difloxacin dropped to less than half that observed in the absence of xylene, indicating that xylene permeablized the MW1200 strain. This idea is further supported by the NPN assay, which showed dye incorporation following exposure to xylene, where little was incorporated in the absence of *o*-xylene.

Although the observed alterations of LPS structure following exposure to xylene did not appear to make the Idaho strain less permeable or more hydrophilic, it is unquestionably much less permeable and more hydrophilic than a similar solvent-sensitive strain that exhibits a hydrophobic cell surface and lacks the ability to alter its LPS in the presence of solvent.

PART V.

SUMMARY AND CONCLUSIONS OF PARTS II-IV

SUMMARY AND CONCLUSIONS

The solvent tolerant *Pseudomonas putida* strain showed an overall increase in total phospholipid following exposure to *o*-xylene. This increase was due to an increase in phospholipid biosynthesis in response to the presence of solvent. It appears that a system exists in this strain that allows for rapid replacement of damaged membrane components. Phosphatidylglycerol (in the turnover experiments) was the phospholipid which was replaced most rapidly, indicating that it may be the membrane component that is the most damaged by xylene. While the solvent-sensitive strain showed a slight increase in membrane biosynthetic rate following exposure to 75 ppm *o*-xylene, it was not statistically significant, and was only 30% as efficient as the solvent-tolerant strain. This indicates that this increased activity plays an important role in solvent tolerance. A summary of the physiological responses of both solvent-tolerant and solvent-sensitive *P. putida* strains is shown in Table 5-1.

Significant changes in the outer membrane were also observed in the solvent-tolerant Idaho strain. The carbohydrate composition and the Lipid A region of the LPS was altered in response to xylene exposure. From a theoretical standpoint, these changes would create a hydrophilic cell surface that is less permeable to hydrophobic substrates. While the ability to alter its surface structure did not make the strain more hydrophilic or less permeable to hydrophobic substrates such as difloxacin and NPN, it was obvious that the

Table 5-1. Summary of responses elicited by *P. putida* MW1200 and *P. putida* Idaho following *o*-xylene exposure.

Affected Parameter	Strain		Time Occured
	MW1200	Idaho	
Phospholipid (PL)	↓ Total PL	↑ Total PL	1-6 h
	No significant change in PL biosynthesis rate	↑ PL Biosynthesis rate	~30 min.
	No significant change in PL turnover rate	↑ PL Turnover rate	~2 h
Phospholipid Fatty Acids (PLFA)	↓ Total PLFA	↑ Total PLFA	1-6 h
	↑ <i>Trans</i> -Unsaturated PLFA	↑ <i>Trans</i> -Unsaturated PLFA	5 min.
		↑ Saturated PLFA	15 min.
Lipopolysaccharide (LPS)	↓ Total LPS	↑ Total LPS	~1 h
	↓ Total LPSFA	↑ Total LPSFA	~1 h
	↑ Hydroxy FA	↑ Hydroxy FA	~1 h
	No change in carbohydrate composition	↑ Amino sugar moieties	nd
		↑ KDO	nd
		↓ Neutral sugars	nd
Permeability	↑ Following <i>o</i> -xylene exposure	No change following <i>o</i> -xylene exposure	nd

nd - not determined

solvent-tolerant strain was up to 100 times less permeable to hydrophobic substrates and 37% more hydrophilic than the solvent-sensitive MW1200 strain. While the role of the changes in LPS structure of the Idaho strain isn't clear at this point, it does appear that the hydrophilic, relatively impermeable outer membrane structure of the Idaho strain is necessary for solvent tolerance.

Although this study has not definitively solved the mystery of solvent tolerance, many aspects of the phenomenon have been elucidated. The efforts of several investigators have shown what is not responsible for solvent tolerance. The results of this study, and the studies of Cruden et al. (1992) and Inoue et al. (1991) show that growth, and solvent tolerance, occurs in the absence of substrate metabolism. Therefore, rapid substrate metabolism and substrate detoxification can be ruled out as tolerance mechanisms. The genetic elements conferring solvent tolerance are not located on a plasmid. Ramos et al. (1995), Cruden et al. (1992), and Inoue et al. (1991) have not been able to isolate plasmids from their solvent tolerant strains. *Trans*-unsaturated fatty acids, although shown to increase phase transition temperature in liposomes (Sikkema et al., 1995) and exhibited as a general protection against mild stress, do not confer solvent tolerance (this study).

From these data, it is possible to propose a model for the solvent tolerance phenomenon. Solvent tolerance involves short-term and long-term responses. The short-term responses include the formation of *trans*-unsaturated fatty acids and the increase in saturated fatty acids of the phospholipid

membrane. These short-term responses allow for transient maintenance of membrane integrity. These responses are present in the solvent-sensitive strain, and would explain the ability to culture cells exposed to lethal doses of solvent (100 ppm) after several hours. *E. coli*, which does not have the ability to make *trans*-unsaturated fatty acids, showed rapid loss of viability within 1-5 minutes following toluene exposure (Deutscher, 1974; Woldringh, 1973).

The long term response begins with increased phospholipid biosynthesis. This allows for replacement of the phospholipid damaged by high concentrations of organic solvent. Increased phospholipid biosynthetic rate would explain the requirement for excess magnesium ions observed by several investigators of solvent-tolerant organisms. The increased phospholipid biosynthesis coincides with outer membrane alterations designed to maintain lipid ordering (the increase in LPS fatty acid content) and exclude hydrophobic substrates (the increase in charged sugars and the increase in hydroxy fatty acid in Lipid A). If extrusion pumps are present (as suggested by Ramos et al. and De Bont et al., 1996) that remove hydrophobic substrates from the cell, these systems would also be responsible for long-term maintenance of solvent-tolerance.

PART VI.

LITERATURE REVIEW AND VITA

LITERATURE CITED

- Ahmed, M., C. M. Borsch, S. S. Taylor, N. Vasquez-Laslop, and A. Neyfakh.** 1994. A protein that activates expression of a multiple efflux transporter upon binding the transporter substrates. *J. Biol. Chem.* **269**:28506-28513.
- Antunes-Madeira, M. C., and V. M. C. Madeira.** 1989. Membrane fluidity as affected by the insecticide lindane. *Biochim. Biophys. Acta.* **982**:161-166.
- Antunes-Madeira, M. C., and V. M. C. Madeira.** 1986. Partition of DDT in synthetic and native membranes. *Biochim. Biophys. Acta.* **861**:159-164.
- Antunes-Madeira, M. C., and V. M. C. Madeira.** 1985. Partition of lindane in synthetic and native membranes. *Biochim. Biophys. Acta.* **820**:165-172.
- Antunes-Madeira, M. C., and V. M. C. Madeira.** 1987. Partition of malathion in synthetic and native membranes. *Biochim. Biophys. Acta.* **901**:61-66.
- Antunes-Madeira, M. C., and V. M. C. Madeira.** 1984. Partition of parathion in synthetic and native membranes. *Biochim. Biophys. Acta.* **778**:49-56.

Aono, R., M. Ito, A. Inoue, and K. Horikoski. 1992. Isolation of a novel toluene-tolerant strain of *Pseudomonas aeruginosa*. Biosci. Biotechnol. Biochem. **56**:145-146.

Aono, R., T. Negeshi, and H. Nakajima. 1994. Cloning of organic solvent tolerance gene *ostA* that determines *n*-hexane tolerance level in *Escherichia coli*. Appl. Environ. Microbiol. **60**(12):4624-4626.

Arnoff, S. C. 1988. Outer membrane permeability in *Pseudomonas cepacia*: diminished porin content in a beta-lactam-resistant mutant and in resistant cystic fibrosis isolates. Antimicrob. Agents Chemother. **32**(11):1636-1639.

Arredondo, R., A. Garcia, and C.A. Jerez. 1994. Partial removal of lipopolysaccharide from *Thiobacillus ferrooxidans* affects its adhesion to solids. Appl. Environ. Microbiol. **60**(8):2846-2851.

Assinder, S. J., and P. A. Williams. 1990. The TOL plasmids: Determinants of the catabolism of toluene and the xylenes. Adv. Microb. Physiol. **31**:1-69.

Baird, W. M., L. Diamond, T. W. Borun, and S. Shulman. 1979. Analysis of metabolism of carcinogenic polycyclic hydrocarbons by position-sensing proportional counting of thin-layer chromatograms. *Anal. Biochem.* **99**:165-169.

Balch, W.E., G.E. Fox, L.J. Nagrum, C.R. Woese, and R.S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. *Microbiol. Rev.* **43**: 260-296.

Bansal, V.S. and G.K. Khuller. 1981. Changes in phospholipids of *Microsporum* species in the presence of ethanol. *Arch. Microbiol.* **130**: 248-249.

Barrow, K.D., J.G. Collins, P.L. Rogers and G.M. Smith. 1983. Lipid composition of an ethanol-tolerant strain of *Zymomonas mobilis*. *Biochim. Biophys. Acta.* **753**: 324-330.

Bateman, J. N., B. Speer, L. Feduik, and R. A. Hartline. 1986. Naphthalene association and uptake in *Pseudomonas putida*. *J. Bacteriol.* **166**:155-161.

Becart, J., C. Chevalier, and J.-P. Biesse. 1990. Quantitative analysis of phospholipids by HPLC with a light scattering evaporative detector-application to raw materials for cosmetic use. *J. High Res. Chromat.* **13**:126-129.

Bell, R. M. 1974. Mutants of *Escherichia coli* defective in membrane phospholipid synthesis: Macromolecular synthesis in an sn-glycerol-3-phosphate acyltransferase K_m mutant. *J. Bacteriol.* **117**(3):1065-1076.

Benschoter, A. S., and L. O. Ingram. 1986. Thermal tolerance of *Xymomonas mobilis*: temperature-induced changes in membrane composition. *Appl. Environ. Microbiol.* **51**(6):1278-1284.

Bishop, W. R., and R. M. Bell. 1988. Assembly of phospholipids into cellular membranes: biosynthesis, transmembrane movement, and intracellular translocation. *Ann. Rev. Cell Biol.* **4**:579-610.

Boden, N., S. A. Jones, and F. Sixl. 1991. On the use of deuterium NMR as a probe of chain packing in lipid bilayers. *Biochem.* **30**:2146-2155.

Brozel, V. S., and T. E. Cloete. 1993. Adaptation of *Pseudomonas aeruginosa* to 2,2'-methylenebis(4-chlorophenol). *J. Appl. Bacteriol.* **74**(1):94-99.

Carruthers, A., and D. L. Melchior. 1983. Studies of the relationship between bilayer water permeability and bilayer physical state. *Biochem.* **22**:5797-5807.

Chamberland, S., F. Maloin, H. R. Rabin, T. Schollardt, T. R. Parr, and L. E. Bryan. 1990. Persistence of *Pseudomonas aeruginosa* during ciprofloxacin therapy of a cystic fibrosis patient: transient resistance to quinolones and protein F deficiency. J. Antimicrob. Chemother. **25**(6):995-1010.

Cox, A. D., and S. G. Wilkinson. 1991. Ionizing groups in lipopolysaccharides of *Pseudomonas cepacia* in relation to antibiotic resistance. Mol. Microbiol. **5**(3):641-646.

Cranney, M., R. B. Cundall, G. R. Jones, J. T. Richards, and E. W. Thomas. 1983. Fluorescence lifetime and quenching studies on some interesting diphenylhexatriene membrane probes. Biochim. Biophys. Acta. **735**:418-425.

Cruden, D. L., J. H. Wolfram, R. T. Rogers, and D. T. Gibson. 1992. Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. Appl. Environ. Microbiol. **58**(9):2723-2729.

Darveau, R. P., and R. E. W. Hancock. 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155**(2):831-838.

de Andres, C., M. J. Espuny, M. Robert, M. E. Mercade, A. Manresa, and J. Guinea. 1991. Cellular lipid accumulation by *Pseudomonas aeruginosa* 44T1. Appl. Microbiol. Biotechnol. **35**(6):813-816.

de Long, E. F., and A. A. Yayanos. 1985. Adaptation of membrane lipids of a deep sea bacterium to changes in hydrostatic pressure. Science. **228**:1101-1103.

de Smet, M. J., G. Eggink, B. Witholt, J. Kingma, and H. Wynberg. 1983. Characterization of cellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. J. Bacteriol. **154**(2):870-878.

de Smet, M. J., J. Kingma, H. Wynberg, and B. Witholt. 1983. *Pseudomonas oleovorans* as a tool in the bioconversions of hydrocarbons: growth, morphology, and conversion characteristics in different two-phase systems. Enzyme Microb. Technol. **5**:352-360.

de Smet, M. J., J. Kingma, and B. Witholt. 1978. The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli*. Biochim. Biophys. Acta. **506**:64-80.

Deutscher, M.P. 1974. Preparation of cells permeable to macromolecules by treatment with toluene: Studies of transfer ribonucleic acid nucleotidyltransferase. *J. Bacteriol.* **118**:633-639.

Ferrante, A. A., J. Augliera, K. Lewis, and A. M. Klibanov. 1995. Cloning of an organic solvent resistance gene in *Escherichia coli*: the unexpected role alkylperoxide reductase. *Proc. Natl. Acad. Sci.* **92**:7617-7621.

Franklin, T.J., and G.A. Snow. 1989. Resistance to antimicrobial drugs. *In* Biochemistry of antimicrobial action. (Barrett-Bee, K.J., and R.D. Nolan, eds.), Fourth Ed., Chapman and Hall, London. p.173-200.

Furukawa, H., J.-T. Tsay, S. Jackowski, Y. Takamura, and C. O. Rock. 1993. Thiolactomycin resistance in *Escherichia coli* is associated with the *emrAB* multidrug resistance efflux pump. *J. Bacteriol.* **175**:3723-3729.

Gibson, D. T., and V. Subramanian. 1984. Microbial degradation of aromatic hydrocarbons. Marcel Dekker, New York.

Gibson, G. T., G. E. Cardini, F. C. Maseles, and R. E. Kallio. 1970. Incorporation of oxygen-18 into benzene by *Pseudomonas putida*. *Biochemistry.* **9**:1631-1635.

Guckert, J. B., C. P. Antworth, P. D. Nichols, and D. C. White. 1985.

Phospholipid ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure in estuarine sediments. *FEMS Microbiol. Ecol.* **31**:147-158.

Guckert, J. B., M.A. Hood and D.C. White. 1986. Phospholipid, ester-linked fatty acid profile changes during nutrient depletion of *Vibrio cholerae*; increases in the *trans/cis* ratio and proportions of cyclopropyl fatty acids. *Appl. Environ. Microbiol.* **52**: 794-801.

Guckert, J. B., D. B. Ringelberg, and D. C. White. 1987. Biosynthesis of *trans* fatty acids from acetate in the bacterium *Pseudomonas atlantica*. *Can. J. Microbiol.* **33**:748-754.

Hancock, I.C., and I.R. Poxton. Bacterial cell surface techniques. *Modern Microbiological Methods*, M. Goodfellow (ed.) 1988, New York, Wiley-Interscience. p. 92-81.

Hancock, R. E. W. 1984. Alterations in outer membrane permeability. *Ann. Rev. Microbiol.* **38**:237-264.

Hancock, R. E. W. 1987. Role of porins in outer membrane permeability. J.

Bacteriol. **169**(3):929-933.

Harayama, S., R.A. Leppik, N. Mermod, P.R. Lehrbach, W. Reineke, and

K.N. Timmis. 1986. Gene order of the TOL catabolic plasmid upper pathway operon and oxidation of both toluene and benzyl alcohol by the *xylA* product. J.

Bacteriol. **167**(2):455-461.

Harayama, S., and K. N. Timmis. 1989. Catabolism of aromatic hydrocarbons

by *Pseudomonas*, p. 151-174. In K. F. Chater and D. A. Hopwood (ed.),

Genetics of bacterial diversity. Academic Press, Ltd., London.

Heipieper, H.-J., H. Keweloh, and H-J. Rehm. 1991. Influence of phenols on growth and membrane permeability of free and immobilized *Escherichia coli*.

Appl. Environ. Microbiol. **57**(4):1213-1217.

Heipieper, H.-J., R. Deifendach, and H. Keweloh. 1992. Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of

phenol-degrading *Pseudomonas* P8 from substrate toxicity. Appl. Environ.

Microbiol. **58**(6):1847-1852.

Heipieper, H.-J., H. J.A.M. de Bont. 1994. Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of the fatty acid composition of membranes. *Appl. Environ. Microbiol.* **60**:4440-4444.

Henderson, R. J., R-M. Millar, J.R. Sargent, and J-P. Jostensen. 1993. *Trans*-monoenoic and polyunsaturated fatty acids in phospholipids of a *Vibrio* species of bacterium in relation to growth conditions. *Lipids.* **28**(5):389-396.

Herrero, A.A., F. Gomez, and M.F. Roberts. 1982. Ethanol-induced changes in the membrane lipid composition of *Clostridium thermocellum*. *Biochim. Biophys. Acta.* **693**: 195-204.

Hubner, W., H.H. Mantsch, and M. Kates. 1991. Intramolecular hydrogen bonding in cardiolipin. *Biochim. Biophys. Acta.* **1066**:166-174.

Hwang, Y.-W., R. Engel, and B.E. Tropp. 1984. Correlation of 3,4-dihydroxybutyl 1-phosphonate resistance with a defect in cardiolipin synthesis in *Escherichia coli*. *J. Bacteriol.* **157**(3):846-856.

Ingram, L. O. 1977. Changes in lipid composition of *Escherichia coli* resulting from growth with organic solvents and with food additives. *Appl. Environ. Microbiol.* **33**(5):1233-1236.

Ingram, L. O. 1986. Microbial tolerance to alcohols: role of the cell membrane. Trends Biotechnol. 4:40-44.

Inoue, A., and K. Horikoshi. 1989. A *Pseudomonas* that thrives in high concentrations of toluene. Nature (London) 338: 264-266.

Inoue, A., M. Yamamoto, and K. Horikoshi. 1991. *Pseudomonas putida* which can grow in the presence of toluene. Appl. Environ. Microbiol. 57: 1560-1562.

In't Veld, G., J. M. Driessen, J. A. F. Op den Kamp, and W. N. Konings. 1991. Hydrophobic membrane thickness and lipid-protein interactions of the leucine transport system of *Lactococcus lactis*. Biochim. Biophys. Acta. 1104:31-39.

Ishii, J., and T. Nakae. 1993. Lipopolysaccharide-promoted opening of the porin channel. FEBS Lett. 320(3):251-255.

Isken, S. and J.A.M. de Bont. 1996. Active export as a mechanism for solvent tolerance in *Pseudomonas putida* S12. GBF-UIB-CSIC-TUB International

Symposium for Biodegradation of Organic Pollutants at Palma, Majorca, June 29- July 3, 1996, abstract # 94.

Jackson, R. W., and J.A. DeMoss. 1965. Effects of toluene on *Escherichia coli*. J. Bacteriol. 90(5):1420-1425.

Jacques, N. A. and A.L. Hunt. 1980. Studies on cyclopropane fatty acid synthesis: effect of carbon source and oxygen tension on the accumulation of methylene hexadecanoic acid by *Pseudomonas denitrificans*. Biochim. Biophys. Acta. 619:453-470.

Jacques, N. A. 1981. Studies on cyclopropane fatty acid synthesis: correlation between the state of reduction of respiratory components and the accumulation of methylene hexadecanoic acid by *Pseudomonas denitrificans*. Biochim. Biophys. Acta. 665(2):270-282.

Jantzen, E. K. B. 1994. Analysis of cellular constituents from gram-negative bacteria., In M. Goodfellow (ed.), Chemical methods in prokaryotic systems. John Wiley and Sons. pp. 21-61.

Jenkins, R.O., G.M. Stephens, and H. Dalton. 1987. Production of toluene *cis*-glycol by *Pseudomonas putida* in a glucose batch-fed culture. *Biotechnol. Bioeng.* 29: 873-883.

Joseleau-Petit, D., F. Kepes, L. Peutat, R. D'Ari, and A. Kepes. 1987. DNA replication initiation, doubling rate of phospholipid synthesis, and cell division in *Escherichia coli*. *J. Bacteriol.* 169(8):3701-3706.

Jung, S., S.E. Lowe, R.I. Hollingsworth, and G. Zeikus. 1993. *Sarcina ventriculi* synthesizes very long chain dicarboxylic acids in response to different forms of environmental stress. *J. Biol. Chem.* 268(4):2828-2835.

Karem, K.L., J.W. Foster, and A.K. Bej. 1994. Adaptive acid tolerance (ATR) in *Aeromonas hydrophilia*. *Microbiology.* 140: 1731-1736.

Kates, M. 1975. *Techniques of Lipidology: isolation, analysis, and identification of lipids.* Elsevier, New York. 610 pp.

Keweloh, H., R. Diefenbach, and H.J. Rehm. 1991. Increase of phenol tolerance of *Escherichia coli* by alterations of the fatty acid composition of the membrane lipids. *Arch. Microbiol.* 157:49-53.

Kieft, T. L., D. B. Ringelberg, and D. C. White. 1994. Changes in ester-linked fatty acid profiles of subsurface bacteria during starvation and dessication in a porous medium. *Appl. Environ. Microbiol.* **60**(9):3292-3299.

King, G. E., R. E. Jacobs, and S. H. White. 1985. Hexane dissolved in dioleoyllecithin bilayers has a partial molar volume of approximately zero. *Biochem.* **24**:4637-4645.

Kropinski, A. M. B., V. Lewis, and D. Berry. 1987. Effect of growth temperature on the lipids, outer membrane proteins, and lipopolysaccharides of *Pseudomonas aeruginosa* PAO. *J. Bacteriol.* **169**(5):1960-1966.

Labischinski, H., G. Barnickel, H. Bradaczek, D. Naumann, E.T. Reitschel, and P. Geisbrecht. 1985. High state of order of isolated bacterial lipopolysaccharide and its possible contribution to the permeation barrier property of the outer membrane. *J. Bacteriol.* **162**(1):9-20.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**(August 15):680-683.

Langworthy, T. A. 1982. Lipids of bacteria living in extreme environments., p. 45-78. *In* F. a. A. K. Bronner (ed.), Current topics in membranes and transport., vol. 17. Academic press, New York.

Lau, P.C.K., Y. Wang, D. Labbe, H. Bergeron, and J. Garnon. 1996. Two-component signal transduction systems regulating toluene and biphenyl/polychlorinated biphenyl degradations in a soil pseudomonad and an actinomycete. *In Pseudomonas: Molecular biology and Biotechnology.* T Nakzawa, K. Furakawa, D. Haas, and S. Silver (eds). ASM Press (in press)

Legakis, N. J., L.S. Tzouvelakis, A. Makris, H. Kotsikafi. 1989. Outer membrane alterations in multiresistant mutants of *Pseudomonas aeruginosa* selected by ciprofloxacin. Antimicrob. Agents. Chemother. **33**(1):124-127.

Lepage, C., F. Foyelle, M. Hermann, and J-P. Vandecasteele. 1987. Changes in the membrane lipid composition of *Clostridium acetobutylicum* during acetone-butanol fermentation: effects of solvents, growth temperature, and pH. J. Gen. Microbiol. **133**:103-110.

Lesse, A. J., A.A. Campagnari, W.E. Bittner, M.A. Apicella. 1990. Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-

sodium dodecyl sulfate-polyacrylamide electrophoresis. *J. Immunol. Meth.*

126(109-117):109-117.

Levy, S. B. 1992. Active efflux systems for antimicrobial resistance. *Antimicro.*

Agents Chemother. **36**:695-703.

Lewis, K., V. Naroditskaya, A. Ferrante, and I. Fokina. 1994. Bacterial

resistance to uncouplers. *J. Bioenerg. Biomembr.* **94**:639-648.

Leying, H. J., K.H. Buscher, W. Cullman, and R.L. Then. 1992.

Lipopolysaccharide alterations responsible for combined quinolone and beta-

lactam resistance in *Pseudomonas aeruginosa*. *Chemotherapy.* **38**(2):82-91.

Lichtenberg, D., M. Rosenberg, N. Sharfman, and I. Ofek. 1985. A kinetic

approach to bacterial adherence to hydrocarbon. *J. Microbiol. Meth.* **4**: 141- 146.

Lomovskaya, O., K. Lewis, and A. Matin. 1995. EmrR is a negative regulator

of the *Escherichia coli* multidrug resistance pump EmrAB. *J. Bacteriol.*

177:2328-2334.

Lovitt, R. W., R. Longin, and J.G. Zeikus. 1984. Ethanol production by

thermophilic bacteria: physiological comparison of solvent effects on parent and

alcohol-tolerant strains of *Clostridium thermohydrosulfuricum*. Appl. Environ. Microbiol. **48**(1):171-177.

Massa, E. M., Vinals, A.L., and R.N. Farias. 1988. Influence of unsaturated fatty acid membrane component on sensitivity of an *Escherichia coli* fatty acid auxotroph to conditions of nutrient depletion. Appl. Environ. Microbiol. **54**(8):2107-2111.

Matsushita, K., O. Adachi, E. Shinagawa, and M. Ameyama. 1978. Isolation and characterization of outer and inner membranes from *Pseudomonas aeruginosa* and effect of EDTA on the membranes. J. Biochem. **83**:171-181.

Mayberry, W. R., and J.R. Lane. 1993. Sequential alkaline saponification/acid hydrolysis/esterification: a one-tube method with enhanced recovery of both cyclopropane and hydroxylated fatty acids. J. Microbiol. Meth. **18**:21-32.

McElhaney, R. N. 1982. Effects of membrane lipids on transport and enzyme activities., p. 317-380. In F. a. A. K. Bronner (ed.), Current topics in membranes and transport., vol. 17. Academic Press, New York.

McIntosh, T. J., R. T. McDaniel, and S. J. Simon. 1980. The organization of *n*-alkanes in lipid bilayers. Biochim. Biophys. Acta. **597**:445-463.

Melchior, D. L. 1982. Lipid phase transition and regulation of membrane fluidity in prokaryotes., p. 263-316. *In* F. a. A. K. Bronner (ed.), Current topics in membranes and transport., vol. 17. Academic Press, New York.

Michea-Hamzehpour, M., Y.X. Furet, and J-C. Pechere. 1991. Role of protein D2 and lipopolysaccharide in diffusion of quinolones through the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents. Chemother. 35(10):2091-2097.

Miller, K. 1985. Effects of temperature and sodium chloride concentrations on the phospholipid and fatty acid compositions of a halotolerant *Planococcus* species. J. Bacteriol., 162(1): 263-270.

Morita, N., A. Shibihara, K. Yamamoto, K. Shinkai, G. Kajimoto, H.

Okuyama. 1993. Evidence for *cis-trans* isomerization of a double bond in the fatty acids of the psychrophilic bacterium *Vibrio* sp. strain ABE-1. J. Bacteriol. 175(3):916-918.

Nakajima, H., K. Kobayashi, M. Kobayashi, H. Asako, and R. Aono. 1995. Overexpression of the *robA* gene increases organic solvent tolerance and

multiple antibiotic and heavy metal ion resistance in *Escherichia coli*. Appl. Environ. Microbiol. **61**:2302-2307.

Neuberger, G. G., and D.C. Johnson. 1987. Comparison of the pulsed amperometric detection of carbohydrates at gold and platinum electrodes for flow injection and liquid chromatographic systems. Anal. Chem. **59**:203-204.

Neyfakh, A. A., V. Bidnenko, and L. B. Chen. 1991. Efflux-mediated multidrug resistance in *Bacillus subtilis*: similarities and dissimilarities with mammalian systems. Proc. Natl. Acad. Sci. USA. **88**:4781-4785.

Nichols, D. S., T.A. McMeekin, and P.D. Nichols. 1994. Manipulation of polyunsaturated, branched-chain, and *trans*-fatty acid production in *Shewanella putrefaciens* strain ACAM 342. Microbiology. **140**:577-584.

Nichols, P.D., J.B. Guckert, and D.C. White. 1986. Determination of monounsaturated fatty acid double bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulfide adducts. J. Microbiol. Meth. **5**: 49-55.

Nieboer, M., J. Kingma, and B. Witholt. 1993. The alkane oxidation system of *Pseudomonas oleovorans*: induction of the *alk* genes in *Escherichia coli*

W3110(pGEc47) affects membrane biogenesis in a distinct cytoplasmic membrane subfraction. *Mol. Microbiol.* **8**(6):1039-1051.

Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.* **49**(1):1-32.

Norris, M. J., D. T. Rogers, and A. D. Russell. 1985. Cell envelope composition and sensitivity of *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Serratia marcescens* to polymixin and other antibacterial agents. *Lett. Appl. Microbiol.* **1**(1):3-6.

Ogino, H., K. Miyamoto, and H. Ishikawa. 1994. Organic solvent-tolerant bacterium which secretes organic solvent-stable lipolytic enzyme. *Appl. Environ. Microbiol.* **60**:3884-3886.

Ogino, H., K. Yasui, T. Shiotani, T. Ishihara, and H. Ishikawa. 1995. Organic solvent-tolerant bacterium which secretes an organic-solvent-stable proteolytic enzyme. *Appl. Environ. Microbiol.* **61**(12):4258-4262.

Pickard, C., J.M. Foght, M.A. Pickard, D.W.S. Westlake. 1993. Oil field and freshwater isolates of *Shewanella putrefaciens* have lipopolysaccharide

polyacrylamide gel profiles characteristic of marine bacteria. *Can. J. Microbiol.* **39**:715-717.

Piddock, L. J., M.C. Hall, F. Bellido, M. Bains and R.E. Hancock. 1992. A pleiotropic, posttherapy, enoxacin-resistant mutant of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **36**(5):1057-61.

Pierucci, O. 1979. Phospholipid synthesis during the cell division cycle of *Escherichia coli*. *J. Bacteriol.* **138**(2):453-460.

Pinkart, H. C., J. W. Wolfram, R. Rodgers, and D. C. White. 1996. Cell envelope changes in solvent-tolerant and solvent-sensitive *Pseudomonas putida* strains following exposure to *o*-xylene. *Appl. Environ. Microbiol.* **62**(3):1129-1132.

Pope, J. M., and D. W. Dubro. 1986. The interaction of *n*-alkanes and *n*-alcohols with lipid bilayer membranes: a ^2H NMR study. *Biochim. Biophys. Acta.* **858**:243-253.

Pope, J. M., L. A. Littlemore, and P. W. Westerman. 1989. Chain-length dependence of n-alkane solubility in phosphatidylcholine bilayers: a H-NMR study. *Biochim. Biophys. Acta.* **980**:69-76.

Raetz, C. R. H., and W. Dowhan. 1990. Biosynthesis and function of phospholipids in *Escherichia coli*. *J. Biol. Chem.* **265**(3):1235-1238.

Ramos, R. J., E. Duque, J.-J. Rodriguez-Herva, and A. Esteve. 1996. Tolerance to organic solvents in bacteria. GBF-UIB-CSIC-TUB International Symposium for Biodegradation of Organic Pollutants at Palma, Majorca, June 29- July 3, 1996, abstract # 96.

Ramos, R. J., E. Duque, M.-J. Huertas, and A. Haidour. 1995. Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. *J. Bacteriol.* **177**(14):3911-3916.

Rosenberg, M., D. Gutnick, and E. Rosenberg. 1980. Adherence of bacteria to hydrocarbons: a useful technique for studying cell surface hydrophobicity. *FEMS Microbiol. Lett.* **9**:29 - 33 .

Russell, N. J., M. Kogut, and M. Kates. 1985. Phospholipid biosynthesis in the moderately halophilic bacterium *Vibrio costicola* during adaptation to changing salt concentrations. J. Gen. Microbiol. **131**:781-789.

Sakagami, Y., H. Yokoyama, H. Nishimura, Y. Ose, and T. Tashima. 1989. Mechanism of resistance to benzalkonium chloride by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **55**(8):2036-2040.

Seydel, U., and K. Brandenburg. 1992. Supramolecular structure of lipopolysaccharides and lipid A. Bacterial endotoxic lipopolysaccharides. Morrison, D.C, and J.L Ryan, eds.(CRC Press, Boca Raton, FL), p. 225-250.

Shibuya, I., C. Miyazaki, and A. Ohta. 1985. Alteration of phospholipid composition by combined defects in phosphatidylserine and cardiolipin synthases and physiological consequences in *Escherichia coli*. J. Bacteriol. **161**:1086-1092.

Sikkema, J., B. Poolman, W.N. Konings, and J.A.M. de Bont. 1992. Effects of the membrane action of tetralin on the functional and structural properties of artificial and bacterial membranes. J. Bacteriol. **174**:2986-2992.

Sikkema, J., F. J. Weber, H. J. Heipieper, and J. A. M. de Bont. 1994.

Cellular toxicity of lipophilic compounds: mechanisms, implications and adaptations. *Biocatalysis*. **10**:113-122.

Sikkema, J., J.A.M. deBont, and B. Poolman. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* **59**: 201-222.

Silver, M. T., and L. Wendt. 1967. Mechanism of action of phenylethylalcohol: breakdown of the cellular permeability barrier. *J. Bacteriol.* **137**:461-468.

Simon, S. A., R. V. McDaniel, and T. J. McIntosh. 1982. Interaction of benzene with micelles and bilayers. *J. Phys. Chem.* **86**:1449-1456.

Sinclair, M. I., P.C. Maxwell, B.R. Lyon, and B.W. Holloway. 1986. Chromosomal location of the TOL plasmid DNA in *Pseudomonas putida*. *J. Bacteriol.* **168**(3):1302-1308.

Sinesky, M. 1974. Homeoviscous adaptation - a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. *Proc. Nat. Acad. Sci.* **71**(2):522-525.

Sledjeski, D. D., and R.M. Weiner. 1991. Hyphomonas spp., Shewanella spp., and other marine bacteria lack heterogenous (ladderlike) lipopolysaccharides. Appl. Environ. Microbiol. **57**(7):2094-2096.

Smith, M.R. 1990. The biodegradation of aromatic hydrocarbons by bacteria. Biodegradation **1**: 191-206.

Smith, M.D., C.I. Masters, and B.E.B. Moseley. 1992. Molecular biology of radiation-resistant bacteria. In Molecular biology and biotechnology of extremophiles (Herbert, R.A and R.J. Sharp, eds.). Chapman and Hall, New York. p258-280.

Taneja, R., and G.K. Khuller. 1980. Ethanol-induced alterations in phospholipids and fatty acids of *Mycobacterium smegmatis* ATCC 607. FEMS Microbiol. Lett. **8**:83-85.

Tsai, C.-M., and C. Frasch. 1981. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Analyt. Biochem. **119**:115-119.

Tsuchido, T., I. Aoki, and M. Takano. 1989. Interaction of the fluorescent dye 1-N-phenyl-naphthylamine with *Escherichia coli* cells during heat stress and recovery from heat stress. J. Gen. Microbiol. **135**:1941-1947.

Uchida, K. 1975. Alteration of the unsaturated to saturated fatty acid ratio of fatty acids in bacterial lipids by alcohols. *Agr. Biol. Chem.* **39**:1515-1516.

Vaara, M. 1992. Agents that increase the permeability of the outer membrane. *Microbiol. Rev.* **56**(3):395-411.

VanBogelen, R. A., P. M. Kelley, and F. C. Neidhardt. 1987. Differential induction of heat-shock, SOS and oxidative stress regulons and accumulation of nucleotides in *Escherichia coli*. *J. Bacteriol.* **169**:26-32.

Vercellone-Smith P., and D. Herson. 1994. The physiological response of *Pseudomonas putida* to toluene stress. Presented at the 94th ASM General Meeting. Las Vegas, May 23-27.

Vercellone-Smith, P., and D. Herson. 1996. Toluene stress induces a carbon starvation response in *Pseudomonas putida* mt-2. Presented at the 96th American Society for Microbiology General Meeting, New Orleans, May 19-23.

Weber, F. 1995. Ph.D. Dissertation. Toluene: waste-gas treatment, toxicity, and microbial adaptation. University of Wageningen, The Netherlands.

Weber, F. J., S. Isken, and J. A. M. de Bont. 1994. *Cis/trans* isomerization of fatty acids as a defense mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. Microbiol. **140**:2013-2017.

Weislander, A., L. Rilfors, and G. Lindblom. 1986. Metabolic changes of membrane lipid composition in *Acholeplasma laidlawii* by hydrocarbons, alcohols and detergents; arguments for effects on lipid packing. Biochem. **25**:7511-7517.

White, D. C., R.J. Bobbie, S.J. Morrison, D.K. Oosterhof, C.W. Taylor, and D.A. Meeter. 1977. Determination of microbial activity of estuarine detritus by relative rates of lipid biosynthesis. Limnol. Ocean. **22**(6):1089-1098.

Wilkinson, S. G., L. Galbraith, and G.A. Lightfoot. 1973. Cell wall, lipids, and lipopolysaccharides of *Pseudomonas* species. Eur. J. Biochem. **33**:158-174.

Wilkinson, S. G. 1988. Gram-negative bacteria, p. 333-348. In C. Ratledge and S. G. Wilkinson (ed.), Microbial Lipids, vol. 1. Academic Press, San Diego.

Woldringh, C. L. 1973. Effects of toluene and phenylethyl alcohol on the ultrastructure of *Escherichia coli*. J. Bacteriol. **114**(3):1359-1361.

VITA

Holly Chubb Pinkart was born in Norwalk, Connecticut on December 23, 1968. She was raised in Norwalk, and attended Silvermine Elementary and West Rocks Middle Schools. She attended Central Catholic High School for two years, then completed her high school education at San Ramon Valley High School in Danville, California, where she graduated in 1986. She entered California State University at Long Beach in the Fall of 1986, and graduated with a Bachelor of Science degree in microbiology and a minor in chemistry in December, 1990. In the fall of 1991, she entered the University of Tennessee graduate school with Dr. David C. White as her advisor and major professor. She will graduate with a Doctor of Philosophy degree in microbiology in December 1996.

The author is married to Wesley Bennett Pinkart. She plans to accept a post-doctoral position with the Environmental Protection Agency in Gulf Breeze, Florida following graduation.