FINAL REPORT

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GRANT TITLE: Effects of shifts in cell surface properties on adhesion and activity in engineered bioluminescent bacteria in biofilms.

AWARD PERIOD: 1 July 1994 - 30 June 1997

OBJECTIVE: To determine how shifts in membrane structure affect attachment, persistence and function of the *Pseudomonas putida* Idaho strain biofilms as the composition of the bulk phase is changed.

APPROACH: An engineered strain of *P. putida* Idaho has been recently constructed which contains a *nah-lux* reporter (*nah* for naphthalene degradation). Light production from this strain will indicate induction of this degradative pathway. This can be used as an indicator of cellular activity, along with monitoring tryptophan fluorescence. Shifts in inner and outer membrane structure can be induced using organic solvents such as xylene and toluene. Changes in attachment response to changes in bulk phase composition will then be monitored through light production and tryptophan fluorescence.

ACCOMPLISHMENTS:

1. *Membrane Shifts*:
Shifts in the inner and outer membranes of two strains of *Pseudomonas putida* (Idaho, solvent tolerant; and MW1200, solvent sensitive) following exposure to solvent were investigated. *Pseudomonas putida* Idaho is 100 × less permeable to *o*-xylene than is the solvent sensitive strain MW1200. Responses to solvent were examined in terms of phospholipid and fatty acid content and composition (inner membrane) and alteration of lipopolysaccharide (LPS, outer membrane). Following *o*-xylene exposure, strain MW1200 exhibited a decrease in total phospholipid content, whereas solvent exposure lead to increases in the amount of phospholipids (Figure 1) in strain Idaho. Both strains produced *trans* fatty acids following exposure to *o*-xylene, however only strain Idaho exhibited an increase in saturated fatty acids. Most likely, these alterations in membrane structure compensate for the increases membrane fluidity induced by the presence of the *o*-xylene within the phospholipids.

Changes in the outer membrane structure making the Idaho strain less permeable to hydrophobic substances than is the MW 1200 strain were also noted. *Pseudomonas putida* strain MW1200 exhibited a decrease in total lipopolysaccharide (LPS) content (~20%) when grown in the presence of *o*-xylene whereas the LPS of the Idaho strain

increased by ~30 %. Changes in the outer membrane of *P. putida* strain Idaho with solvent exposure were evident from the scanning electron micrographs of the bacteria with and without solvent (Figure 2). Concomitantly, there was a 13 % decrease in the carbohydrate content of the LPS from the Idaho strain when grown in the presence of *o*-xylene compared to the cells grown in the absence of *o*-xylene. The carbohydrate composition of the Idaho strain was shifted from one that contained primarily neutral sugars (glucose and mannose) to one that contained primarily charged sugar moieties (amino sugars) (Figure 3). This composition shift changed the cell surface as well as it's permeability properties, with strain Idaho maintaining a hydrophilic cell surface less permeable than the MW1200 strain to difloxacin and 1-*N*-phenylnaphthylamine. Also, stable biofilms of strain Idaho formed more quickly and showed greater strength of adhesion in the presence of organic solvent than was observed in the absence of solvent. This increase in adhesion for the Idaho strain was most likely due to the alteration in LPS structure following exposure to xylene.

The cell surface hydrophobicity of both strains MW1200 and Idaho did not change following exposure of *o*-xylene over a range of concentrations. However, there was a significant difference in the cell surface hydrophobicity between the two strains ($p<0.0001$) with Idaho maintaining far lower hydrophobicity than did MW1200 (Figure 4). A summary of responses elicited by *P. putida* strains MW1200 and Idaho is shown in table 1.

2. *Phospholipid Biosynthesis*:

To determine differences in the biosynthetic rate between solvent-tolerant Idaho strain and solvent-sensitive *P. putida* MW1200 in batch culture, phospholipid biosynthesis was measured with P^{32}-orthophosphoric acid tracer experiments in the presence and absence of 75 ppm *o*-xylene. In the absence of xylene exposure cells showed little turnover of phospholipid. In MW1200, cardiolipin showed the most rapid turnover in the presence and absence of organic solvent. The Idaho strain showed most rapid turnover of phosphatidylglycerol in the absence of *o*-xylene but in the presence of 75 ppm solvent showed phosphatidylglycerol to have the greatest turnover (Figure 5). In all cases, cardiolipin turnover was at a very much lower rate in the Idaho strain than in the solvent sensitive strain. In both strains, phosphatidylglycerol generally showed the greatest incorporation of label and showed a higher rate of turnover than phosphatidylethanolamine in all cases except *P. putida* Idaho at *o*-xylene levels of 200 ppm. Although phosphatidylglycerol is second in abundance to phosphatidylethanolamine in the cell membrane, it is turned over faster in the presence of solvent, most likely because it is more readily damaged by the *o*-xylene than is the phosphatidylethanolamine.

3. *nah-lux reporter*:

The *nah-lux* construction was made and resides on a transposon. The construct was successfully inserted onto the chromosome of *P. putida* strain Idaho, however, although stable the transformant produced light under all conditions and not just with the presence of inducer molecules. Consequently, it was not useful for studies of changes in attachment response following changes in bulk phase composition.

SIGNIFICANCE: By investigating the impact of organic solvent on the membrane structure of a solvent tolerant (strain Idaho) and a solvent sensitive (strain MW 1200) *P. putida*, many aspects of solvent tolerance and how it impacts on adhesion have been elucidated. In addition to structural changes in the membrane of solvent-tolerant *P. putida* Idaho (*i.e.*, in types and amounts of phospholipid fatty acids present), the rate of lipid turnover in the tolerant strain was significantly greater in the presence of organic solvent than in the absence of organic solvent. No increase in lipid turnover was observed in the solvent-sensitive strain in the presence of *o*-xylene.

An increased rate of membrane biosynthesis is highly beneficial to the Idaho cells as it allows rapid repair of membrane damage by exposure to organic solvents, leading to increases in solvent tolerance and up to 100-fold decreased permeability to organic solvent. An inducible membrane repair mechanism exists in *P. putida* Idaho which allows rapid repair of damaged membrane components. The significance of the changes in the structure and amount of LPS in the outer membrane of the Idaho strain isn't completely clear, however, the relatively hydrophilic relatively impermeable outer cell membrane of the Idaho strain compared to that of the MW 1200 strain, does seem to be necessary for high solvent tolerance and results in improved adhesion of this strain.

PUBLICATIONS AND ABSTRACTS :

1. Pinkart, H.C. and D.C. White (1995). Lipopolysaccharide structure of solvent tolerant *Pseudomonas putida* Idaho. Abstract, 95th General Meeting of the American Society for Microbiology. Washington, D.C.

2. Pinkart, H.C., J.W. Wolfram, R. Rogers, and D.C. White (1996). Cell envelope changes in solvent-tolerant and solvent-sensitive *Pseudomonas putida* strains following exposure to *o*-xylene. Applied and Environmental Microbiology **62**: 1129-1132.

3. White, D.C., H.C. Pinkart, and D.B. Ringelberg. (1996). Biomass measurements: Biochemical approaches. *In* Manual of Environmental Microbiology, 1st Ed. (C.H. Hurst, G. Knudsen, M. McInerney, L. D. Stetzenbach, and M. Walter, eds). American Society for Microbiology Press, Washington, D.C. pp. 91-101.

4. Pinkart, H.C. (1996). Cell envelope dynamics and solvent tolerance in *Pseudomonas putida.* Ph.D. Thesis. University of Tennessee, Knoxville.

5. Pinkart HC and DC White (1997). Phospholipid biosynthesis and solvent tolerance in *Pseudomonas putida* strains. Journal of Bacteriology **179** (13): 4219-4226.

6. Pinkart, H.C. and D.C. White. Lipids in *Pseudomonas. In* Biotechnology Handbooks: *Pseudomonas.* (T. Montie Ed). Plenum Publishing Co, Ltd. Middlesex, London (IN PRESS).

Table 1: Summary of the responses exhibited by *P. putida* MW1200 and *P. putida* Idaho following exposure to *o*-xylene.

	Strain		Time Occurred
	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
	↑ Trans-Unsaturated PLFA	↑ Trans-Unsaturated PLFA	5 min.
		↑ Saturated PLFA	15 min.
Lipopolysaccharide	↓ 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 o-xylene exposure	No change following o-xylene exposure	ND

ND Not Determined.

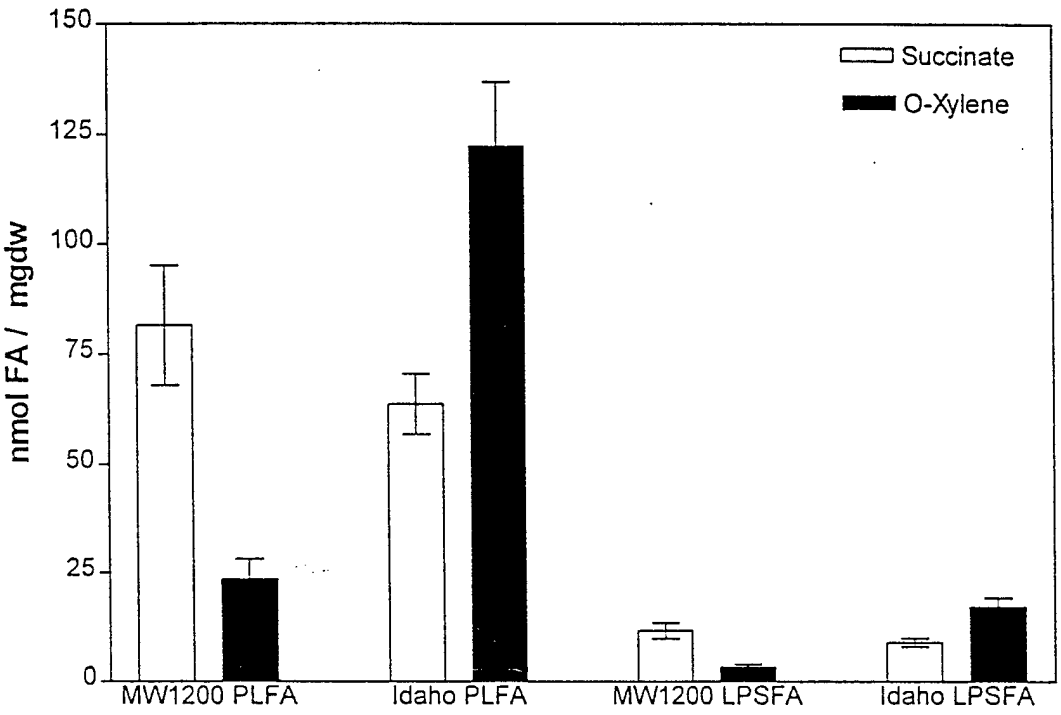
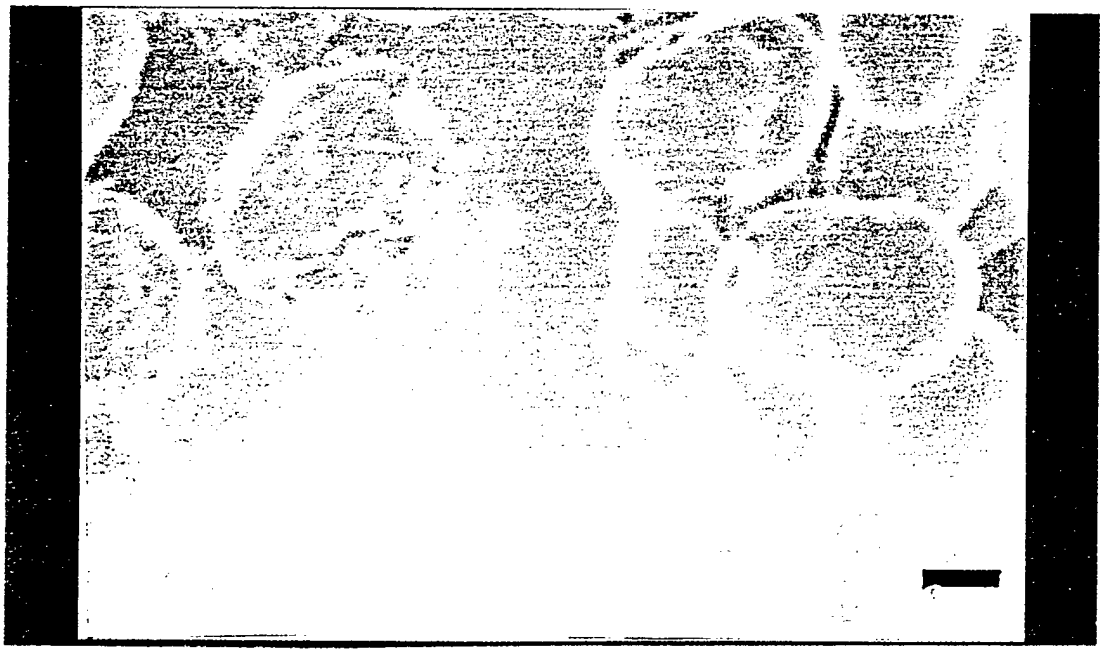


Figure 1: A comparison of the fatty acid content of *P. putida* Idaho and *P. putida* MW1200 in the absence and presence of *o*-xylene. Error bars represent 1 standard deviation, n=5. The *o*-xylene concentration for *P. putida* MW1200 was 75 ppm and was 200 ppm for *P. putida* Idaho. PLFA, phospholipid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



A

B

Figure 2: SEM micrographs of *P. putida* strain Idaho, A) before exposure to *o*-xylene and B) after exposure to *o*-xylene (200 ppm).

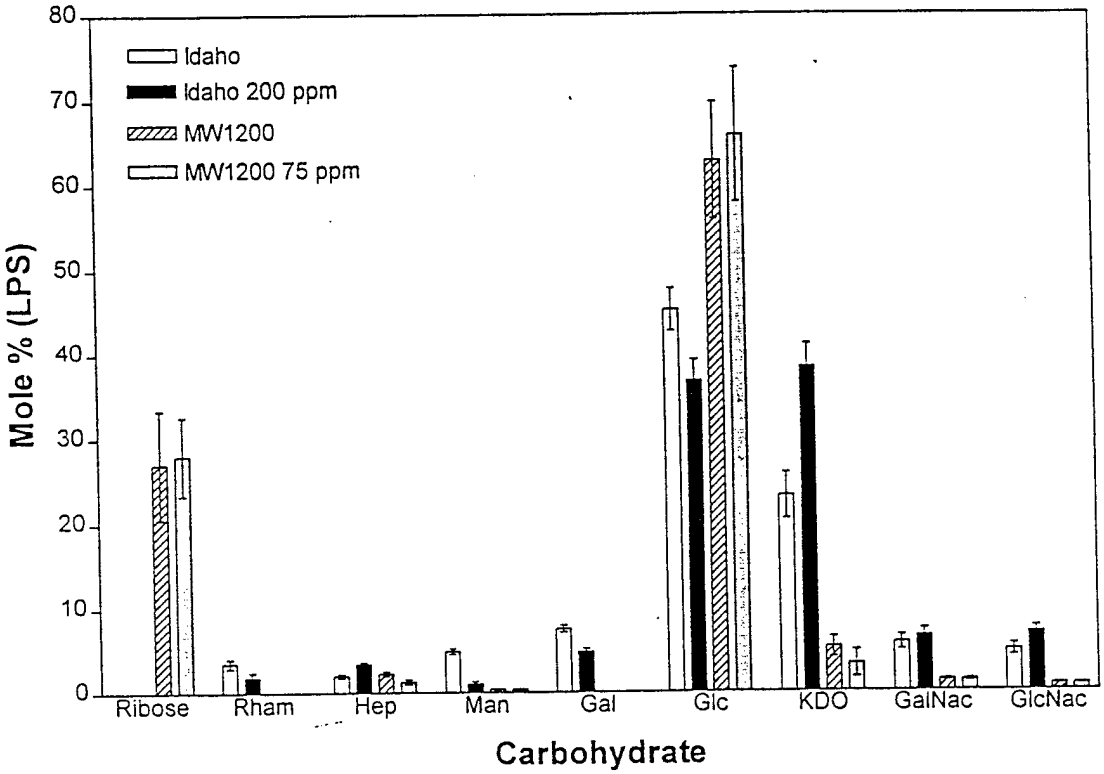


Figure 3: Carbohydrate composition of LPS from *P. putida* MW1200 and *P. putida* Idaho in the presence and absence of *o*-xylene. Rham, rhamnose; Hep, heptose; Man, mannose; Gal, galactose; KDO, 2-keto-3-deoxyoctulose; GalNac, glucosamine. Error bars represent 1 standard deviation, n=3.

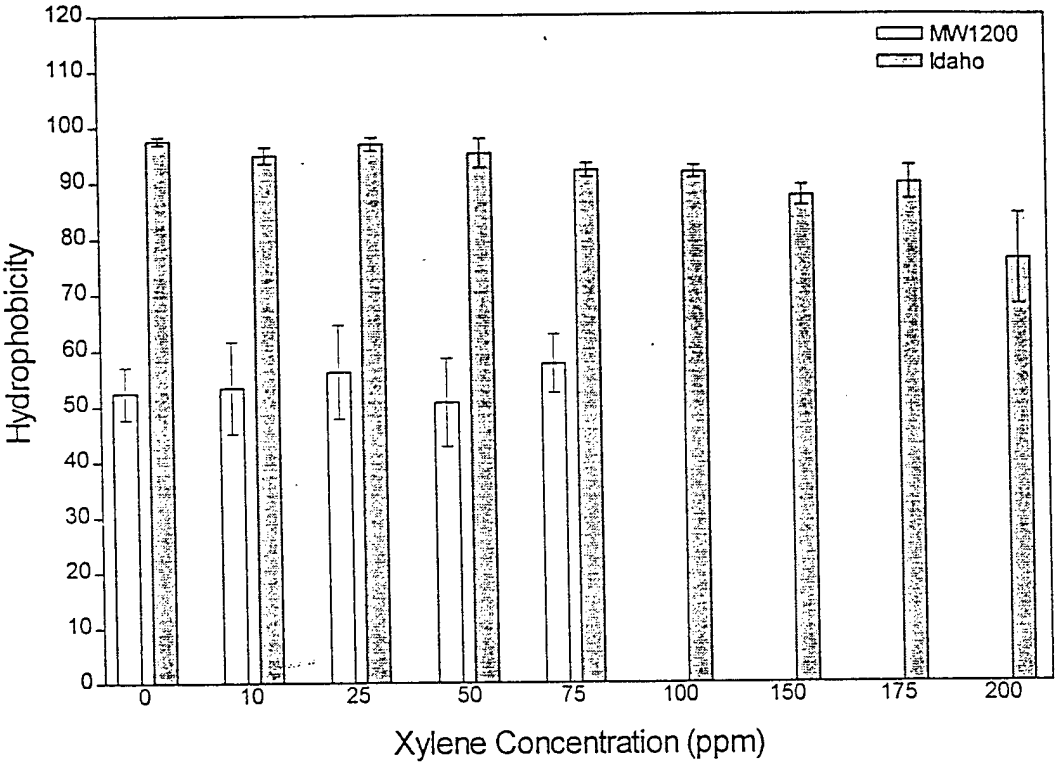


Figure 4: Bacterial adhesion to hydrocarbons (BATH) assay performed on *P. putida* MW1200 and *P. putida* Idaho. Hydrophobicity represents the percentage of cells remaining in the aqueous phase following incubation with hexadecane (0= most hydrophobic, 100= most hydrophilic). Error bars equal one standard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

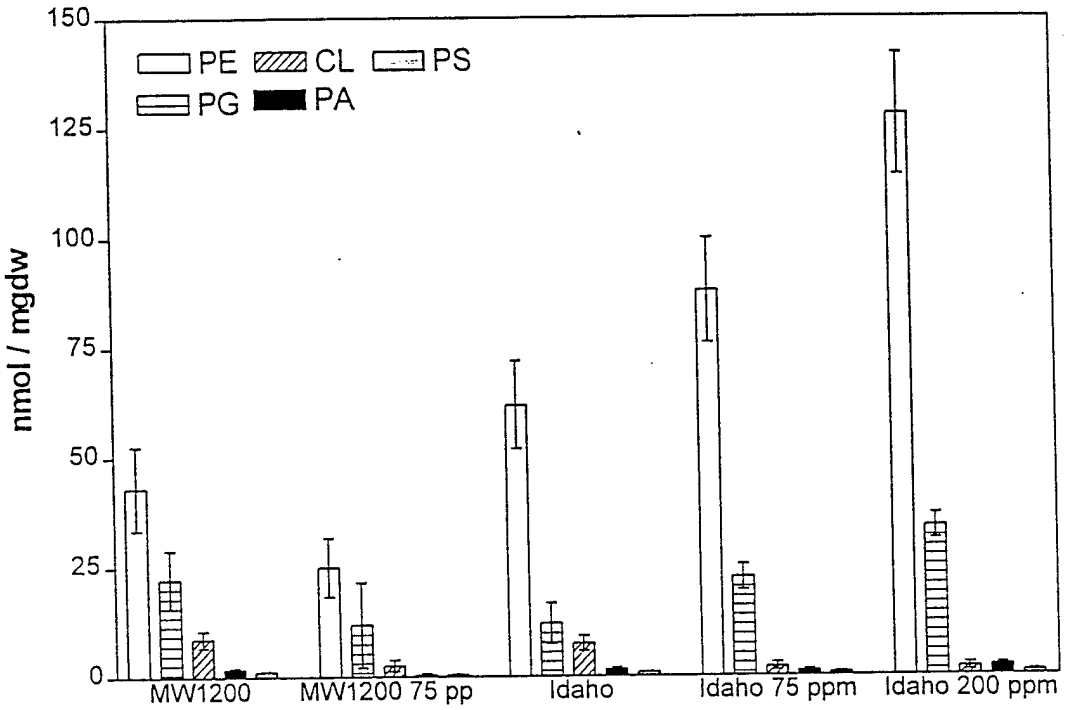


Figure 5: Phospholipid composition of *P. putida* MW1200 and *P. putida* Idaho grown in the presence and absence of *o*-xylene. PE, phosphtidylethanolamine; PG, phosphtidylglycerol; CL, cardiolipin; PA, phosphatidic acid; PS, phsophatidylserine. Units are expressed as nanomoles of phospholipid per milligram cell dry weight. Error bars represent 1 standard deviation, n=3.

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13. ABSTRACT (Maximum 200 words)

Shifts in the membrane composition of two strains of *Pseudomonas putida* (solvent sensitive strain MW1200 and solvent tolerant strain Idaho) were studied following exposure to solvent (*o*-xylene). Responses to solvent were examined in terms of phospholipid and fatty acid content (increasing in strain Idaho, and decreasing in strain MW1200), and synthesis and composition of lipopolysaccharide (again increasing in strain Idaho and decreasing in strain MW1200). Possibly due to the alteration in LPS structure, stable biofilms of strain Idaho formed more quickly in the presence of solvent than was observed in the absence of solvent. Also, changes in the biosynthesis rate of the phospholipids in the two strains were measured. Both strains exhibited an increase in biosynthesis rate in the presence of *o*-xylene with the greatest increase in strain Idaho. The increased rate of biosynthesis is highly beneficial to the Idaho cells enabling rapid repair of membrane damage and, therefore, an increased solvent tolerance with up to an 100 fold decrease in permeability to solvent.

Although a *nah-lux* construction was made and successfully inserted onto the strain Idaho chromosome, the transformant produced light under all conditions. Consequently, it was not usable for attachment response studies following changes in the bulk phase composition.

14. SUBJECT TERMS
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