1990

Characterization of the outer membrane of Pseudomonas putida.

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CHARACTERIZATION OF THE OUTER MEMBRANE OF *PSEUDOMONAS PUTIDA*

by

Edward George Saravolac

A DISSERTATION

Submitted to the Faculty of Graduate Studies and Research through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

Windsor, Ontario, Canada

1990

ISBN 0-315-61861-2

NCP 1730

ABSTRACT

CHARACTERIZATION OF THE OUTER MEMBRANE OF *PSEUDOMONAS PUTIDA*

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Edward George Saravolac

The outer membrane of *Pseudomonas putida* is prepared using both lysozyme digestion and sucrose density gradient separation of French pressed whole cell suspensions of the bacteria. The sucrose density gradient preparation gives greater yields and is more suited to large scale preparations of the outer membrane of *P. putida*. Examination of the outer membrane preparations by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed that it contains 9 major protein bands. These proteins are similar but not identical to the outer membrane proteins of *P. aeruginosa*.

One particular protein, Band E (42.9 kDa), is induced by growth of *P. putida* on glucose, maltose and lactose but repressed by growth on organic acids including gluconic acid and the citric acid cycle intermediates citrate, malate and succinate. Cell surface protein labelling using ^{125}I autoradiography shows that most outer membrane proteins including band E are expressed at the cell surface. The defluorination of 4-deoxy-4-fluoro-D-glucose by whole cells *P. putida*, was induced by growth of cells on glucose and repressed by growth on succinate. However, when cells are grown on gluconate defluorination is still induced. This suggests that band E is not responsible for the observed defluorinating activity observed in outer membrane preparations of *P. putida*. The inhibition of the defluorinating activity of outer

membranes by antibiotics and the concommitant inhibition of whole cell contamination after 24 hours of incubation suggests that the previously observed defluorination of 4FG by outer membranes of *P. putida* is caused by contaminating whole cells.

Band E is extracted from outer membrane preparations using ionic and non-ionic detergents. Extraction of band E with non-ionic detergents requires the use of EDTA. Band E is purified from a Lubrol PX-EDTA extract using DEAE Sephacel ion-exchange chromatography. By an identical procedure a glucose inducible protein (D1) is isolated from the outer membrane of *P. aeruginosa*. A comparative amino acid analysis indicates that proteins D1 and E are almost homologous with respect to amino acid content. Circular dichroism spectroscopy indicated that band E is very high in β-sheet content. A purified band E is used to immunize rabbits for the production of antisera against band E. Western blot analysis shows that the antisera obtained is specific not to band E but to lipopolysaccharide.

It is the customary fate of new truths to begin as heresies and end as superstitions.

Thomas Henry Huxley

ACKNOWLEDGEMENTS

I appreciate the fact that this page affords me the opportunity to make a long list of thanks to the many people who have made this work possible. First I would like to thank my supervisor Dr. Norman F. Taylor for supporting this work and especially for indulging me the many scientific diversions and preliminary experiments it took to produce this project. His expert editorial assistance was particularly gratefully received in the production of this report.

Thanks also go to the Social Services of Windsor, Ontario Student Assistance Program, the Department of National Defence, the Natural Sciences and Engineering Research Council and all of the other government agencies who have provided assistance through the years.

Many members of the department past and present deserve thanks especially members of the "Mutus group": Jaswinder Grewal, Betsy Palmer and Jerome Wagner deserve thanks for their friendship, advice and bartering skills. The "Lee group"; Chris Talpas for his advice and opinions and Tak Leung who performed the amino acid analyses of band E and D1. Thanks are also due to "Thibert group" especially Bouchra Harake for isoelectric focusing work and Peter Catomeris for helping me discover the wonders of I^{125}.

I wish to express my gratitude to members of our own lab. To Diego Sbrissa for the provision of 4FG and wisdom in the formative years and for help with certain figures; to John Williams for our various collaborations and critical reading of parts of this report and to Max Tejada and Dina Francescutti for staring and smiling during what must

have been an endless procession of gels and speeches about glucose inducible proteins.

I owe a debt of thanks to the members of the Department of Biological Sciences especially Dr. Cotter and Dr. Fackrell for advice and for the use of equipment. In this regard a special thanks goes to Rick Barrette who has proved to be a fine friend, able colleague, and the shrewdest negotiator I have ever met.

Dr. Raj Karrupiah (Univeristy of Michigan) deserves credit for providing the facilities and collaborative effort in the production of antisera to band E.

To the staff of the Chemistry and Biochemistry Department: Dave Hill, Jim Olsen, Mike Feurth, Jerry Vriesaker, Terry Edwards deserve thanks for keeping the place running so I could get on with my work. A special word of appreciation goes to Maeve Doyle, Kathy Feruth and Donna Dee for expert and sympathetic secretarial assistance.

Thank you to the members of the examining committee Dr. Cotter, Dr. Theibert, and Dr. Reithmeier for their patience and advice while reading and correcting this report. A special thank you to Dr. Bulent Mutus who has been a good friend and tireless source of sound advice.

Finally, but certainly not least I thank my best friend and wife Anita for maintaining my life support systems, physical, mental and spiritual during these four long years. I will be forever trying to deserve the love and care she has given me.

DEDICATION

Dedicated to Dorothy Renaud and

to the memory of George C. Bott.

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LIST OF ABBREVIATIONS

D-Ala	D-alanine
L-Ala	L-alanine
ATP	adenosine 5'-triphosphate
^{o}C	degrees Celcius
CD	circular dichroism
cm	centimetre
CM	cytoplasmic membrane
BCIP	5-bromo-4-chloro-3-indolyl phosphate
dia.	diameter
m-DAP	meso-diaminopimelic acid
DTT	Dithiothreitol
DSP	(3,3'-dithio-bis-(propionic acid) N-hydroxy-succinimide ester
EDTA	ethylenediaminetetraacetic acid
3FG	3-deoxy-3-fluoro-D-glucose
4FG	4-deoxy-4-fluoro-D-glucose
g	gram or gravity
Ga	gauge
GBP	glucose binding protein
GlcNAc	N-acetylglucosamine
D-Glu	D-glutamic acid
h	hour
hv	light
kDa	kilodalton
KDG	3-deoxy-D-arabino-2-hexulosonic acid

KDO	3-deoxy-3-D-manno-2-octulosonic acid
Km	Michaelis-Menten Constant
L	litre
LPS	lipopolysaccharide
mA	milliampere
mg	milligram
mL	millilitre
M	molar
Mw	molecular weight
MurNAc	N-acetylmuramic acid
min	minute
MBP	maltose binding protein
MCP	maltose chemotaxis protein
mM	millimolar
mm	millimeter
NBT	Nitro blue tetrazolium
N	normal
nm	nanometer
NMR	nuclear magnetic resonance spectroscopy
PBS	phosphate buffered saline
pS	picosiemen
psi	pounds per square inch
Tris	tris(hydroxymethyl)aminomethane
TCA	trichloroacetic acid
SDS	sodium dodecylsulfate

SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
ug	microgram
uM	micromolar
ul	microlitre

INTRODUCTION

Pseudomonads are normally free living bacteria found in water, soil or on plants (1). They exist as gram negative rods and are strict aerobes with the exception of species which utilize nitrate for anaerobic fermentation. Pseudomonads metabolize sugars via 3-deoxy-D-arabino-2-hexulosonic acid (KDG) using the Entner-Doudouoff pathway and are unable to ferment glucose to pyruvate via glycolysis because they lack phosphofructokinase (2).

Pseudomonas putida belongs to a group of saprophytic, fluorescent pseudomonads including *P. aeruginosa* and *P. fluorescens* which are characterized by the production of fluorescein, a yellow-green pteridine pigment (11). Pseudomonads are well known for their ability to degrade and dehalogenate a number of natural and synthetic compounds which include naphthalene (3), chlorobenzene (4), fluoroacetate (5) and 4-deoxy-4-fluoro-D-glucose (6). Also characteristic of pseudomonads is their resistance to antibiotics, a property which has been attributed to the low permeability of their outer membrane. Pseudomonads are not normally considered to be pathogenic but *P. aeruginosa* is often found as an opportunistic pathogen in infections where the host is immunologically compromised, eg. burn victim, immunosupressive therapy, or chronic lung disease particularly cystic fibrosis (1,7). Such infections are especially difficult to control because in addition to the intrinsic low permeability of the outer membrane, pseudomonads also carry resistance plasmids which code for lactamases capable of degrading permeable antibiotics (8). *P. putida* and *P. fluorescens* on the other hand have been

shown to be important in the stimulation of plant growth because of their ability to sequester Fe^{3+} using an outer membrane-mediated siderophore binding system which causes the inhibition of other deleterious plant root colonizing species of microorganisms (9).

The Gram Negative Bacterial Cell Envelope.

The cell envelope of gram negative bacteria consists of four layers which include the cytoplasmic and outer membrane (Figure 1). These membranes enclose the cell wall or peptidoglycan and the periplasmic space or periplasm (10). Together these layers form a rigid structure which maintains the shape of the bacterium and is responsible for several functions which include the uptake of nutrients, the production of energy, resistance to osmotic forces, delimitation of the cytoplasm and exclusion of deleterious agents.

The bacterial cytoplasmic membrane is a complex phospholipid bilayer in which a large number of proteins are associated. Typically the gram negative cytoplasmic membrane contains approximately 65% protein and 35% lipid (12). The predominant lipid in bacterial membranes is phosphatidylethanolamine (12). The cytoplasmic membrane contains hundreds of proteins which include oxidase enzymes, electron transport proteins (cytochromes), oxidative phosphorylation systems, active transport systems both for the uptake and secretion of solutes and ions and the proteins which direct the synthesis, transport and processing of membrane and other external proteins (13). The cytoplasmic membrane is permeable to water and dissolved gases (that are uncharged in solution), but is impermeable to larger polar molecules and ions. Impermeablity to ions and

especially protons allow bacteria to generate an electrochemical gradient across the cytoplasmic membrane. Bacteria, like the mitochondria generate the electrochemical gradient by pumping protons out of the cytoplasm as electrons from substrate oxidation flow through the electron transport chain. (15,16). Bacteria harness the electrochemical gradient or proton motive force (17) to generate ATP via the Proton/ATP'ase, energize transport of solutes against concentration gradients (see below), or rotate flagella for motility (1). This form of energy production is particularly important in the pseudomonads which ultimately rely solely on the oxidation of substrates for the generation of energy (1).

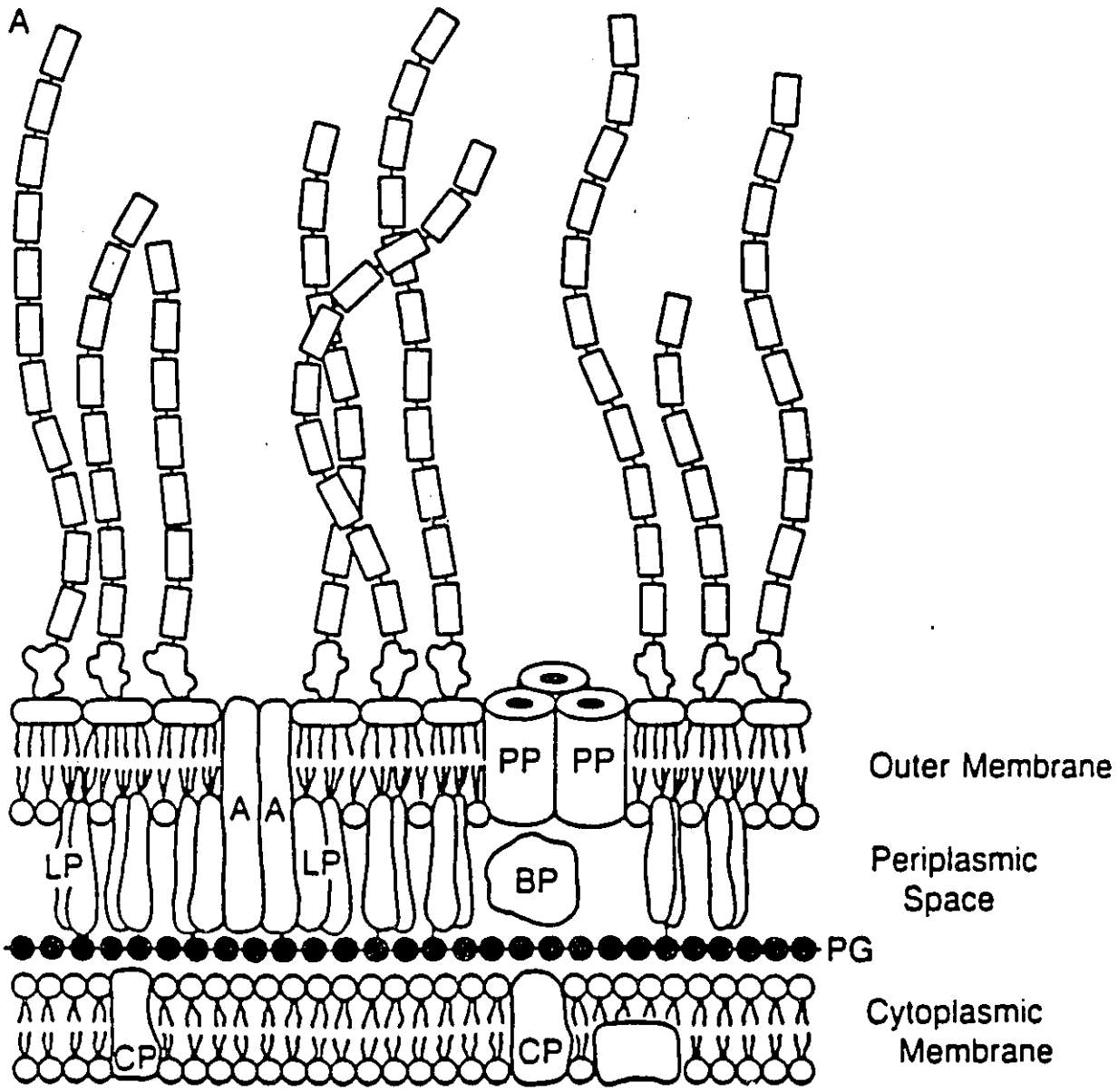
The periplasmic space is the volume enclosed by the inner and outer membranes of the cell envelope and forms a matrix of polypeptides and saccharides with net negative charges (10). This compartment accounts for 5 to 20 % of the total cell volume according to various estimates (13,17,18). The periplasm is nearly iso-osmotic with the cytoplasm and the osmotic pressure normally 3.5 bars and up to 8 bars is maintained across the outer membrane (18). Gram negative bacteria adapt to rapid changes in osmolarity by accumulating net negative charges in the periplasm. This is accomplished by synthesis of membrane derived oligosaccharides (MDO's) which are turnover products of the outer membrane (55). During osmotic shock of whole cells a number of proteins thought to be located in the periplasm are released (19). These "periplasmic proteins" include a number of enzymes (ie. phosphatases, nucleases and β-lactamases) and probably most important, binding proteins to a number of solutes including glucose, maltose, dicarboxylic and amino acids (10,18-20).

Figure 1

The molecular organization of the outer membrane of gram-negative bacteria showing the cytoplasmic membrane, periplasmic space and outer membrane.

Note the asymmetric distribution of lipopolysaccharide (LPS) and phospholipid (PL) in the outer membrane. Three types of outer membrane proteins are included: trimeric porin protein (PP), outer membrane transmembrane protein (A), and the major lipoprotein (LP). A solute transport (permease) binding protein (BP) is shown in the periplasm, as is the thin peptidoglycan cell wall (PG). A cytoplasmic membrane protein (CP) is also represented. Reproduced with permission (23).

Figure 1


A
PP
PP
A
A
LP
LP
BP
PG
CP
CP
Outer Membrane
Periplasmic Space
Cytoplasmic Membrane

The characteristics of several periplasmic binding proteins are summarized in Table 1.

These solute binding proteins are associated with "shock sensitive" periplasmic permeases ie. those transport systems which are inactivated because of the loss of an essential periplasmic component. Typically the binding proteins are capable of binding solutes with dissociation constants in the order of 0.1 to 1 uM and may be present in the periplasm at concentrations up to 1 mM (13,14,23). Binding proteins have a single binding site and a change in conformation upon binding the ligand can be observed by fluorescence measurements, ultraviolet absorption, NMR or by following the change in isoelectric point of the free or bound protein (14). In addition to sites for binding solute the binding proteins also have sites for interaction with permease proteins in the outer and cytoplasmic membranes (23). The maltose transport system of *Escherichia coli* is amongst the best characterized of the periplasmic binding protein dependent systems and will be further discussed below.

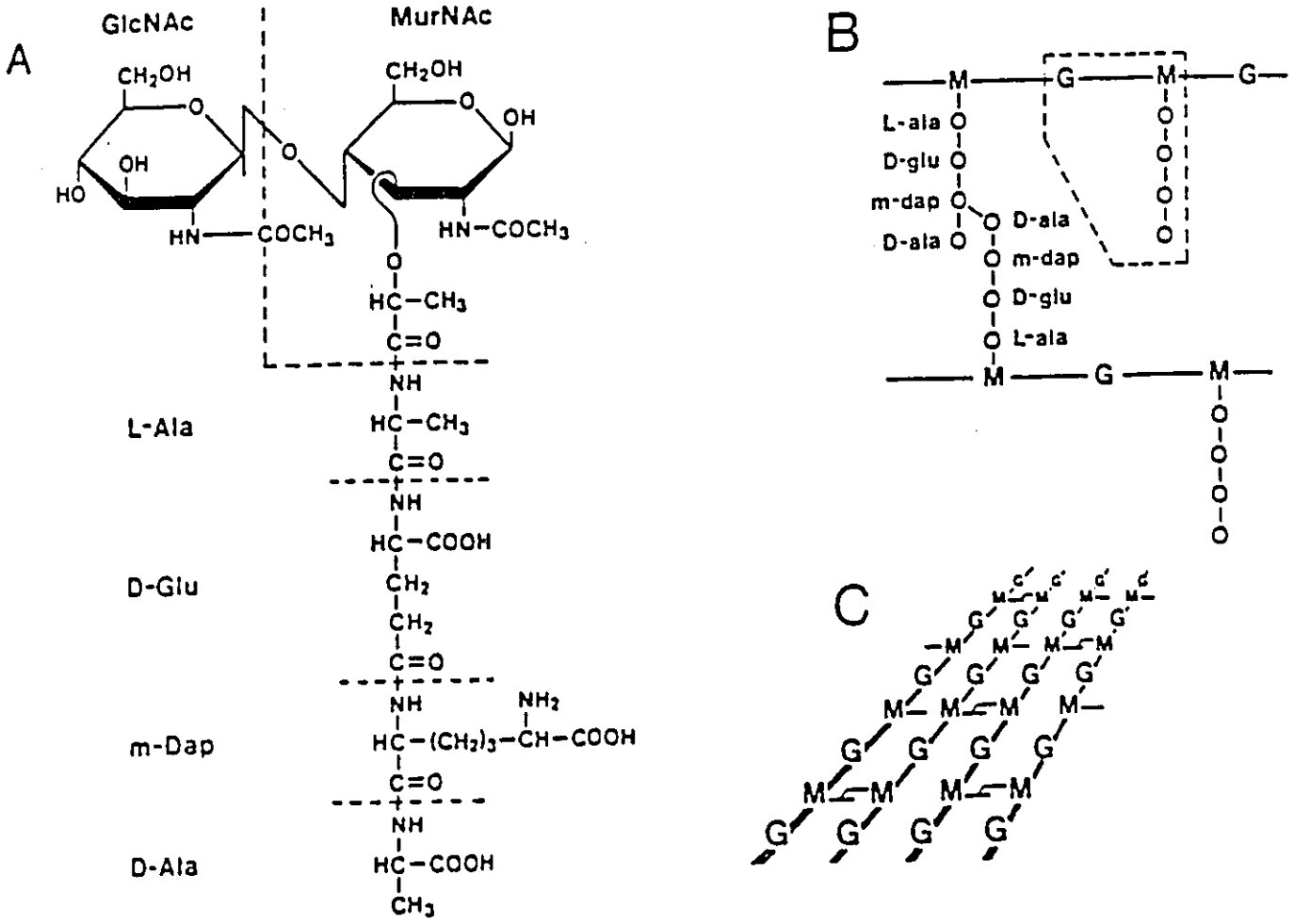
The structure which allows the bacterial cell to contain its cytoplasm against the osmotic pressures pushing against the cell envelope is the cell wall. The cell wall owes its great strength to the peptidoglycan. Peptidoglycan defines the linear network of polysaccharide chains or glycan strands of alternating units of N-acetylglucosamine β1-4 linked to N-acetylmuramic acid (Figure 2) (1,13). These glycan strands can be up to 200 units in length and make up 10 to 20% of the gram negative cell wall (13). Glycan strands are cross-linked to one another by a tetrapeptide covalently bound to the carboxyl group of a muramic acid residue. In *E. coli* the tetrapeptide is in the sequence L-alanine,

TABLE 1

Periplasmic Binding Proteins of Gram Negative Bacteria

Ligand	Mw of Binding Protein (daltons)	Dissociation Constant (uM)	Organism	Ref.
Ions				
Sulfate	31,000	20	*S. typhimurium*	13
Phosphate	41,000	0.08	*E. coli*	"
Phosphate	37,000	0.34	*P. aeruginosa*	27
Amino Acids				
Leucine		0.6		13
Isoleucine	36,000	0.6	*E. coli*	"
Valine		10		"
Glutamine	25,000	0.3	*E. coli*	"
Lysine		3		"
Arginine	27,000	1.5	*E. coli*	"
Ornithine		5		"
Histidine	26,000	1	*S. typhimurium*	"
Sugars				
Arabinose	38,000	2	*E. coli*	"
Ribose	30,000	0.2	*E. coli*	
Maltose	37,000	160	*E. coli*	"
Galactose		1	*S. enteriditus*	"
Glucose	35,000	0.5	*E. coli*	"
"	44,500	0.36	*P. aeruginosa*	20
"	?	< 1.0	*P. putida*	24
Lectin PA-I Galactose N-Acetyl-galactose	13,700	?	*P. aeruginosa*	23
Lectin PA-II L-Fucose		0.67		
D-Mannose	11,400	3225	*P. aeruginosa*	23
Dicarboxylic acids Succinate Malate	?	?	*P. aeruginosa*	21

Figure 2



Structure of Peptidoglycan from Gram Negative Bacteria

A) Structure of one of the lysozyme degradation products showing the basic unit containing the two aminosugars N-Acetylglucosamine (GlcNAc) and N-Acetylmuramic acid (MurNAc) and a tetrapeptide containing the peptidoglycan-specific amino acid meso-diaminopimelic acid (m-dap).

B) Detail of the peptidoglycan structure showing chains of alternating GlcNAc and MurNAc, sometimes interconnected by a peptide bridge. The framed part details the structure shown in (A).

C) Perspective of the peptidoglycan sacculus.

Reproduced with permission (28).

D-glutamate, meso-diaminopimelic acid and D-alanine (1,13). Like N-acetylmuramic acid, diaminopimelic acid is unique to the bacterial cell wall. In this way, the cross-linked peptidoglycan strands form a large macromolecular structure, the sacculus, which serves as the delimiting structure for the bacterium (13,28). The peptidoglycan is covalently linked to the outer membrane lipoprotein (69); several other outer membrane proteins, particularly the porins, are non-covalently associated with the cell wall (13,25,26). Because of this close association between outer membrane and peptidoglycan, the outer membrane is often considered to be part of the cell wall complex.

The Outer Membrane.

The outer membrane of gram negative bacteria provides the ultimate surface for contact with the environment. Its most important function is to provide a selective permeability barrier which allows relatively small hydrophilic solutes like nutrients and ions to cross into the periplasm. The unique architecture of the outer membrane provides the gram negative bacteria protection to harsh environmental conditions. For example the outer membrane provides resistance to life in the gut of animals which assault the bacteria with a battery of disruptive agents like bile salts, fatty acids, glycerides and digestive enzymes including proteases, lipases and glycosidases (28). The size-selective permeability of the outer membrane also provides protection against host defense systems making it impermeable to lysozyme, B-lysin, leukocyte proteins and many antibiotics including penicillin, novobiocin, rifamycins and others (11,29).

The outer membrane also provides a surface where the bacteria can interact with its environment. Mechanisms for the secretion of proteins, toxins and polysaccharides are also present in the outer membrane. In addition the outer membrane provides sites for phage binding and structures like the pili through which bacteria can communicate by transferring genetic material coding for the resistance to antibiotics, heavy metals or antibodies (8,28). The outer membrane also participates in the active uptake of nutrients and ions like iron and phosphate when environmental concentrations are so low that they are growth limiting (32,33).

The outer membrane was first isolated from *E. coli* in the late 1960's by Miura and Mizushima by using isopycnic sucrose density gradient centrifugation of outer membranes obtained from sphaeroplast lysates (30,31). This was soon followed by the isolation of outer membranes from a number of other gram negative bacteria including *S.typhimurium* (34), *P. aeruginosa* (30), *Neisseria gonorrhoeae* (35) and *Vibrio cholerae* (36).

The sphaeroplast-lysate method (30,31) of outer membrane preparation involves suspending bacterial cells in a solution of sucrose to support osmoticaly fragile sphaeroplasts, EDTA to permeabilize the outer membrane and lysozyme for digestion of the peptidoglycan. Upon incubation the outer membranes are shed and can be harvested by differential and sucrose density gradient centrifugation. Hancock and Nikaido (38) devised a method which allowed the separation of the inner and outer membrane without the use of EDTA. Cells of *P. aeruginosa* were first disrupted using a French press in the presence of lysozyme and nucleases. Alternatively the cell suspension could be disrupted using sonication

(48,49). The outer membrane could then be separated from the broken cell suspension by sucrose density gradient centrifugation.

A corollary of the French press-density gradient method was developed by Filip et al (39) and involves the use of detergents such as N-Lauroylsarkosinate (Sarkosyl) to solubilize the contaminating cytoplasmic membrane fragments after disruption by French pressing. The insoluble outer membrane "ghosts" (typically with the cell wall intact) could then be separated from the lysate by centrifugation (40,41). These outer membrane ghosts have not been well studied but appear to be suitable for studying the effects of pilin binding (41). In addition, detergent solubilization methods cause the release of some outer membrane proteins into the soluble fraction and it is unlikely that these preparations are characteristic of native outer membrane (28,42).

Outer membranes of *Vibrio* and *Neisseria* have been isolated using a lithium chloride-lithium acetate preparation (35,37). For this technique cells are suspended in a slightly acidic lithium chloride-lithium acetate solution and membrane vesicles are generated by shaking the cell suspension at 45^{o}C in a flask containing 3 mm diameter glass beads. Outer membranes are then harvested by differential centrifugation.

From analytical and structural studies it was shown that the outer membrane is composed of lipid, lipopolysaccharide, protein and, if not treated with lysozyme, peptidoglycan. Characteristic of the outer membrane is the asymmetric distribution of the lipid and lipopolysaccharide. The inner layer of the outer membrane bilayer is composed of phospholipids (18,29). Phospholipids cover about 50 % of the inner surface of enteric bacteria outer membrane and like the cytoplasmic

membrane phosphatidylethanolamine is the predominant species (18,44).

Lipopolysaccharide (LPS) forms the outer layer of the outer membrane complex and covers about 40% of the cell surface with the remaining 60% covered by protein (18). The characteristic properties of the outer membrane are attributed to the LPS. LPS is the major permeability barrier, it is responsible for resistance to phagocytosis, to serum, and serves as a receptor for the absorption of some bacteriophages (46). The structure of the lipopolysaccharide of pseudomonads is similar to that of the Enterobacteriaceae and is composed of 3 regions: Lipid A (endotoxin), rough core oligosaccharide and the O-antigenic side chain (Figure 3).

Lipid A, (Figure 3A) the hydrophobic component of LPS is quite distinct from its phospholipid counterpart in the inner surface. Where phospholipids contain only two fatty acids linked to a glyceryl backbone, lipid A is composed of an D-glucosaminyl-ß(1-6)-D-glucosamine backbone to which five to seven fatty acids are linked via ester and amide bonds (18). In addition some fatty acids are linked via the characteristic 3-acyl-oxy-acyl structure to the C3 hydroxy group of another fatty acid (29) (Figure 3A). In *P. aeruginosa* the lipid A contains five fatty acids 3-hydroxydecanoate (20.5%), dodecanoate (11.9%), and 2- and 3-hydroxydodecanoate and hexadecanoate (33% and 30.2% respectively)(47). The disaccharide backbone is usually phophorylated on each glucosamine residue and often di-or tri-phosphorlyated (57).

The oligosaccharide core is attached to lipid A through 3-deoxy-D-*manno*-2-octulosonic acid (formerly, 2-Keto-3-deoxy-octulsonic acid or KDO). Both the core oligosaccharide and o-antigen polysaccharide contain sugars and amino sugars which are unique to gram negative bacteria, and

Figure 3

Structure of Lipopolysaccharide and Lipid A Core
of Gram Negative Bacteria

A) Detailed Structure of Lipid A from *E. coli* K-12 (Ref. 45).

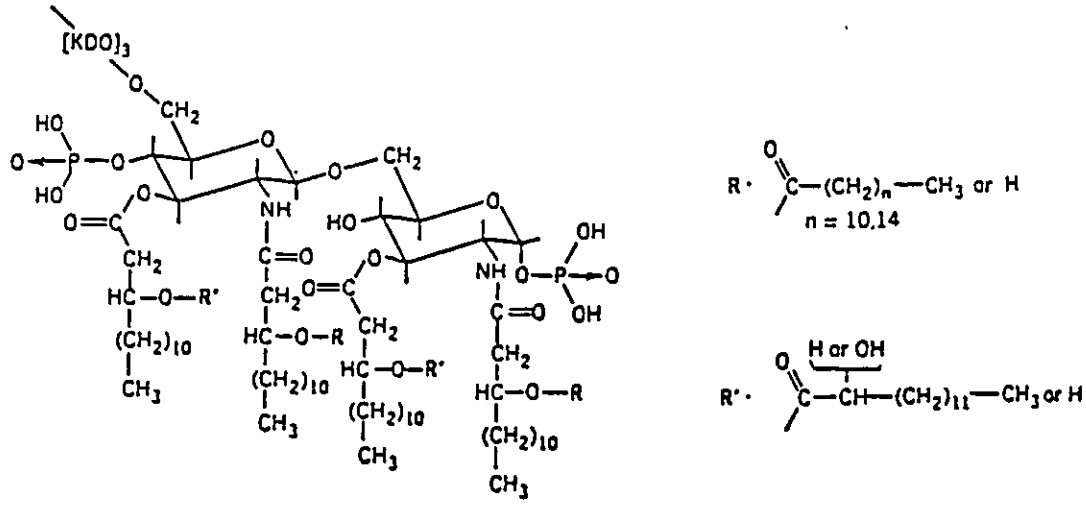
B) Various oligosaccharide core (partial) structures from *P. aeruginosa*. Where p is covalently bound phosphate, Glc is glucose, Rha is rhamnose, GalN is 2-acetamido-2-deoxygalactose (galactosamine), L-Ala is L-alanine, Hep is heptose and KDO is 3-deoxy-D-manno-2-octulosonic acid.

C) Various o-antigen structures from *P. aeruginosa*. Where DL-FucNAc is 2-acetamino-2,6-dideoxy-(DL)-galactopyranose (N-Acetylfucosamine), D-GalNAcAN is 2-acetamino-2-deoxy-D-galacturonamide, D-GalNFmA is 2-formamido-2-deoxy-D-galacturonic acid, D-QuiNAc is 2-acetamino-2,6-dideoxy-D-glucopyranoside (N-acetylquinovosamine) and $BacNAc_2$ is 2,4-diacetamino-2,4,6-trideoxyglucose (N,N'-deacetylbacillosamine).

References in brackets.

Figure 3

A



B

Glcp(α1→6)Glcp

Glcp(β1→2)Rhap(α1→6)Glcp···GalN(···3)Hepp(1→4/5)KDO(2→4/5)KDO→ Lipid A (56)

L-Ala

Rha
α
1
6
Glc(α1→6)Glc(β1→3)GalN(α1→?)Hep(1→4/5)KDO(β2→4/5)KDO→ Lipid A (50)
2
L-Ala

C

[→3)L-FucNAc(α1→3)D-FucNAc(β1→2)D-Glc(β1→]n (54)

[→4)D-GalNAcAn(α1→6)D-GalNFmA(α1→3)D-QuiNAc(β1→3)L-Rha(α1→]n (53)

[→3)Rhap(β1→6)GlcpNAc(α1→4)GalNAc(α1→3)BacpNAc2(α→]n (51)

configurations of these which are both species and strain specific. The core oligosaccharide for *P. aeruginosa* has been elucidated and typically contains glucose, rhamnose, heptose, and alanine in the approximate molar ratios 3:1:1:1:1 (50,56) and proposed structures of the oligosaccharide are illustrated in Figure 3B. Recently, the structures of a number of o-specific polysaccharides have been determined (51-54). Several of these acidic polysaccharides have identical monosaccharide composition but differ in the arrangement of the repeating tetra-and pentasaccharide units (Figure 3C).

The LPS carries a substantial net negative charge as a result of the phosphate and acidic groups on the lipid A and polysaccharide chains. Several lines of evidence suggest that divalent cations like Ca^{2+} and Mg^{2+} appear to cross-link the LPS molecules which contributes to the overall stability and integrity of the outer membrane. This is true of both pseudomonads and enteric bacteria (10, 58-60). The addition of EDTA is known to cause the release of LPS from the outer membrane (10) due to the chelation of divalent cations. A combination of the large organic cation Tris(hydroxymethyl)aminomethane (Tris) and EDTA is used to weaken the outer membrane to make cells more susceptible to lysozyme treatment and this is an important step in the production of cytoplasmic and outer membrane vesicles in *E. coli* (30), *Salmonella* (34) and *Pseudomonas* (31). Polycationic compounds such as polymyxin B or oligolysines and aminoglycosides like gentamycin and streptomycin are also known to disrupt the outer membrane by displacing the divalent cations, but unlike EDTA they do not result in the release of LPS into the medium (10,60).

Early studies of antibiotic resistance in *P. aeruginosa* demonstrated

that growth of cells in Mg^{2+} deficient medium or in the presence of polymyxin resulted in the acquisition of resistance of the bacteria to aminoglycosides, polymyxin B and EDTA (58,63). Certain polymyxin-resistant mutant strains of *P. aeruginosa* were observed to have a 24 fold increase in the level of outer membrane protein H1 and concomitant decrease in Mg^{2+} in the cell envelope (58). Hancock and Nicas (58) demonstrated that there is an increase in the production of outer membrane protein H1 when *P. aeruginosa* is grown under limiting (0.02mM) Mg^{2+}. The induction of H1 in low Mg^{2+} medium was repressed by the addition of (0.5 mM) Mg^{2+}, Ca^{2+}, Mn^{2+} or Sr^{2+} but not by the same concentrations of Zn^{2+}, Ba^{2+}, Sn^{2+}, Al^{3+} or Na^{+} (59). In addition, growth of *P. aeruginosa* in the presence of H1 repressible cations respectively showed that each became the major cell envelope-associated cation (59). Nicas and Hancock (58,59) proposed that protein H1 acts to replace divalent cations in the critical outer membrane locations in the LPS where attack by polycationic antibiotics and chelators cause disruption of the cell envelope (66).

Isolated outer membranes of gram negative bacteria are usually characterized by their protein content which is detected on SDS-PAGE. Normally, the outer membrane proteins of a given species have a distinct pattern of a limited number of major proteins (30,31,35,36,42,64). Reproducible patterns of outer membrane proteins in SDS-PAGE may require heating for several minutes in 1-2% SDS (18,65).

Some proteins in the outer membrane may have a structural role. In *P. aeruginosa* protein H1, as previously described, appears to stabilize the LPS in the absence of divalent cations and when over expressed in the

outer membrane confers increased resistance to antibiotics (66). Another structural protein in the outer membrane of *P. aeruginosa* is lipoprotein I (67,68). Lipoprotein I, like its counter part Braun's lipoprotein in *E. coli*, has covalently bound fatty acids (68,69). Some evidence suggests that lipoprotein I may not be covalently linked to the peptidoglycan like Braun's lipoprotein. However both proteins share an identical sequence at the site for attachment as in the *E. coli* protein (26,67,69). Recent studies indicate that lipoprotein I is also an important candidate for the production of a vaccine against *P. aeruginosa* as it cross-reacts with all serotype strains (67).

Porins.

The outer membrane of gram negative bacteria also contains a high number of diffusion pore proteins known as porins (70). Porins exist as water filled channels which generally act as molecular sieves allowing the selective permeation of solutes through the outer membrane according to size (18,29,38,71,72). Consistent with the pore structure these proteins are trans-membrane and are non-covalently associated with the peptidoglycan and lipopolysaccharide (26,72,73). Porins are found in high copy number in the outer membrane and may number up to 10^5 per cell (18). Unlike cytoplasmic membrane proteins which usually consist of bundles of trans-membrane alpha helixes, porins are rich in β-pleated sheet structure and range in size from 30-50 kDa (18,28,74,77). Several porins have been shown to be arranged in the outer membrane as trimers by gel permeation chromatography and treatment with chemical cross-linkers (75,76).

Porins, because of their association with the LPS and peptidoglycan are tightly bound to the outer membrane. The treatment of whole cells or outer membranes of *P. aeruginosa* with non-ionic detergents such as Triton X-100 does not result in the extraction of porins but will extract some proteins which are not "peptidoglycan associated" (26). Hancock and others have demonstrated that extraction of porins from Triton treated whole cells could be achieved using 2% Triton X-100 in the presence of 10 mM EDTA (26,33,72,78). Porin proteins F, P, and D1 extracted with Triton were subsequently purified by ion-exchange chromatography on DEAE resins and eluting the proteins with 50-500 mM linear gradients of NaCl in detergent concentrations decreased to 0.1%. Recently maltoporin was extracted from SDS washed *S. typhimurium* using the non-ionic detergent Genapol X-80 and purified by passage through a hydroxyapatite column (80).

Alternatively ionic detergents may be used to extract porins but their use precludes ion-exchange chromatography for subsequent purification. Nikaido and his associates (75) demonstrated that porin F from *P. aeruginosa* could be extracted from outer membranes with 2% cholate in 1 M NaCl and 10 mM EDTA (at room temperature) or from whole cells with 2% lithium dodecyl sulfate in 1 M LiCl at 37°C. The ionic detergent extracts were purified by gel permeation chromatography in the respective detergent (75). The ionic detergent-gel permeation method is widely used for the purification of gram negative porins with modifications that include extraction with heating up to 60°C (77,79).

Porins have been isolated and characterized from a number of gram negative species. Hancock (81) summarized the properties of porins from

Table 2

Properties of Porin Proteins of Gram Negative Bacteria

Porin Protein	Species	Molecular Weight (Monomer)	Native Oligomeric State	SDS Stable Oligomers	β-sheet Structure	Peptidoglycan Associated	LPS Associated	Conditions Favoring Production
F	*P. aeruginosa*	39,000	Trimer	-(+)	+	+	+	
P	"	48,000	Trimer	+	+	-	+	Pi-limitation
D1	"	46,000	Trimer	-		-	+	Glucose
D2	"	45,500 (74)						
OmpF	*E. coli*	32,703	Trimer	+	+	+	+	Low osmolarity
OmpC	"	36,000	Trimer	+	+	+	+	High osmolarity
LamB	"	47,932	Trimer	+	+	+	+	Maltose
PhoE	"	36,782	Trimer	+		+	+	Pi-limitation
OmpF	*S.typhimurium*	39,300	Trimer	+	+	+	+	Low osmolarity
OmpC	"	39,800	Trimer	+	+	+	+	High osmolarity
OmpD	"	38,000	Trimer	+	+	+	+	
PhoE	"	34,000				+		Pi-limitation
LamB	"	46,000 (80)				+		Maltose
I	*N. gonorrhoeae*	34,000	Trimer	-				
I	*N. meningitidis*	37,000		+/-		+		
Por A	"	45,000 (64)						
E	*Y. pestis*	33,000		+		+		Low growth temperature

Adapted from Reference 81, other references in brackets.

several species and many of these are listed in Table 2. Most of the major outer membrane proteins and porins of *P. aeruginosa* have been characterized.

Properties and Function of Porins

Recently Hancock (81) described several methods by which the function of porins as pore-forming proteins could be determined (Figure 4). Each of these methods requires the reconstitution of outer membranes in artificial lipid or lipid-LPS membranes. The first reconstitution of a porin was performed by Nakae (82) who mixed the total outer membrane fraction of *S. typhimurium* with phospholipid and LPS. Soon after, the total outer membrane fraction of *P. aeruginosa* was reconstituted using a similar method (38). The determination of the outer membrane as a permeability barrier involved mixing outer membrane and LPS with phospholipids dried on the surface of a test tube. The membranes were randomized by sonication and caused to enclose a suspension of permeant ^{14}C-sucrose and "impermeant" tritiated saccharide (2 kDa inulin to 50 kDa dextran). The vesicles thus formed were diluted, incubated for a period of time and the extent retention of sucrose vs. saccharide was used to determine the exclusion limit for the outer membrane (Figure 4A).

Using this technique outer membranes from *S.typhimurium* (82), *E. coli* (83) and *Proteus morganii* (84) were shown to have exclusion limits of about 600 daltons but the outer membrane of *P. aeruginosa* had a substantially larger exclusion limit of 3000-9000 daltons (38). In agreement with this result, Triton-EDTA purified reconstituted porin F from *P. aeruginosa* was also determined to be approx. 6000 daltons (72).

Figure 4.

Schematic representation of four model membrane systems (81).

A. Proteolipsosome radioisotope efflux. Liposomes (L) are reconstituted from phospholipids, LPS and porin proteins in the presence of a ^{3}H dextran of high molecular weight (20,000 - 70,000 daltons) and ^{14}C sucrose (342 daltons). Dilution of the medium external to the liposomes creates a concentration gradient across the liposome membrane, allowing ^{14}C sucrose to leak out. H^{3}-dextran, being larger than the exclusion of the porin, is retained within the liposome.

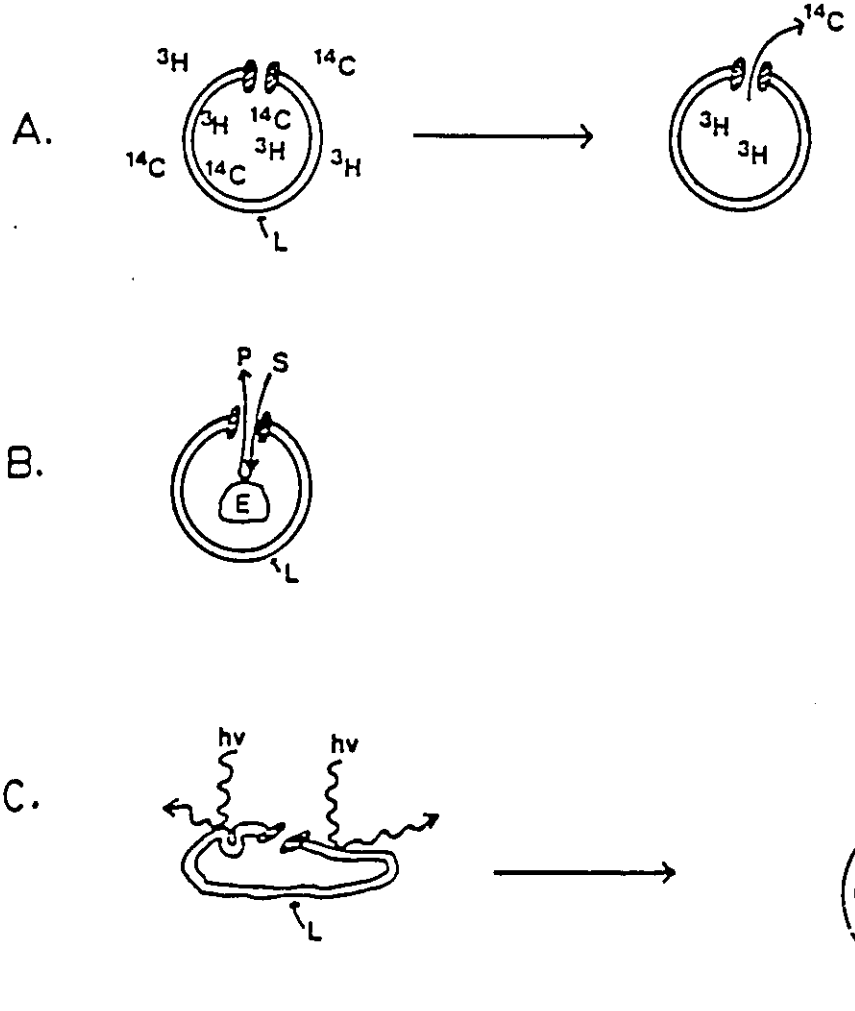
B. Enzyme-Containing proteoliposome assay. Used in studies of maltoporin of *E. coli*. Liposomes are reconstituted as above but in the presence of an enzyme (E) and removed from excess enzyme by gel filtration chromatography. Permeability is measured by assessing the rate of conversion of the substrate (S) to product (P).

C. Liposome Swelling Assay. Proteoliposomes are reconstituted in the presence of an impermeant solute of high osmolarity. The multilamellar liposomes are of non-uniform shape and efficiently scatter light (hv). Addition of a permeant solute (P) of equivalent osmolarity to the external mileau will cause a concentration gradient across the liposome membrane. The permeant solute thus flows through the porin into the liposome creating an increase in internal osmolarity. Consequently, water (H_2O) flows in to maintain the iso-osmolarity exterior and interior. This results in liposome swelling, in at least the most external liposome bilayer and the liposome is less able to scatter light. Thus the rate of swelling is related to the rate of solute influx.

D. Black lipid bilayer assay. The apparatus involves a Teflon chamber (C) divided into two compartments by a Teflon divider (D) containing a small hole. The chamber is filled with a bathing salt solution (BS) and electrodes (E) dip into the salt solutions in each compartment. One electrode is connected to a voltage source (V), and the other is connected to a measuring device (M). A lipid membrane (M) is formed across the hole in the Teflon divider, and reconstitution of single porin molecules into the membrane can be followed by current increases due to movement of ions (K^{+} and Cl^{-}) through the porin channels.

Reproduced with permission (81).

Figure 4


A.
^{3}H
^{14}C
^{3}H
^{14}C
^{14}C
^{3}H
^{14}C
^{3}H
^{3}H
L
^{14}C
^{3}H
^{3}H
B.
P
S
E
L
C.
hv
hv
L
hv
hv
P
H_2O
P
H_2O
D.
V
M
BS
D
K^+
Cl^-
E
M
C

Porin F purified from SDS extracted *P. aeruginosa* outer membranes was reconstituted by a similar method except that the vesicles were caused to enclose a non-permeant unlabelled saccharide (75). The vesicles were diluted into iso-osmotically prepared buffer containing one of a number of permeant saccharides (Figure 4C). The uptake of the permeant saccharide caused by the chemical gradient occurs concomitantly with the influx of water to maintain the osmotic equilibrium. The influx of water causes the liposome to swell and results in a change in the optical density of the vesicle suspension. Using the liposome swelling technique Nikaido and his co-workers (75) showed that with *P. aeruginosa* porin the rate of swelling was much less dependent on solute size than was the rate for *E. coli* porin (ie. larger pore) and that a given amount of *P. aeruginosa* porin was 40 fold less permeable to small solutes than the same amount of *E. coli* porin. The latter result was taken to suggest that only a small proportion of the *P. aeruginosa* porins were "open" at any given time (75,85).

Membrane conductance measurements are another method for determining the permeability of porin proteins (Figure 4D) (81, 85-89). In conductance studies a Teflon barrier separates compartments containing salt solutions (ie. KCl, NaCl, NH_4Cl, etc.). Electrodes are placed in either side of the barrier. The barrier has a small (approx. 0.1-2 mm^2) hole which is painted with a lipid dissolved in n-decane which appears black when a bilayer has formed. A porin protein of known concentration, suspended in a detergent solution is added to one or both compartments of the salt bath. An increase in the conductance is observed as the porin inserts itself into the lipid membrane.

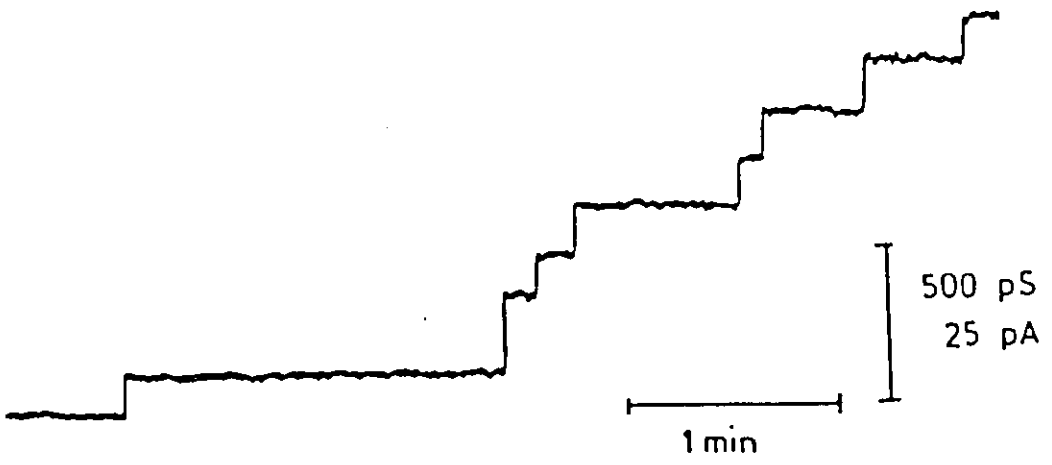
Measurement of membrane conductance revealed a number of properties of porins. When small amounts of porin are added to the aqueous solutions bathing the lipid membrane, membrane conductance increases in a stepwise fashion (85,87,89)(Figure 5). These increases in conductance are interpreted to be the result of the incorporation of individual porin channels ie. single channel conductance (Λ). Benz and his colleagues measured the single channel conductance of porins from several species (85,87,89,91). For example porin from *E. coli* had a single channel conductance of 10^{-11} S whereas that of porin F from *P. aeruginosa* was 4.8 x 10^{-10} S (measured in 0.1 M NaCl, pH 6.5) (85). Assuming that the pore is a cylinder with a length of 6 nm (the length of the *E. coli* Omp F channels as determined by X-ray diffraction measurements) (90) and that the pore interior has the same specific conductance as the bulk aqueous phase, the average diameter of the pore formed by a porin could be determined from the single channel conductance, Λ , using the formula:

$$\Lambda = \sigma r^2 \pi / l \qquad (1)$$

where σ is the specific conductivity of the bulk solution, r is the radius of the channel, l is the length of the channel and π has its usual meaning. The channel diameter for a number of porins determined by this method in 1 M KCl are shown in Table 3 (91). While the assumptions for equation (1) are probably not strictly valid, the diameters calculated from single channel conductance are in good agreement with diameters measured by liposome swelling studies (81).

Black lipid membrane studies were also used to evaluate the extent of channel selectivity of cations vs. anions by determining the zero

Figure 5



Measurement of Single Channel Conductance. Stepwise conductance increases after the addition of small amounts (10^{-11} M) of Pho E porin (*E. coli*) to the salt solution (0.1 KH_2PO_4) bathing a lipid bilayer membrane. The individual steps represent single porin trimers reconstituting into the membrane and giving rise to conductance increases.
Reproduced with permission (81).

Table 3

Comparison of Porin Channel Diameters and Zero Current Membrane Potentials from Gram Negative Bacteria. (91)

Pore	Λ^a (nS)	diameter (nm)	V_m^b (mV)
E. coli			
OmpF (B)	2.1	1.2	27
OmpF (K-12)	1.9	1.1	26
OmpC	1.5	1.0	50
PhoE	1.8	1.1	-24
LamB	2.7	1.4	30
S. typhimurium			
OmpD	2.5	1.3	46
OmpF	2.2	1.2	54
OmpC	2.4	1.3	50
P. aeruginosa			
F	5.6	2.2	20
P	0.28	0.6	-58
Y. pestis			
E	1.7	1.1	45

a Average single channel conductance Λ measured in 1M KCl, pH 6.
b Zero current membrane potential measured in the presence of a ten fold KCl concentration gradient. V_m is the electrical potential of the dilute side (0.01 M) minus the potential at the concentrated side (0.1M).

current potential (81,91). To determine a zero current potential, a bilayer apparatus is set up (Figure 4D) under a current to allow the insertion of 10-100 porins in the membrane. The applied current is stopped and the apparatus is adjusted to measure membrane potential. An aliquot of concentrated salt (KCl, LiCl, potassium acetate) is added to one side and an equal volume of water is added to the other. A chemical potential is thus established which will drive the movement of ions but, if the channel is ion selective, ie. cation selective, more cations will move to the dilute side of the membrane causing an electrical potential to be established. When the electrical potential balances the opposing chemical potential, the zero current potential (V_m) is achieved (81). For cation selective channels, V_m will become increasingly positive and for anion selective channels it will become increasingly negative as a function of salt concentration (81,91)(Table 3).

Porins of P. aeruginosa.

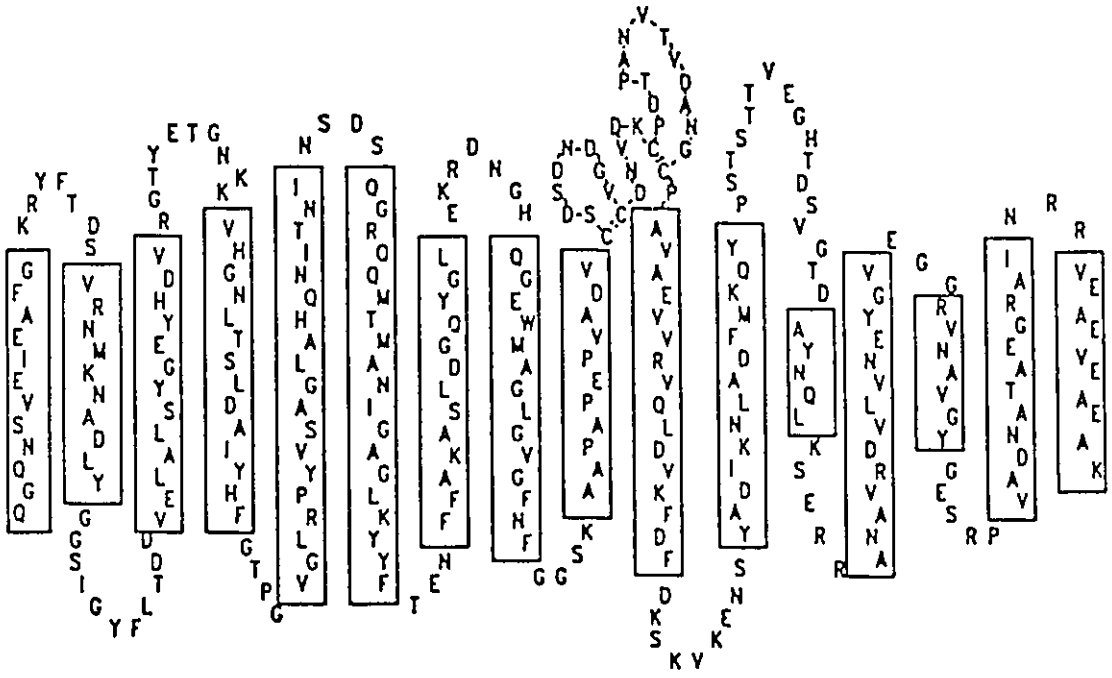
Porin F.

Porin F is the most well studied of the outer membrane proteins of *P. aeruginosa*. Mizuno and Kageyama (31) first noted that protein F was resistant to solubilization by SDS and 0.1 M NaCl and suggested that the protein was peptidoglycan associated. Further studies with Triton X-100 and EDTA confirmed this observation (26) and led to techniques for extraction and purification (72,75) as previously described. Evidence from cross-linking studies with the bifunctional (2-mercaptoethanol cleavable) cross-linking agent DSP suggests that porin F exists as a trimer in its native state in the outer membrane (76). The trimers could

be detected when porin F was purified after extraction with Triton X-100 but not after treatment with with SDS (76). This observation is consistent with results from gel filtration chromatograpy in SDS where purified porin F eluted only as a single peak of protein at 35 kDa, the monomer molecular weight (75).

Porin F is characterized by its heat and 2-mercaptoethanol modifiable mobility on SDS-PAGE (31,65). Addition of 2-mercaptoethanol to sample buffer with prolonged heating during SDS-PAGE sample preparation (ie. 95°C for 20 min) results in the conversion from a faster (F) 33 kDa migrating band to a slower (F^*) 42 kDa running form of the protein (65). A similar shift in porin F mobility was observed when outer membrane proteins were treated with trichloroacetic acid (31) or phenol (31,38,65). Hancock and others (65,74,81) have suggested that the heat modifiable mobility of the protein was due to resilient β-sheet structure and that the 2-mercaptoethanol modification was due to the breakage intramolecular disulfide bonds resulting in a more open, slower moving conformation of the protein. Using circular dichroism, porin F was estimated to have about 62% β-sheet structure (74) although the role of secondary structure in heat stability remains to be elucidated. Recently, the amino acid sequence of porin F was determined (92). The sequence shows little overall homology with known porin sequences from *E. coli* but there is a short stretch of sequence (30 amino acids) highly homologous with a region in Omp A of *E. coli*. Siehnel et al (74) have used the sequence to build a conceptual model of porin F (Figure 6) to show how the β-sheet regions, predicted by the method of Paul and Rosenbusch (93) would sit in the outer membrane.

Figure 6



Conceptual model of Omp F (porin F) of P. aeruginosa based on the amino acid sequence and method of Paul and Rosenbusch (92). Regions predicted to have β structure are boxed and are assumed to sit within the membrane. Reproduced with permission (74).

A number of monoclonal antibodies have been produced which are directed against porin F and protein H2 (94-96). The antibodies have been used to show that porin F is expressed on the surface of *P. aeruginosa* (95,96) and as such provide a method for screening clinical isolates representing the 17 serotypes of the species (95). Western blot analysis of porin F run on SDS-PAGE revealed the presence of some higher oligomers which were not completely denatured by SDS supporting the previous evidence for porin F oligomeric structure (95). Surprisingly, monoclonal antibodies to porin F cross reacted with *P. putida* and *P. syringae* (but not *P. fluorescens*) suggesting a close structural relationship of the homologous outer membrane proteins between these species (96). Earlier studies (94) indicated that antibodies directed against *P. aeruginosa* protein H2 cross reacted with outer membranes from both *P. putida* and *P. fluorescens*. The immunological activity of porin F as a common antigen for *P. aeruginosa* is being exploited to produce a vaccine against *P. aeruginosa* in mice (97-99). The high antigenicity of LPS is a major concern in vaccine production but the authors demonstrated that mice (97) or rats (98) immunized with porin F purified from cells of a particular LPS serotype protected them from infection with *P. aeruginosa* of several other serotypes. Most recently, *P. aeruginosa* porin F expressed in *E. coli* (which has no pseudomonad LPS), was used successfully to immunize mice against two *P. aeruginosa* serotypes (99).

A number of apparently conflicting results have recently caused a controversy concerning the function of porin F. As previously discussed, black lipid bilayer and liposome studies of reconstituted porin F (and total outer membrane proteins) of *P. aeruginosa* showed that the porin

formed large (2.2 nm) water filled channels with an exclusion limit for saccharides of about 3000-9000 daltons (72,75,85). However, the apparent exclusion limit of the outer membrane is inconsistent with the very low permeability of the outer membrane of *P. aeruginosa* (whole cells) to small hydrophilic antibiotics (100 fold lower than the outer membrane of *E. coli*) (100). The question of *P. aeruginosa* outer membrane permeability was re-examined by determining the extent of permeation of mono-, di- and oligosaccharides across the outer membrane of whole cells. These studies showed that the outer membrane was permeable to pentoses, but limited the permeation of disaccharides like sucrose (102-103). In agreement with these results electron microscopy was used to show that high osmolal oligosaccharides like stachyose, raffinose and sucrose but not ribose caused shrinkage of whole cells because of the impermeability of the outer membrane to saccharides greater than monomer size (104). Using liposome swelling assays, Nakae and his associates showed that there was no difference in curves of swelling rate vs. saccharide Mw. with liposomes reconstituted with total outer membrane proteins or porin F deficient outer membranes (105). These authors suggested that porin F may not be a permeability pore and using the same technique they reconstituted each individual porin from *P. aeruginosa* outer membrane and recently claimed that outer membrane proteins C (Mw, 70,000), D2 (Mw, 46,000), and E (Mw, 43,000) were the only proteins which were responsible for producing monosaccharide permeable pores (106).

Contrary evidence supporting the role of porin F as the major permeability pore comes from studies of defective or porin F deficient cells of *P. aeruginosa*. Studies with defective porin F reconstituted into

proteoliposomes suggested that porin F mediated the outer membrane permeability of antibiotics to which *P. aeruginosa* was succeptable (101). Porin F deficient mutant cells had reduced permeability to chromogenic β-lactam antibiotics but the antibiotic susceptibility, compared to the porin F sufficient cells, was unchanged (107,108). This is in contrast to the 8-32 fold increased resistance in porin deficient mutants of *E. coli* and other enteric bacteria (109-112). It should be noted however that other porin proteins (apart from porin F) may be present as noted above (106). Taking into account the number of channels in the porin F sufficient cells, Nicas and Hancock (107) estimated that over 99% of porin F molecules are closed in the *P. aeruginosa* outer membrane. Recently, porin F has been cloned and expressed in *E. coli* (113). The cloned protein was purified from *E. coli* and tested for porin function by incorporation into black lipid membranes. With improved detection methods (for the resolution of small conductance changes), over 99% of porin F from *E.coli* or *P. aeruginosa* were found to produce very small (0.32 nS, 0.06 nm) pores, too small for the passage of small molecules including monosaccharides (113).

The assignment of the primary role of porin F as a permeability pore may be incorrect, and the pore formation of the very small minority of protein F molecules could be secondary to its role in maintaining the stability of the outer membrane (74). It has been known for some time that porin F deficient mutants grow poorly in both minimal and rich media and require high concentrations (50-200 mM) of NaCl, KCl, glucose, sucrose or succinate to osmotically stabilize the outer membrane (74,107,108,114). Woodruff and Hancock (114) recently demonstrated that

porin F is not only partially homologous (92) in sequence to Omp A of *E. coli* but also cross reacts with polyclonal antisera to Omp A. Omp A has been demonstrated to have a structural role in stability and shape maintenance (with Braun's lipoprotein) in *E. coli* (115). Microscopic (115) and electron-microscopic (116,117) observations have shown that porin F deficient *P. aeruginosa* mutants are more succeptable to osmotic shock, rounding, blebing of the outer membrane and breakage than the porin sufficient parent cells. Thus porin F may function primarily to stabilize the outer membrane and anchor it to the peptidoglycan.

Porin P.

When cells of *P. aeruginosa* are grown under phosphate limiting conditions a 48,000 dalton protein (Protein P) is expressed in the outer membrane (33). This protein exists as a trimer in the outer membrane (76) and unlike porin F, the oligomeric structure remains intact when purified in SDS by gel filtration (33,118). Notably, protein P is very stable, resistant to proteolysis, or denaturation with detergents and heating. Circular dichroism spectra estimate that the secondary structure consists of 65 % β-sheet (26 % β-turn, 3% α helix), which only decreases to 55% β-sheet after boiling in 0.1 % SDS for 10 min (118). Protein P was reconstituted into black lipid membranes and formed small, 0.6 nm pores (Table 3) which were 100 times more selective for anions than cations as determined by zero current membrane potentials (33,74,89,91).

Protein P has a phosphate binding site (measured indirectly as the ability of phosphate to inhibit conductance in KCl) with a Kd of 0.34 mM representing an affinity for phosphate at least 100 times greater than

for chloride or other anions (120). Results from the chemical modification of available lysine ϵ-amino groups by acetylation, carbamylation and methylation suggest that the binding site of protein P contains 3 lysine groups in a narrow channel which result in a high affinity site for phosphate (119,120). Porin P has recently been crystallized (118) and x-ray diffraction studies should confirm the proposed structure of the binding site. Growth on limiting phosphate also induces a high affinity-periplasmic binding protein (Kd = 0.34 uM) dependent-phosphate transport system (27) analogous to the Pst, phosphate transport system of *E. coli* (122) and protein P has been proposed to be involved in this phosphate uptake system (27,74). Polyclonal antisera to the trimeric form of protein P cross-reacted on western blots with phosphate-starvation-induced proteins in several pseudomonad and enteric species, but only to the oligomeric forms (121). This suggests that there is a conserved structural (antigenic) relationship found only in the functional proteins.

Protein D1.

Protein D1 is induced in *P. aeruginosa* by growth on D-glucose, and non-metabolizible inducers like D-galactose, 2-deoxy-D-glucose, and methyl α-D-glucopyranoside with the addition of pyruvate as an energy source but is repressed by growth on citrate, succinate, acetate and D-gluconate (31,78). This pattern of induction and repression is also shared by the high affinity glucose transport (see below) system and the periplasmic glucose binding protein in *P. aeruginosa* (20,21,123,124). Protein D1 is an SDS-labile trimer in the outer membrane (76), and its

mobility on SDS-PAGE is heat modifiable from an apparent 35.5 kDa when incubated in SDS at 37°C, to 46 kDa when incubated at 65°C or over (65,78). In liposome swelling assays, reconstituted D1 was shown to be selective for permeability to a number of carbohydrates with rates of permeation a function of solute size. The channel showed some selectivity for D-glucose, as L-glucose and the 2-OH and 4-OH epimers of glucose, D-mannose and D-galactose had much reduced rates of permeation relative to D-glucose (125). Hancock and Carey (78) provided evidence to suggest that protein D1 does not bind radiolabelled glucose using the method for the periplasmic glucose binding devised by Stinson et al (22). Two points should be noted however, firstly, no attempt was made to test for binding of glucose when the protein was in its native state in the outer membrane. Detergent solubilization may have denatured the binding activity. Secondly, as with porin P (above) and LamB of *E. coli* (126), binding studies with specific porin proteins are usually conducted in reconstituted systems which involve indirect measurement of the inhibition of conductivity of small ions. Protein D1 most likely has a role in high affinity transport of glucose analogous to the operation of LamB in the maltose transport system of *E. coli* (14).

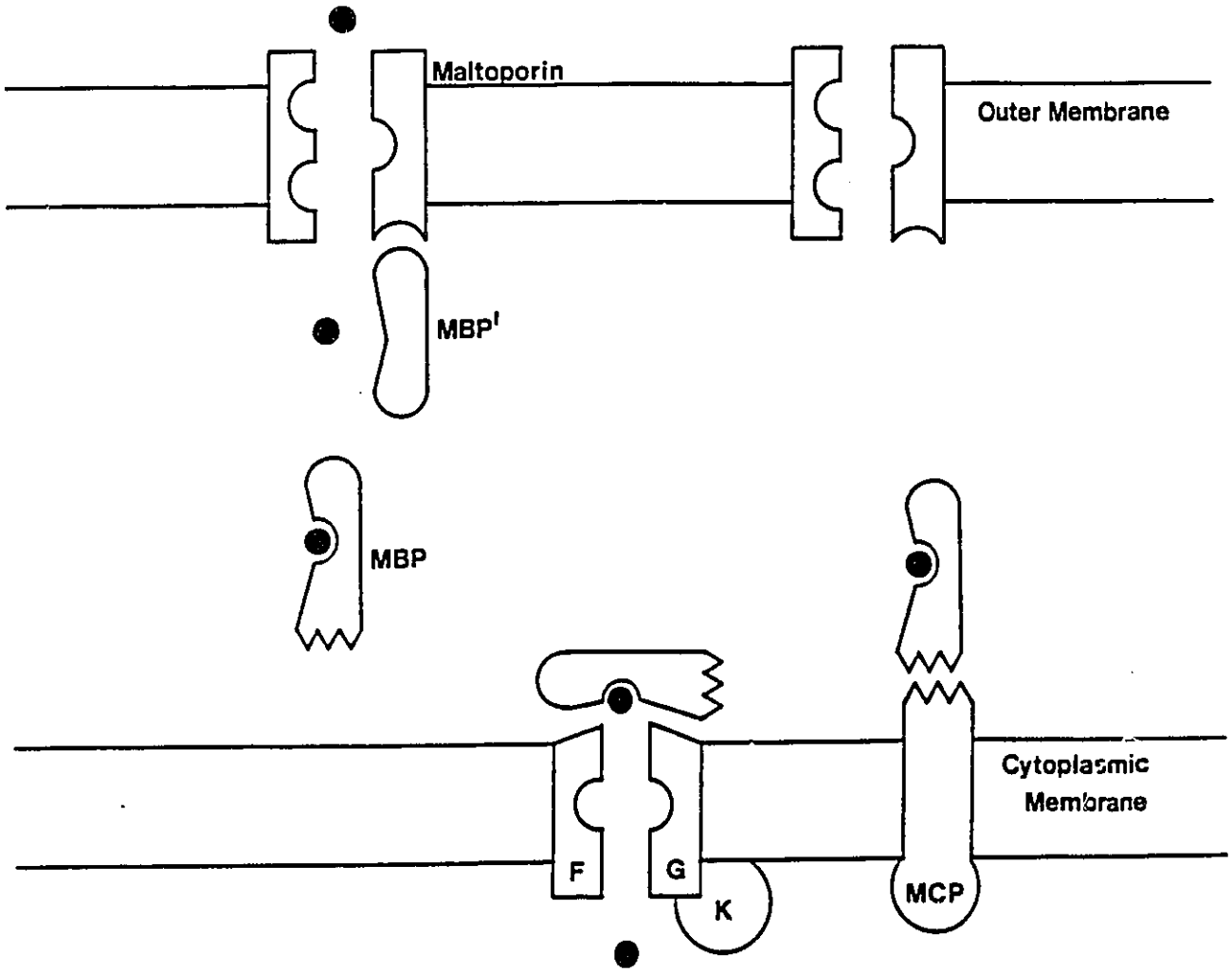
LamB and the Maltose Transport System of E. coli.

LamB is both the receptor site for the bacteriophage and a selective porin involved in the diffusion of maltose and maltodextrins across the outer membrane of *E. coli* and *S. typhimurium* (14,80). LamB or maltoporin forms trimers (145 kDa) in the outer membrane which are stable to detergents and heating up to 60°C (127). Studies of the channel

architecture have indicated that each trimer consists of 3 independent channels which are not formed at the interface between the monomers (128). Binding of maltodextrins is measured indirectly by a number of methods which include measurement of the inhibition of channel conductance in lipid bilayer membranes by the addition of various concentrations of maltodextrins to the bathing solutions (126). From this type of measurement, maltoporin bound maltosaccharide with a Kd of 10 mM for maltose and and 6.7 x 10^{-5}M for maltohexose (18). Binding has also been measured with immobilization of cells on starch-Sepharose or by using fluorescent amylopectin analogues (128).

LamB is part of a shock sensitive maltose transport system (Figure 7) which mediates the accumulation of maltose into the cell against 10^5-fold concentration gradients (14). Mutants lacking maltoporin are impaired in chemotaxis for maltose and uptake when the maltose concentration is below 0.1 mM (129,130). The maltose uptake system includes a periplasmic maltose binding protein (MBP) which has one site for maltose binding per protein and domains for binding to maltoporin and the cytoplasmic membrane components for uptake and for chemotaxis (14). Genetic studies have dissected the components for maltose transport and in addition to lamB and MBP, three additional proteins are required for translocation across the membrane F, G, and K. (19). Reconstitution studies of the maltose transport have recently been made possible by the use of a MBP mutant where MBP is tethered to the cytoplasmic membrane of *E. coli* (132,133). With the tethered MPB in cytoplasmic membrane vesicles, it has been shown that translocation of maltose across the membrane requires hydrolysis of ATP on the mal K domain (132). This

Figure 7



Location of the maltose transport system in the cell envelope of *E. coli* (adapted from reference 14). MBP and MBP' are the substrate loaded and free forms of the maltose binding protein. F, G and K are the gene products from *malF*, *malG* and *malK*. MCP is the signal transducer for maltose chemotaxis. There are about 40,000 molecules for MBP in the cell envelope, most of them are associated with the trimeric maltoporin. The amount of FGK receptor is small, 500-1000 per cell.

confirmation of ATP as the direct energy source distinguishes the shock sensitive-binding protein dependent uptake systems from those requiring proton symport (14) (ie, lactose permease) or group translocation by the phosphotransferase system (133).

Aspects of Carbohydrate Transport and Metabolism in *Pseudomonas*

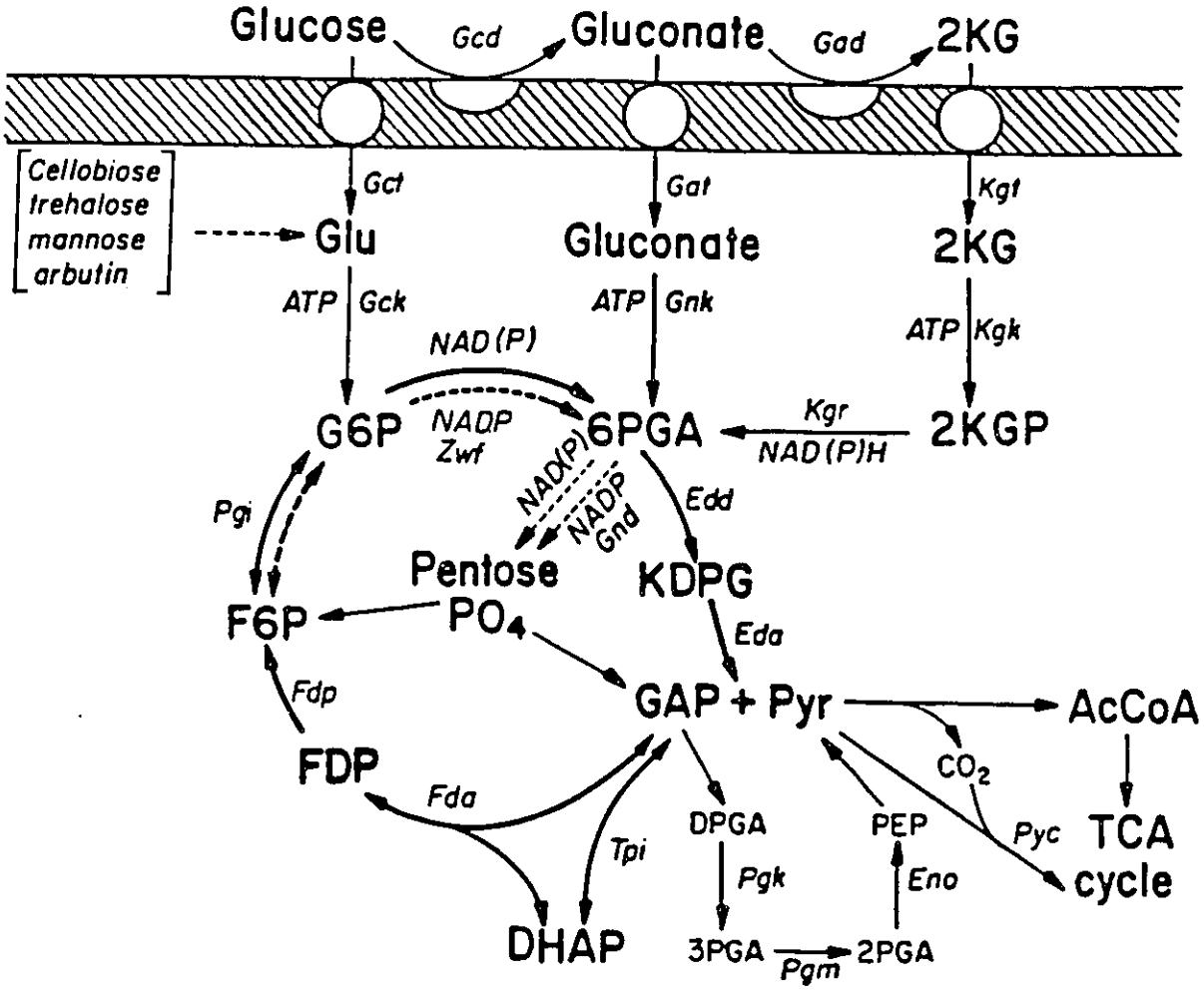
Glucose transport and metabolism in the fluorescent pseudomonads (*P. aeruginosa*, *P. fluorescens* and *P. putida*) is distinct from that of the enteric bacteria in a number of respects. Firstly, pseudomonads lack phosphofructokinase and thus are unable to ferment glucose using glycolysis (2,134). Secondly, glucose uptake proceeds through two alternative pathways, an oxidative or phosphorylative route (134) and not through a phosphotransferase system (133). Figure 8 illustrates the pathways of glucose dissimulation in pseudomonads. Glucose is either actively transported directly and phosphorylated intracellularly to glucose-6-phosphate or oxidized to gluconate which may be further oxidized to 2-ketogluconate and actively transported (135,161). Growth under limiting oxygen (136,137), low temperature (138) or limiting glucose concentrations (139), favors the glucose uptake by the intracellular phosphorylative route (134). This results in two forms of glucose uptake, a high affinity uptake (Km= 8uM) related to the phosphorylative pathway and a low affinity transport for gluconate, (Km 1 mM) produced by the action of glucose dehydrogenase (124,141). Glucose uptake and metabolism are regulated by metabolite and catabolite repression. Growth of pseudomonads on citric acid cycle intermediates severely represses enzymes of glucose metabolism (140) and components of

Figure 8

Enzymes Related to Glucose Utilization in Pseudomonads

Dashed lines indicate features characteristic of *Pseudomonas cepacia*. All other reactions shown here have been demonstrated in *P. aeruginosa*. Sugars and intermediates are of the D-configuration. Abbreviations: Gcd and Gad, membrane associated glucose and gluconate dehydrogenases; Gct, Gat and Kgt represent transport systems for glucose, gluconate and 2-ketogluconate respectively; Gck, Gak and Kgk represent ATP-dependent kinases for glucose, gluconate and 2-ketogluconate respectively; Kgr, 2-keto-6-phosphogluconate reductase; Zwf and Gnd, glucose-6-phosphate (G6P) and 6-phosphogluconate (6PGA) dehydrogenases; Edd and Eda, 6PGA dehydratase and KDPG aldolase; Tpi, triose phosphate isomerase; Fda, fructose 1,6-diphosphatea aldolase; Fdp, fructose 1,6-diphosphatase; and Pgi, phosphoglucoisomerase; Pyc, pyruvate carboxylase.
Adapted from reference (134).

Figure 8


Glucose
Gcd
Gluconate
Gad
2KG
Cellobiose
trehalose
mannose
arbutin
Gct
Gat
Kgt
Glu
Gluconate
2KG
ATP
Gck
ATP
Gnk
ATP
Kgk
NAD(P)
G6P
NADP
Zwf
6PGA
Kgr
NAD(P)H
2KGP
NAD(P)
NADP
Gnd
Edd
Pgi
Pentose
PO4
KDPG
F6P
Eda
Fdp
GAP + Pyr
AcCoA
FDP
CO2
Fda
DPGA
PEP
Pyc
TCA
cycle
Tpi
Pgk
Eno
DHAP
3PGA
Pgm
2PGA

the glucose transport system (123,124,141,146). In whole cells gluconate as a growth carbon source, or produced from glucose extracellularly by glucose dehydrogenase represses glucose transport (142,143), the glucose binding protein (21) and outer membrane protein D1 (78).

Deoxyfluoroglucose analogues have been used to probe the uptake and metabolism of carbohydrates in *P. putida* (6,144-146). Whole cells and cytoplasmic membranes of *P. putida* are capable of oxidizing 3-deoxy-3-fluoro-D-glucose (3FG), and 3-deoxy-3-fluro-D-gluconate (3FGA) with the retention of the carbon-fluoride bond (144,145). In addition, cytoplasmic membrane vesicles were capable of transporting 3FG (146). Cytoplasmic membranes of *P. putida* also oxidize 4-deoxy-4-fluoro-D-glucose (4FG) but incubation of whole cells of *P. putida* with 4FG results in the cleavage of the carbon-fluoride bond (6) and subsequent metabolism to 2,3-dideoxyribonic acid (162). This defluorinating activity was glucose inducible and succinate repressible. Fractionation of the *P. putida* cell suspensions suggested that the defluorinating activity resided in the isolated outer membrane (6).

Objectives.

1. Prepare and characterize outer membranes of *P. putida* grown on various carbon sources and identify a glucose inducible protein.
2. Isolation and characterization of a glucose inducible protein (Band E) from *P. putida* and comparison with protein D1 from *P. aeruginosa*.
3. Establish whether the isolated outer membrane and/or its glucose inducible protein (Band E) is involved in the defluorination of 4-deoxy-4-fluoro-D-glucose.

4. Prepare polyclonal antibodies to Band E , characterize them by Western blot analysis and investigate whether they inhibit glucose uptake in whole cells of *P. putida*.

MATERIALS AND METHODS

Materials.

D-[U-14C]-4-Deoxy-4-fluoro-glucopyranose (10,600 dpm/umole) and D-4-deoxy-4-fluoro-glucopyranose were prepared by Dr. D. Sbrissa in this laboratory and were provided by Dr. N.F. Taylor. D-[6-^{3}H]-glucose (38 Ci/mmole) and Na^{125}I (200 uCi/100ul) were obtained from ICN Radiochemicals (Irvine, Ca.). Ammonium persulfate (electrophoresis grade), silver stain reagent kit, 2-mercaptoethanol, N,N,N',N'-tetramethyl-ethylenediamine, (3,3'-dithio-bis-(propionic acid) N-hydroxysuccinimide ester (DSP), Dalton Mark IV low molecular weight standards, SDS 6H high molecular weight standards, ribonuclease A (bovine pancreas), lysozyme chloride, B-octyl glucopyranoside, Tween 20, Lubrol PX, Triton X-100, cholic acid, deoxycholic acid, N-lauroylsarkosinate (Sarkosyl), agar, bovine serum albumin (Fraction V), Flavin adenine dinucleotide, Folin and Ciocalteau's phenol reagent, 2-keto-3-deoxyoctonate (KDO) ammonium salt, crystal violet, Gram saffranin, chloramphenicol, gentamycin, kanamycin, penicillin and DEAE Sephacel were obtained from Sigma Chemical Company (St. Louis Mo.). Electrophoresis grade sodium dodecyl sulfate, acrylamide, glycine, tris(hydroxymethyl)aminomethane and Bradford dye reagent kit were obtained from BioRad (Richmond Ca.). dithiothreitol, and N,N-methylene-bis-acrylamide were obtained from Aldrich Chemical Company (Milwaukee Wi.). Microbiological grade gelatin, nutrient agar, nutrient broth, proteose peptone, and beef extract were obtained from Difco Laboratories (Detroit, Mi.). Sephadex G-25 was obtained from Pharmacia Fine Chemicals

AB (Uppsala, Sweden). Deoxyribonuclease I was obtained from Boeringer Mannheim (Montreal, Quebec). Dupont Formula 963 aqueous scintillation cocktail was obtained from NEN Research Products (Boston, Ma.). Biogelwrap was obtained from Biodesign (N.Y., N.Y.). *Pseudomonas putida* 12633 and *P. aeruginosa* 15692 were obtained from the American Type Culture Collection. (Rockville, Md.). *P. fluorescens* 277 and *P. cepacia* 296 were obtained from Windsor Public Health Laboratories (Windsor, Ont.). Iodo-beads iodination catalyst was obtained from Pierce Chemicals (Rockford, Il.). Nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate blot qualified bovine serum albumin and alkaline phosphatase conjugated goat Anti-rabbit IgG were part of the Can/Blot Western Blotting System obtained from Bio/Can Scientific Inc. (Mississauga, Ont). Ultrapure phenol (BRL, Bethesda Md.), Fugi RX X-ray Film (Fugi Photo Film Co., Japan) and Gelman Biotrace RP charge modified nylon membrane (Gelman Sciences, Ann Arbour, Mi) were a generous gift of Dr. P.D.N. Hebert, Dept. of Biology, University of Windsor. Other common salts and reagents were obtained from Fisher Scientific Company (Fairlawn, N.J.) or BDH (Toronto, Ont.) and were of ACS or reagent grade.

Methods.

Cell Culture.

Cultures of *Pseudomonas putida* and *Pseudomonas aeruginosa* were usually rehydrated on nutrient broth (proteose peptone and beef extract) and cultures were stored on nutrient agar (nutrient broth + 2% agar) slants at 4°C. The cultures were also rehydrated using the semi-defined

mineral-salts media of Davis and Mingioli (155) which contained per litre:

K_2HPO_4	7.0 g
KH_2PO_4	3.0 g
NH_4SO_4	1.0 g
$MgSO_4.4H_2O$	0.1 g
Yeast Extract	0.2 g
Trace Solution A	1.0 ml
Trace Solution B	1.0 ml

The solutions of trace elements were prepared according to the procedure of Barnett and Ingram (156) and contained the following per 100 ml:

Trace Solution A

$FeSO_4.4H_2O$	40 mg
$MnSO_4.4H_2O$	40 mg
NaCl	1000 mg

Trace Solution B

$ZnSO_4.7H_2O$	20 mg
$CuSO_4.5H_2O$	4 mg
$CoCl_2.6H_2O$	4 mg
$CaCl_2$	500 mg
$Na_2MoO_4.2H_2O$	5 mg
KI	30 mg

Liquid media was prepared by dissolving the mineral salts in 900 ml of distilled deionized water and sterilizing by autoclaving. The carbon source (glucose, gluconate, malate, citrate or succinate) was prepared as a 2 % solution autoclaved separately and added aseptically to the mineral salts. Solid media was prepared in a similar manner except that double strength solutions of mineral salts and agar (4%) were autoclaved separately, mixed with carbon source and aliquoted into sterile test tubes. Nutrient broth and agar were prepared according to the manufacturers directions.

Cultures of *P. putida* (and *P. aeruginosa*) were routinely maintained in 125 ml flasks containing 50 ml of media with 0.2 % glucose or succinate. The cells were aseptically transferred daily by adding one inoculating loopful of culture to a fresh flask. For large scale cultures cells were grown in either the 4 L flasks containing 2 L of mineral salts media per flask or in the 12 L fermentor. The 2 L cultures were inoculated with 6-10 ml of 4-8 hour culture and incubated at 30 ^{o}C shaking at 150 rpm (Orbit Incubator Shaker Labline Instruments, Melrose Park Ill.) for 14-15 h. Flasks were used primarily to produce cells for small scale membrane preparations and uptake studies. The 12 L fermentor (Model SF-116 New Brunswick Scientific, Edison, N.J.) was used to prepare cultures for large scale membrane preparations. Mineral salts for 12 L were dissolved in 11.8 ml of distilled water and sterilized in the fermentor according to the manufacturer's directions. The carbon source (24 g) was dissolved in 150 ml of distilled deionized water, autoclaved separately and added to the fermentor while the mineral salts were still hot. The fermentor was innoculated with a 4-8 hour 50 ml culture and the

cells were incubated at 30 ^{o}C, stirring at 400 rpm, and aerated with air at 1.5 l per minute under a slight positive pressure. Normally cells were harvested after 14-15 hours of growth. At this point the cells were at the mid- to late logarithmic phase of growth with an optical density @ 620 nm of 0.9-1.1 (Spectronic 20 Spectrophotometer, Bausch and Lomb Corporation, Rochester, N.Y.). The cells were harvested by centrifugation at 4000 xg, 20^{o}C for 20 minutes (Beckman J-6 centrifuge, Beckman Instruments Inc., Palo Alto, Ca.). Typically the cell yield from a 12 L batch was 35-40 g wet weight.

Periodically the cultures were checked for purity by the following criteria. Initially the cultures were inspected microscopically at 1000 X under oil immersion after staining with the Gram stain. The cells were Gram-stained by first allowing an inoculating loopfull of cell suspension to dry on a glass microscope slide. The cells were heat fixed onto the slide by passing through a low flame 3 times and allowed to cooled. The slide was flooded with Gram crystal violet stain and allowed to sit for 1 minute. The crystal violet was washed away with Gram iodine and allowed to sit for an additional minute. The slide was washed for 15 seconds with Gram alcohol and stained with Gram saffranin for 1 minute and then washed with water. The slide was blotted dry and is ready for observation. *P. putida* are small gram negative (red-pink) rods under the microscope. The gram negative rods must also produce a fluorescent pigment. This was observed by briefly holding the culture under an ultraviolet lamp and observing a light blue pigment which is tinted green under visible light. Finally the fluorescent rods were distinguished from other fluorescent pseudomonads (i.e. *P. aeruginosa* and *P. fluorescens*) by observing growth

on nutrient gelatin (Nutrient Broth in 4 % gelatin). *P. putida* unlike other fluorescent pseudomonads does not have the ability to hydrolyse gelatin. Therefore after 24 hours of growth on nutrient gelatin (30°C), the media remains solid after cooling to 4 °C for 15-20 minutes (1,157).

Outer Membrane Preparation .

(i) Outer membranes were previously produced by D'Amore and Taylor (6) using a modification of the method of Mizuno and Kageyama (31). This preparation was used in preliminary studies to measure defluorination by these preparations. After harvesting 12 L of cell culture at 4000 x g, the pellets were washed in 500 ml of 100 mM potassium phosphate buffer pH 7.1 and 10 mM $MgSO_4$. The cells were resuspended in ice-cold 20 % sucrose (360 ml), and the following ice-cold solutions were added while stirring: 180 ml 2 M sucrose, 90 ml 100 mM $MgSO_4$, 90 ml 100 mM potassium phosphate, pH 7.1. To this 360 mg of lysozyme chloride and 576 mg dithiothreitol were added to the suspension which was incubated at 30°C for 60 min. Both 3 mg of DNA'se and RNA'se were added to the suspension after 30 min. The sphaeroplasts were removed by centrifugation at 13,000 x g and 4°C. Outer membranes were harvested at 90,000 x g, 4°C for 2 hours. The outer membranes were washed and resuspended in 100 mM potassium phosphate, pH 7.1.

(ii) Another preparation was a modification of the method of Hancock and Carey (65). After harvesting cells from a 12 L fermentor batch at 20 °C the pellet was resuspended in 500 ml 100 mM Tris-HCl, pH 7.0, and 10 mM MgSO4 and recentrifuged at 4000 x g for 20 min., 20 °C. The pellet was resuspended in the same buffer to a final volume of 120 ml and DNA'se (10

mg) and RNA'se (10 mg) were added. The cells were loaded in 3 batches into a pre-chilled (4°C) French pressure cell (American Instrument Company, Silver Spring, Md.) and each batch was pressed three times at 16,000 to 20,000 psi. The suspension changed from an light beige to dark brown during this treatment. Unbroken cells and debris were immediately removed by centrifugation (twice if required) at 4000 x g for 20 minutes at 4°C. The supernatant was carefully decanted to avoid carry over of the cell pellet. Crude membranes were harvested at 18-20,000 x g for 90 minutes at 4°C. This fraction was enriched in outer membrane. The supernatant was decanted and the crude membrane pellet was resuspended with a teflon rod in 22 ml of 20% sucrose and repeatedly homogenized with a 5 ml syringe (5.1 cm x 16 Ga needle). Six 3.5-4 ml aliquots of this suspension were layered over sucrose density gradients containing 4 ml 60 % sucrose layered over 4 ml of 70% sucrose. The gradients were prepared at room temperature, placed in a SW 41 Ti rotor (Beckman Instruments, Palo Alto, Ca.) and centrifuged at 40,000 rpm for 12-18 hours at 4°C. After density gradient centrifugation the bottom 4-4.5 ml of each gradient was removed using a 5 ml (5.1 cm x 16 Ga needle) syringe and diluted in 25 ml of distilled, deionized water. The outer membranes were harvested at 90,000 x g (TY 30 rotor, Beckman Instruments) for 2 hours at 4°C and washed twice in 75 ml distilled, deionized water. The outer membranes were resuspended in water to a protein concentration of 5-10 mg/ml. Alternatively the gradient was fractioned by puncturing the bottom of the polyallomer centrifuge tube with a 16 Ga needle and collecting dropwise fractions through the needle using a fraction collector (Instrument Specialties Company, Lincoln, Nb.). The fractions

were analyzed for protein by the Lowry method (153,154) and KDO by the method of Kharkanhis (151).

Induction/Repression Studies.

Cells were grown in 4 L of culture media with the appropriate carbon source in flasks as outlined above. The cells were washed in 175 ml 100 mM Tris-HCl, pH 7.0, with 10 mM $MgSO_4$ and resuspended in 25 ml of the same buffer containing DNA'se (3 mg) and RNA'se (3 mg). The suspension was disrupted with a French press and outer membranes were isolated as described above using two tubes of 60 to 70 % sucrose gradients per 4 L of culture media.

Protein Assay.

Proteins were assayed using the BioRad dye reagent kit which is based on the method of Bradford (152) or the Lowry method (153,154) with BSA as the standard. It should be noted that with a given sample of outer membrane or whole cell suspension the Lowry assay often yielded a protein concentration about 2.5 times higher than the Biorad kit. In addition nonionic detergents like Lubrol PX, Triton X-100 and 8-octyl glucoside strongly interfered with the BioRad Comassie Blue reagent. In the Lowry assay EDTA formed a precipitate which could be removed by brief centrifugation in an analytical centrifuge. Standard curves for the BioRad and Lowry methods are in Appendices I and II respectively.

2-Keto-3-Deoxyoctonate (KDO) Assay.

Samples (50 ul) in buffer or water were suspended in a final volume

of 1 ml of 0.18N sulfuric acid and assayed directly. Samples from sucrose density gradients were precipitated in 1 ml of 10 % Trichloroacetic acid at 4°C, vortexed and centrifuged at 15,000 x g in an Eppendorf microcentrifuge (Brinkman Instruments Company, Westbury, N.Y.). The TCA was removed with a Pasteur pipette and the pellet was washed twice with 1 ml of distilled deionized water at 4°C and resuspended in 0.18N sulfuric acid. The samples were sonicated in a bath sonicator (Fisher Scientific, Fairlawn, N.J.) for up to 30 minutes to disrupt the pellets. KDO was hydrolysed by heating for 30 minutes in polypropylene microcentrifuge tubes in a boiling water bath. To 0.5 ml aliquots of the hydrolysate, 0.25 ml of 0.04M periodic acid in 0.125N sulfuric acid was added and the mixture was incubated at room temperature for 20 minutes. After the addition of 2.6% sodium arsenite in 0.5N HCL (0.25 ml), the tubes were vortexed and allowed to sit until the brown color disapeared. When the samples were colorless 0.6 % thiobarbituric acid (0.5 ml) was added and the samples were boiled in a water bath for 15 minutes. While still hot, dimethylsulfoxide (1.0 ml) was added to each sample. The samples were allowed to cool before measurement of absorbance at 548 nm. KDO, suspended in 0.5 ml 0.18N sulfuric acid was used as the standard (Appendix III).

Enzyme Assays

Glucose and gluconate dehydrogenase were determined by the method of Jurtzhuk et al (149) using a 1 ml reaction mixture containing 50 mM Tris-HCl, pH 7.0, 1 mM $MgSO_4$, 10 mM Glucose, 0.165 mM phenazine methosulfate (PMS), 0.04 mM 2,6-dichlorophenol indophenol (DCIP), 1 mM

KCN, and 70 uM FAD. The membrane fractions of known protein concentration were added to initiate the reaction and the decrease in absorbance at 600 nm is measured. Enzyme activity was calculated as the nm of DCIP reduced per min per mg of protein. The mM extinction coefficient of 22 was obtained from Dawson *et al* (150).

SDS-PAGE Analysis

The electrophoresis method of Lugtenburg et al (147) was used to analyze the protein content of outer membrane preparations and extracts. The Lugtenberg method uses an SDS concentration two times higher than the popularly used Laemmeli gel system (148) and is commonly employed in outer membrane studies. The procedure for casting the polyacrylamide is found in Appendix (IV). The samples were prepared in a sample buffer system with or without the reducing agent dithiothreitol (DTT). "Sample buffer" is based on the formula of Lammeli (Appendix IV). A reduced sample was prepared by mixing 5-25 ul of sample with 25 ul of 1M DTT in 4 % SDS sample buffer occasionally with the addition of an extra 25 ul of 4% SDS sample buffer depending upon protein concentration. Non-reduced samples were prepared by mixing 25 ul of sample with 25-50 ul 4 % SDS sample buffer. The samples were heated to 95 ^{o}C for 20 minutes and allowed to cool before being loaded onto the gel. Normally for routine analysis of protein content an 11 % acrylamide gel was run with a 5 % stacking gel. Two types of gel were run a large "BRL-type" (Bethesda Research Laboratories, Bethesda, Md.) 16 x 16 cm large gel or the "Hoefer-type" (Hoefer Scientific Instruments, San Francisco, Ca.) 9 x 8

cm "mini"-gel. Both types of devices were manufactured in the University of Windsor, Central Research shop. The BRL-type gels were run for 4-4.5 hours and the Hoefer-type mini-gels were run for 1 hour both at 25-30 mA constant current fixed and stained overnight in a solution containing 50 % methanol, 10 % acetic acid and 2.5 % Coomassie Brilliant Blue R 250. The gels were destained in 50 % methanol and 10 % acetic acid until the backround stain was removed. For storage the gels were dried using the Biogel Wrap system (Biodesign of New York, Carmel, N.Y.). Holecular weight analysis was performed using a Hi Pad Digitizer DT-11A (Houston Instruments, Houston, Tx.) with the DNAGEL program by Tobas Kieser (John Innes Institute, Norwich, U.K.) and Peter Grewe (University of Windsor, Windsor, Ontario) or calculated from a standard curve (Appendix V) prepared on Sigmaplot version 2.03 (Jandel Scientific, Corte Madere, Ca).

DSP Cross-linking Analysis(76).

Outer membrane protein (20 ug) was incubated with 0-4000 ug of DSP per mg of protein in a microcentrifuge tube for 5 minutes at room temp. The reaction was quenched with 5 ul of 1 M Tris-HCl pH 8.5, diluted in the same volume of 4% SDS non-reducing sample buffer and heated for 10 minutes at 88°C. The entire sample volume was loaded into each well on an 11 % Lugtenberg gel with a 3% stacking gel. After running for 4.5 to 5.5 hours at 30 -25 mA. The gel was stained in Comassie Brilliant Blue stain.

Defluorination Assay.

Defluorination of 4FG by outer membrane preparations was assayed after 24 hour incubation at 30°C in sterile capped 10 ml culture tubes.

The 1 ml reaction mixture contained 400 ul outer membranes (1-2 mg/ml in distilled deionized water), 495 ul 50 mM potassium phosphate, pH 7.0, 100 ul 0.1% antibiotic (chloramphenicol, kanamycin, gentmycin or water control), and 5 ul 200 mM 4FG. Whole cell defluorination of 4FG was performed with 10 mg of cells suspended in 3 ml of 50 mM potassium phosphate buffer pH 7.1 with 1 mM 4FG and incubated stirring at 30°C. Using either method the suspensions were measured for fluoride ion release using the fluoride electrode (Orion Research, Cambridge Ma.). A calibration curve for the fluoride electrode is shown in Appendix VI.

Detergent extraction and Band E Purification.

(i) Preliminary extraction studies have been carried out in small volumes (2-4 ml) to determine the optimal detergent concentration for extraction of band E from the outer membrane preparation. Using final protein concentrations of 0.75 to 1.5 mg per ml (BioRad method), the outer membranes were extracted with 0.025 % to 2 % of ionic or non-ionic detergents which include sodium dodecyl sulfate, sodium N-laurylsarkosinate (Sarkosyl), cholic acid, deoxycholic acid, Triton X-100, Lubrol PX, Tween 20 and B-octylglucoside, each in the presence or absence of 10 mM EDTA. The extraction was carried out in 50 mM Tris-HCl, pH 7.0, with stirring or shaking at 30°C. After incubation, the outer membrane was centrifuged at 200,000 x g, for 15 minutes at 4°C the supernatant was removed and the pellet was resuspended to the incubation volume. Both pellet and supernatant were analyzed by SDS-PAGE.

ii) Band E was most selectively extracted from the outer membrane by

incubating the outer membranes (1 mg/ml protein, Lowry method) in a mixture containing 2% Lubrol PX, 10 mM EDTA and 20 mM Tris-HCl, pH 8.0, with stirring at room temperature for 30 minutes. Lubrol is a non-ionic detergent which does not absorb at 280 nm (unlike Triton-X 100) so that protein purification can be followed by measuring absorbance at this wavelength. The undissolved outer membrane pellet was removed by centrifugation at 90,000 x g for 1 h. The detergent extract was treated succesively with 30 %, 40%, and 90% saturated ammonium sulfate solution for 15-20 min at room temperature with stirring to remove contaminating proteins and the latter precipitated fraction contained primarily band E. Each precipitate was recovered by centrifugation at 15,000 xg for 20-30 minutes at 20°C when a pellet was formed on top of the subnatant. The precipitates were resuspended in a minimal volume of distilled deionized water and desalted on a (1.5 x 26 cm) column of Sephadex G-25 which was equilibrated with 0.2 % Lubrol PX, 10 mM EDTA , 20 mM Tris-HCl pH 8.0 and 50 mM NaCl (column buffer). The 280 nm absorbing fractions (3 ml) of the first peak were pooled and loaded onto a column (1.5 x 18 cm) of DEAE Sephacel equilibrated in the same buffer. The column was washed in 60-80 ml of column buffer and eluted with a 50 mM to 400 mM NaCl linear gradient in column buffer (150 ml). Dropwise fractions (3 ml) were collected and each was measured for absorbance at 280 nm. The fractions containing band E were pooled and stored at 4°C. An identical procedure was followed for the extraction and purification of protein D1 from *P. aeruginosa*.

Electrophoretic purification of D1.

The DEAE Sephacel purified protein D1 preparation contained residual contaminating bands of protein. About 750 ug of protein D1 (300 ul) was added to 100 ul of 4% SDS non-reducing sample buffer (SDS-PAGE) and heated for 20 min at 95°C. The protein was loaded in 40 ul aliquots onto an 11% Lugtenburg SDS-PAGE mini-gel (Appendix IV) and run at 27 mA for 1 hour. The gel was rinsed in deionized water and proteins were visualized in ice-cold 0.25M KCl in 1 mM dithiothreitol for 5 min (169). White bands of protein were observed and the bands corresponding to D1 were cut from the gel. The gel slices were rinsed briefly in distilled deionized water and D1 was eluted from the gel for 5 h at 10 mA using a Bio-Rad Model 442 Electroeluter (Bio-Rad, Richmond, Ca.) fitted with a 3500 Da cut-off membrane cap in SDS-PAGE tank buffer (Appendix IV). SDS was removed by dialysis against distilled water for 48 hr at 4°C.

Lipopolysaccharide Extraction.

A preparation of electrophoretically pure Band E (or protein D1 from the peak fraction of DEAE Sephacel chromatography (above) was added in 0.5 ml aliquots to microcentrifuge tubes. To each tube 0.5 ml of 90 % phenol (BRL ultrapure) was added and the samples were heated for 15 minutes at 70°C. The samples were cooled on ice for 10 - 15 minutes and centrifuged in the bottom of a Sorvall SS-34 rotor at 5000 x g for 15 min at 4° C (Ivan Sorval Inc., Newtown, Ct.). The upper aqueous layer was removed and the lower protein and phenol layers were re-extracted against an addtional 0.5 ml of water at 70°C as above. The protein-phenol layer was then extracted twice with cold (4°C) acetone (1 ml) and cold

diethyl ether (1 ml) to remove phenol from the protein. Precipitated protein was harvested after each extraction in a microcentrifuge at 15,000 x g for 15 minutes at 4°C. Sonication was used to resuspend the precipitate in distilled deionized water. A significant amount of the sample remained insoluble in water and was suspended (by sonication) before protein, or KDO determination or used for antibody preparations.

Polyclonal Antibody Preparation.

Adult Female New Zeland white rabbits were used for the preparation of polyclonal antibodies directed against purified-phenol extracted band E. For immunization band E was suspended in an equal volume of Freunds incomplete adjuvant. The mixture was suspended into a thick emulsion by vortexing. The rabbits were immunized with this suspension by injecting 0.1 ml of it into the thigh muscle with a 1 ml tuberculin syringe and a 25 Ga x 5/8 " needle. The innoculation site was cleaned with 95 % ethanol before injection. Typically the rabbits were immunized three times in two week intervals and tested for antibody production 6 weeks after the first injection (160). Blood was collected from the rabbits by bleeding from the central ear artery. The ear was shaved and the artery was dilated by stroking the skin over the artery with a cotton swab soaked in xylene. Using a 12 Ga x 3/4", 12" tubing butterfly needle the artery was punctured and blood was collected into sterile vacutainers. The blood was allowed to coagulate by sitting on ice overnight and the clot was removed by centrifugation in a Dynac II benchtop centrifuge (Becton-Dickson, Rutherford, N.J) at room temperature for 20 - 40 minutes. Serum was removed from the vacutainer and stored in a sterile polystyrene cell

culture centrifuge tube or aliquoted into 2 ml cryovials for storage at -20°C.

Ouchterlony Double Diffusion Test(158).

To determine if there was a significant titre of antibodies against band E, antisera was routinely tested using the ouchterlony double diffusion test. A molten solution of agarose (50-100 ml) was prepared by autoclaving 1% agarose in 50 mM sodium phosphate, pH 7.1, 150 mM NaCl (phosphate buffered saline, PBS) with 0.05-0.2% sodium azide. Microscope slides were overlayed with 3-4 ml of the molten solution and allowed to harden by cooling. Wells were cut into the agarose using a gel cutter (template) which produced a hexagonal pattern with a center well. Usually 10 ul of antisera was placed in the centre well and surrounding wells were loaded with various samples including purified and partially purified band E. The agarose gels were allowed to incubate at room temperature in parafilm sealed petri dishes and precipitin bands were usually observed after incubation overnight. The precipitin bands were recorded using a Polaroid MP 4 land camera (Polaroid Corporation, Cambridge, Ma.).

Western Blot Analysis for Antisera Specificity.

Samples of outer membrane and purified outer membrane protein were run on an 11% Lugtenburg (147) mini-gel at 25-30 mA for 1 hr until the tracking dye was eluted from the gel. The stacking gel was cut away and the resolving gel was equilibrated in transfer buffer (48 mM Tris, 39 mM Glycine, 0.0375 SDS, pH 9.3) for 30 min. One sheet of BioTrace RP nylon

membrane (Gelman Sciences Inc., Ann Arbour, Mi.) and 4 sheets of Whatman 3MM chromatography paper were soaked for 30 min. or more in transfer buffer and placed in a Bio-Rad Transblot-SD Semi-Dry Electrophoretic Transfer Cell according to the manufacturer's directions (Bio-Rad, Richmond, Ca.) with the gel and the transfer was allowed to run at 15 V for 45 minutes. The membrane was rinsed in 25 ml TBST (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% Tween) and then incubated shaking in 25 ml 2% blot quality BSA in TBST for 80 min. The membrane was transfered to 25 ml TBST containing an appropriate dilution (1:500 to 1:1000) of the test antiserum and incubated with shaking for 30 min at room temperature. The membrane was washed 3 times in 50 ml TBST for at least 5 minutes. The alkaline phosphatase conjugated goat anti-rabbit antisera was added to 25 ml of TBST and incubated with the membrane for 30 min. The membrane was washed 3 times with TBST as above and incubated with substrate solution containing 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$ 165 ul NBT and 82.5 ul BCIP in a final volume of 25 ml. The membrane was incubated stirring until an adequate color was observed on the membrane and then the reaction was stopped by rinsing the membrane in distilled deionized water. The membranes were dried for storage in Biogelwrap.

Circular Dichroism Spectroscopy.

A circular dichroism spectrum was determined in the Department of Biochemistry, University of Toronto, with the help of Joe Casey the laboratory of Dr. Reinhart Reithmeier. The spectra were measured on a JASCO J-41A Spectropolarimeter. A 0.1 cm path length quartz cuvette contained 0.75 ug/ml purified band E in 0.06 % Lubrol PX, 6.3 mM Tris-HCl

pH 8.0 and 31 mM NaCl. The spectra were measured from 260 to 200 nm.

Cell Surface Iodination with ^{125}I.

Proteins expressed at the outer surface of the cell were determined by labelling with ^{125}I by the method of Richardson and Parker (37). Whole cells of *P. putida* (both succinate and glucose grown) were harvested as previously described, washed twice in 50 mM PBS with 10 mM $MgSO_4$ at 20°C and resuspended to 0.2 g/ml wet weight. Iodo-beads were washed twice in PBS (0.5 ml per bead) and allowed to dry on a piece of Whatman # 54 filter paper. One bead was placed per microcentrifuge tube and 100 ul of PBS was added to each with a 5 ul aliquot of a $Na^{125}I$ (specific activity 2.7 mCi/100 ul) working stock. The solution was incubated at room temperature for 5 min. Iodination was initiated by adding 100 ul of the above cell suspensions to the reaction vial and incubating at room temperature for 10 minutes. The reaction was terminated by removing the suspension from the vial with a pasteur pipette into a centrifuge tube containing 20 ml of PBS containing 1 mg/ml NaI. The suspension was centrifuged at 10,000 x g for 20 min, the supernatant was discarded and the pellet was washed by suspending four more times with the above buffer and centrifuging to remove unbound ^{125}I and washed once in 0.25 M Tris-HCl, pH 6.8. The labelled cells were treated with reducing or non-reducing SDS-PAGE sample buffer (Appendix IV) and 50,000 to 100,000 cpm of sample was applied per well and run by the Lugtenburg (147) procedure as described above. When electrophoresis was completed the gel was placed on Whatman 3 MM filter paper and dried in a BioRad Model 483 slab gel drier (BioRad, Richmond, Ca.) in vaccuo for 3 hours at 80°C. The dried

gel was placed in an autoradiography cassette (Cronex Cassette with HiPlus intensifying screens, Dupont Canada, Mississauga, Ont.). The gel was covered with Fugi RX X-ray film (Fugi Photo Film Co., Japan), incubated at -70°C and developed after 24-72 hr exposure.

Glucose Binding Studies.

i) Equilibrium Dialysis: Binding of D-glucose to outer membranes and purified band E was measured using the equilibrium dialysis method of Stinson et al (20,21). Dialysis bags (0.8 cm dia. x 8 cm , Sigma Chemical Co. St. Louis, Mo.) were filled with 0.3 ml of an outer membrane preparation or outer membrane protein solution of known concentration. The bag was sealed with dialysis bag clips (Spectrum Medical Products Los Angeles, Ca.) and placed in a beaker with 5 ml of 10 mM Tris-HCl pH 7.5 with 1 mM $MgCl_2$ (TM buffer) per bag. An aliquot of D-[6-^{3}H]-glucose (30 Ci/mmole) was added to a final conc of 0.1 - 10 uM and the bag was allowed to incubate at 4°C for 48 hr. When incubation was complete the bag was removed from the buffer, rinsed very quickly with distilled water and blotted onto Whatman #1 filter paper. Aliquots (0.1 ml) were removed from inside and outside the bag and counted for radiolabel by scintillation counting. Binding occured when radiolabel inside the bag was greater that that outside the bag. Equilibration of labelled glucose is tested by using a bag filled with 0.3 ml TM buffer ensuring that the radiochemical concentration was the same inside and outside the bag at the end of the incubation period.

ii) Ultracentrifugation Assay: Binding to the particulate outer membrane was determined by suspending outer membranes in 100 mM Tris-HCl, pH 7.0,

to a known protein concentration with D-[6-^{3}H]-glucose (100,000 cpm/ml) to a final volume of 2 ml. Usually 2-3 100 ul aliquots of the suspension was removed and counted to determine the "Total" glucose concentration. The outer membrane suspension was centrifuged at 200,000 x g for 30 minutes, 20^{o}C, to remove the bound glucose. Again 2-3 100ul aliquots were removed and counted to determine the "Free" glucose concentration. Bound glucose was calculate using Bound = Total - Free.

Liquid Scintillation Counting.

For the radiochemical experiments described previously, the appropriate sample (on a membrane filter or in solution) was added to a 20 ml polypropyline scintillation vial (Fisher Scientific Co., Fairlawn, N.J.) containing 10 ml of Dupont Formula 963 scintillation cocktail. The vials were shaken and placed in a Beckman LS-7500 liquid scintillation counter (Beckman Instruments, Palo Alto, Ca.) to count for a pre-programmed time (usually 1-10 min).

PicoTag Amino Acid Analysis.

A 1.5 nmole (Lowry protein assay - 45,000 mw) aliquot of phenol extracted, purified band E was added to a small sample tube. The tube was placed in a larger reaction vial containing 200 ul of a 6N HCl solution with 1 % phenol (Pierce Chemicals, Rockford, Il.). The reaction vial was evacuated and purged thrice with nitrogen and the protein was then hydrolysed under a slight vacuum at 105^{o}C for 24 hours. After cooling HCl was removed in vacuo. The samples were redried (to adjust pH) under vacuum after the addition of 20 ul of ethanol:water:triethylamine

(2:1:1). The amino acids were derivitized by adding 20 ul of a fresh solution of methanol:triethylamine:water:phenylisothiocyanate (PITC) 7:1:1:1 to the redried samples. The suspensions were allowed to stand at room temperature and the mixture was dried under vacuum. The samples were resuspended in 100 ul of sample diluent (710 ul sodium phosphate, pH 7.40 with 5 % acetonitrile). The derivitized amino acid sample was filtered through a 0.45 um Millipore filter (Millipore Products Division, Bedford, Ma.) and 15 - 20 ul was injected onto a Pico-Tag C_{18} HPLC column (3.9 cm x 15 cm, Waters part # 88131). The derivatives were eluted on a 10 minute Waters #5 gradient of 94% A, 0% B, and 6% C to 56% A, 16% B, and 28 % C and the 3 minutes of 0% A, 4% B, and 60 % C (where A = 19 g $CH_3COONa.3H_2O$ + 0.5 ul triethylamine per litre pH 6.40; B = H_2O and C = Acetonitrile. The derivatives were detected by UV absorbance at 254 nm (Model 441 absorbance detector, Waters Associates Inc., Milford, Ma.) and quanified on a Waters 745 Data Module (Waters Associates Inc., Milford, Ma.).

RESULTS AND DISCUSSION

The present research was initiated as a follow up of the results published by D'Amore and Taylor (6) which suggested that the outer membrane of *P. putida* was the cell fraction responsible for fluoride release from 4-deoxy-4-fluoro-glucose (4FG) upon incubation for 24 hours at 30 °C. These studies demonstrated that defluorination was induced in outer membranes from glucose grown cells and repressed in outer membranes from succinate grown cells. Preliminary studies were performed in order to reproduce the fluoride release from 4FG with the outer membrane and to determine if a glucose inducible-succinate repressible protein was responsible for this activity. In co-operation with D. Sbrissa outer membrane fractions were prepared by the lysozyme-digestion procedure of Mizuno and Kageyama (31) as modified by D'Amore and Taylor (6). Both the yields of outer membrane protein and defluorinating activities of these outer membrane preparations were lower than reported by D'Amore (Table 4). Although the extent of dehydrogenase contamination from the cytoplasmic membrane was similar to that of D'Amore's preparations, in our hands the outer membrane prepared by the lysozyme digestion procedure could be reproduced qualitatively if not quantitatively.

It was decided that the lysozyme preparation was inherently inefficient because the outer membrane of pseudomonad species is reported to have a permeability limit for molecules of 200-6000 daltons (72,104). At a molecular weight of 14,300 daltons, lysozyme would cross the outer membrane to hydrolyze the cell wall only very slowly. It should be noted that Mizuno and Kageyama (31) used EDTA in their sphaeroplast preparation

Table 4.

Comparison of Outer Membrane Preparations of *P. putida* and 4FG Defluorinating Activity.

Preparation	Outer Membrane Protein Yield (mg) per L of Cell Culture	Defluorination Activity (nmole F^- released per mg protein)	Glucose Oxidase Activity (nmole Glucose oxidized per mg protein)
Sphaeroplast Preparation			
D'Amore (6)	1.3	352	4
This study[a]	0.3-1.1	55-253	5.3
French Press			
This Study	15.8	97	17.1

[a] includes preliminary studies performed with D. Sbrissa.
4FG defluorination and glucose oxidase assays were performed as described in Materials and Methods.

to permeabilize the outer membrane to lysozyme. This was not used in D'Amore's preparation (6) and may have contributed to the low yield. In addition, the limited yield observed by D'Amore (16 mg per 12 L cell culture, up to 13 mg per 12 L in our hands) made large scale incubations with outer membranes produced by this membrane unfeasible.

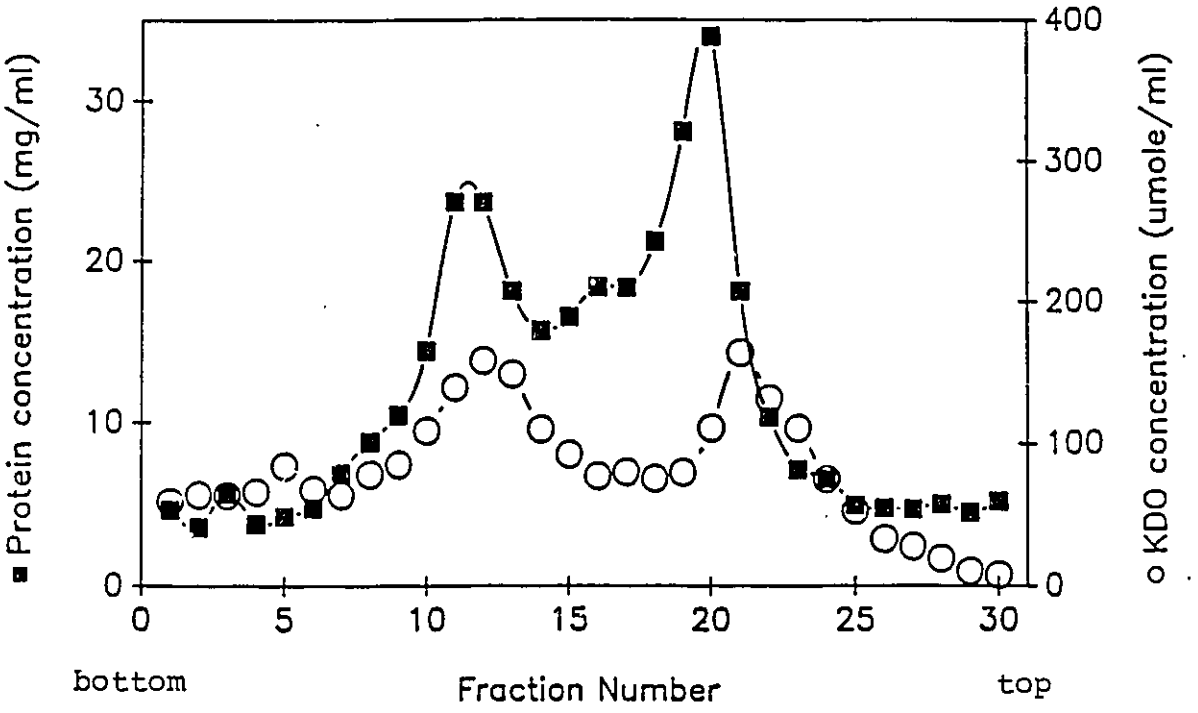
The French press-density gradient preparation was a more efficient method of providing outer membrane in good yields. For the preparation of outer membrane from *P. putida* the method of Hancock and Carey (65) was modified slightly. The crude cell envelope harvested from the French pressed cells was enriched in outer membrane by centrifugation at 20,000 x g. This step reduced the amount of cytoplasmic membrane which was loaded onto the sucrose density gradient and prevented the formation of a cytoplasmic membrane pellet over the 61 % sucrose layer. This made possible fractionation of the density gradient by collecting dropwise fractions through a needle puncturing through the bottom of the centrifuge tube. Since lysozyme was not used in this preparation the resulting outer membranes contained peptidoglycan.

A remarkable increase in the outer membrane yield was noted with the French Press-Density gradient preparation. From a typical gradient preparation (Table 4) about 35 g wet weight of *P. putida* whole cells were disrupted and the yield of outer membrane ranged from 108 mg to 291 mg of protein (Lowry assay, bovine serum albumen standard) per 12 L cell culture. The outer membrane yield depended primarily on the yield from the cell culture. This represents a 12 fold increase in yield over the lysozyme digestion preparation of D'Amore (6). The increased yields made large scale preparations practical for defluorination studies.

While the outer membranes of *P. aeruginosa* prepared on the sucrose-density gradient have been characterized often in the literature, in only a few instances were preparations of *P. putida* outer membrane described (9,32,95,121). In such cases, these descriptions only highlighted a particular protein (ie. porins F and P) which was common amongst several pseudomonad species (95,121). In figure 9 the distribution of protein and 2-keto-3-deoxy-octulosonic acid (KDO) throughout the 20%-61%-70% sucrose density gradient, is illustrated. The two peaks of protein and KDO correspond to bands of membrane material visible at the 61%-70% and 20%-61% sucrose interfaces of the sucrose density gradient. In this case KDO is used as a marker for the lipopolysaccharide in outer membrane material. An SDS-polyacrylamide gel was run on fractions throughout the sucrose density gradient. A pattern of 9 major bands were observed in fractions throughout the gradient (Figure 10). This relatively small number of protein bands is observed primarily in the fractions from the highest density. This is typical in outer membranes from *P. aeruginosa* and other gram negative bacteria (30,31,32,34,163). The peak intensity of these bands corresponds to the first peak of protein and KDO assayed from the gradient (Figure 9) in the location of the 61%-70% sucrose interface. Because of the characteristic brown color in the gradient and large number of proteins observed on SDS-PAGE the 2nd peak of protein and KDO corresponds to the cytoplasmic membrane fraction. This less dense fraction contains contaminating lipopolysaccharide and outer membrane presumably due to membrane mixing during French pressing. The cytoplasmic membrane fraction in this preparation had a glucose dehydrogenase activity 2-4 times higher that of the peak outer membrane fraction (data

Figure 9

Distribution of Protein and KDO From *P. putida* Cell Envelope Through the 61%-70% Sucrose Density Gradient.



The gradient was prepared as outlined in Materials and Methods. The open circles indicate protein concentration (Lowry assay) in mg/ml and the closed squares indicate KDO concentration in micromoles/ml.

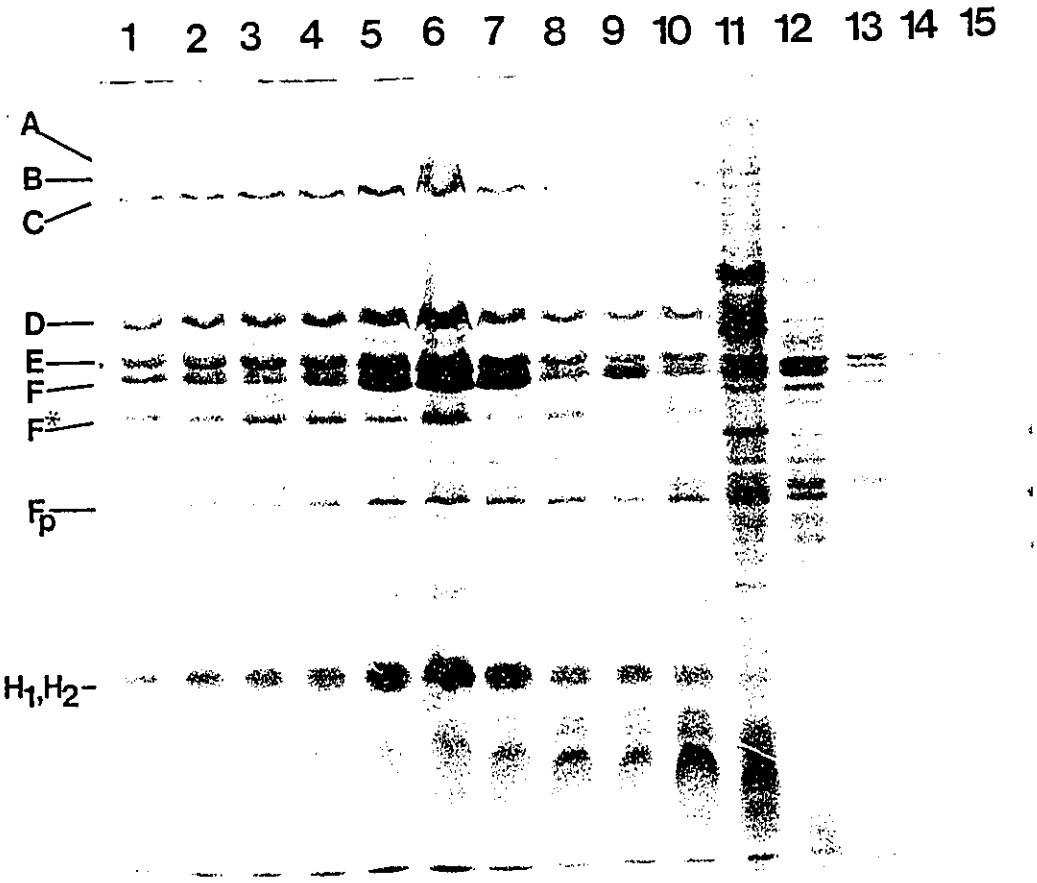
Figure 10

SDS-PAGE of Fractions from the 61%-70% Sucrose Density Gradient Preparation of Outer Membrane from Glucose Grown P. putida Cell Envelope.

Samples of odd numbered gradient fractions were diluted 1:5 in reducing sample buffer, treated as described in Materials and Methods and aliquots (10 ul) were added to each lane as indicated below.

Lane 1: Fraction 1.
Lane 2: Fraction 3.
Lane 3: Fraction 5.
Lane 4: Fraction 7.
Lane 5: Fraction 9.
Lane 6: Fraction 11.
Lane 7: Fraction 13.
Lane 8: Fraction 15.
Lane 9: Fraction 17.
Lane 10: Fraction 19.
Lane 11: Fraction 21.
Lane 12: Fraction 23.
Lane 13: Fraction 25.
Lane 14: Fraction 27.
Lane 15: Fraction 29.

Figure 10



not shown).

For the purpose of discussion and comparison with other species , a nomenclature for the outer membrane proteins of *P. putida* on SDS-PAGE is being proposed here. This nomenclature is based on that presently used for *P. aeruginosa* (31,65) but does not necessarily correspond to proteins with similar characteristics and/or molecular weights shared by both species. The accepted nomenclature of certain *P. aeruginosa* outer membrane proteins (31,65) is shown in Figure 11. The assignment of the outer membrane proteins for *P. putida* for the purpose of this report are shown in Figure 10 and 11. The molecular weights of the *P. putida* proteins assigned in Figure 10 and 11 were calculated from standard proteins (Appendix V) on SDS-PAGE and are listed in Table 5.

The outer membrane proteins of *P. aeruginosa* have been characterized by their modified mobility on SDS-PAGE when treated with heat and reducing agents (31,65). Band F in the outer membrane of *P. putida* is very similar to the band F or porin F from the outer membrane of *P. aeruginosa*. Both proteins demonstrate decreased mobility to a higher molecular weight form in the presence of 2-mercaptoethanol or dithiothreitol. In the absence of reducing agent, porin F from *P. aeruginosa* runs as a 33 kDa band (Figure 11, lane 4)(65). In *P. putida* up to two bands are observed for band F when the outer membrane is not treated with reducing agent (Figure 10; Figure 11, lane 3). We have given the nomenclature F for the reduced form of band F in *P. putida* with apparent Mw 40.6 kDa (Figure 10; Figure 11, lane 1), F* for the first non-reduced band of apparent Mw 37.5 kDa and F** for the second non-reduced and highest mobility band of apparent Mw 34 kDa (Figure 11, lane

Figure 11

SDS-PAGE of Thiol-Modifiable Proteins in Outer Membrane Preparations from Various Pseudomonad Species

Lane 1: Dithiothreitol reduced *P. putida* outer membrane
Lane 2: Dithiothreitol reduced *P. aeruginosa* outer membrane
Lane 3: Non-reduced *P. putida* outer membrane
Lane 4: Non-reduced *P. aeruginosa* outer membrane
Lane 5: Dithiothreitol reduced *P. fluorescens* outer membrane
Lane 6: Dithiothreitol reduced *P. cepacia* outer membrane
Lane 7: Non-reduced *P. fluorescens* outer membrane
Lane 8: Non-reduced *P. cepacia* outer membrane

Arrows indicate the thiol-modifiable protein in each preparation.

Figure 11

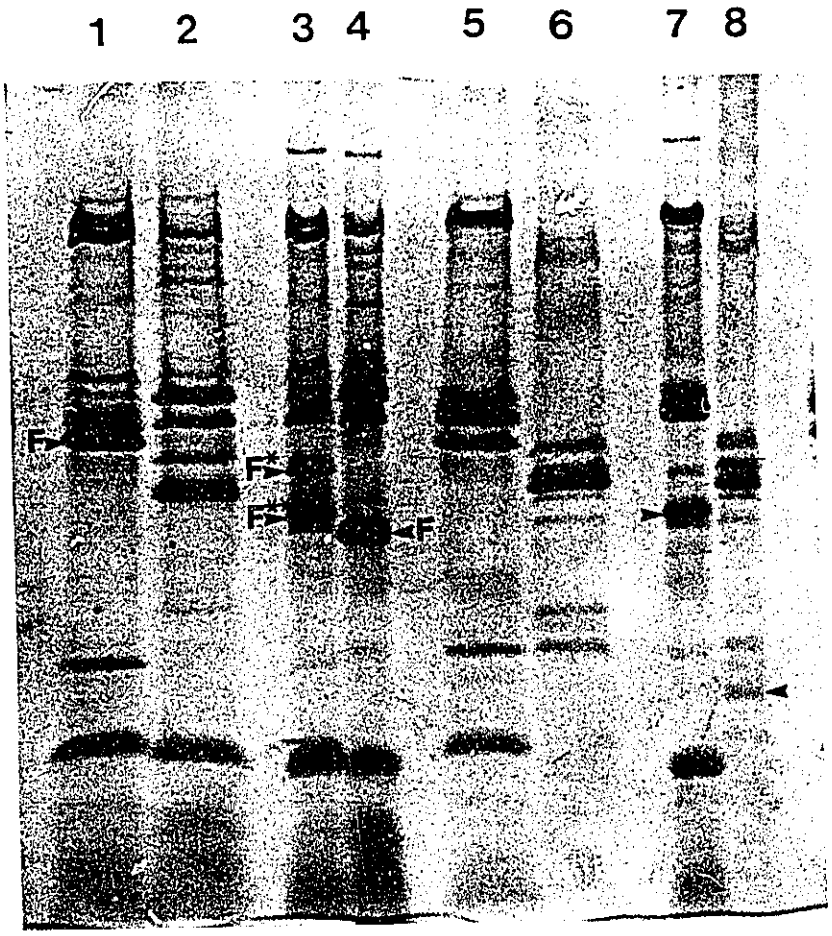


Table 5

Nomenclature and Molecular Weight of P.putida
Major Outer Membrane Proteins.

Protein Band	Molecular Weight (KDa)		
	M.W	+/-	(n)
A	106	1.19	(62)
B_1	96.7	1.05	(48)
B_2	93.6	0.77	(48)
B	93.0	1.64	(21)
C	85.9	0.62	(86)
D	49.3	0.31	(92)
E	42.9	0.31	(67)
F	40.6	0.24	(80)
H_1, H_2	18.46	0.25	(40)
A^*	124	1.53	(11)
F^*	37.5	0.39	(13)
F^{**}	34	0.20	(4)

Molecular weight determination by digitization of dried gels as described in Appendix IV. M.W. - molecular weight in kilodaltons, +/- 95.5 % or 2o confidence limit, n - number of measurements.

3). This is in contradiction to the nomenclature used by Hancock and Carey (65) who used F for the high mobility band and F* for the reduced form in *P. aeruginosa*. We have decided that the most denatured form of the protein should carry the root nomenclature (i.e. F). Similar thiol modifiable proteins can be observed in outer membranes of *P. fluorescens* and *P.cepacia* (Figure 11). Thiol-modifiable proteins have also been reported in *P. cepacia* (163) (Figure 11) and *P. syringae* (164).

Hancock and Cary (65) postulated that the altered mobility of the porin F is caused by intramolecular disulfide bonds. Thus when the protein is reduced, the cleavage of the disulfide bonds allows the conformation of the protein to open and have increased frictional drag and hence decreased mobility by electrophoresis. The sequence data for porin F from *P. aeruginosa* has identified 4 cysteine residues which are likely candidates for the formation of such disulfide bonds (92). The highest mobility form of band F in *P. putida*, F**, appears to result from some conformational, and not disulfide stabilized structure as the addition of DMSO to the sample buffer causes a change from F** to F* without the addition of reducing agent. (not shown) The relationship between porin F of *P. aeruginosa* and band F of *P. putida* was recently demonstrated by Mutharia and Hancock (94-96) who developed monoclonal antibodies to the porin F of *P. aeruginosa*. These antibodies were shown by western blotting to bind to a 39,000 Dalton outer membrane protein of *P. putida* and to a common 31,000 Dalton proteolytic fragment (formed during outer membrane preparation) of protein F found in both species (96). This protein (Fp) may be present in the outer membrane preparations in Figures 10 and 12.

Pseudomonad outer membrane proteins are also characterized by induction and repression with carbon sources and limiting ions. Outer membranes of *P. putida* were prepared from cells grown on a variety of carbohydrates and organic acids. As shown in Figure 12, band E of molecular weight 42.8 kDa is repressed by growth on organic acids such as the citric acid cycle intermediates succinate, citrate and malate and by gluconate which is central to carbohydrate metabolism (134). This protein is induced by growth on glucose and non-metabolizable carbon sources such as maltose and lactose. With the latter two carbon sources growth was presumably supported by yeast extract in the media. The conditions for induction and repression of band E are summarized in Table 6. A similar protein D1 has been isolated and characterized from the outer membrane of *P. aeruginosa* (78).

With this identification of a glucose inducible/succinate repressible outer membrane protein it was now possible to establish whether this protein was directly responsible for defluorination of 4FG. In addition, the larger quantities (150-300 mg outer membrane protein/ 12 L of cell culture) of outer membrane made available allowed the possibility of using the preparation to isolate the defluorinated product. Studies were initiated to test the 4FG defluorinating activity of the outer membranes isolated on the density gradient. Microscopic examination of Gram stained preparations established that fresh outer membrane fraction were essentially free from contamination by whole unbroken cells. The fraction defluorinated 4FG significantly (Table 4) but the specific activity varied considerably between preparations and was usually much lower than that reported by D'Amore. After 24 hour

Figure 12

SDS-PAGE of Outer Membranes Prepared from P. putida Grown On Various Carbon Sources.

Reduced samples were prepared as described in Materials and Methods.

Lane 1: Outer membrane from succinate grown cells (succinate innoculum)
Lane 2: Outer membrane from malate grown cells (succinate innoculum)
Lane 3: Outer membrane from citrate grown cells (succinate innoculum)
Lane 4: Outer membrane from glucose grown cells (glucose innoculum)
Lane 5: Lysozyme prepared Outer membrane from glucose grown cells (glucose innoculum)
Lane 6: Outer membrane from succinate grown cells (glucose innoculum)
Lane 7: Outer membrane from malate grown cells (glucose innoculum)
Lane 8: Outer membrane from citrate grown cells (glucose innoculum)
Lane 9: Outer membrane from proteose peptone grown cells (glucose innoculum)
Lane 10: Outer membrane from glucose grown cells (glucose innoculum)
Lane 11: Outer membrane from gluconate grown cells (glucose innoculum)
Lane 12: Outer membrane from lactose grown cells (glucose innoculum)
Lane 13: Outer membrane from maltose grown cells (glucose innoculum)

Figure 12

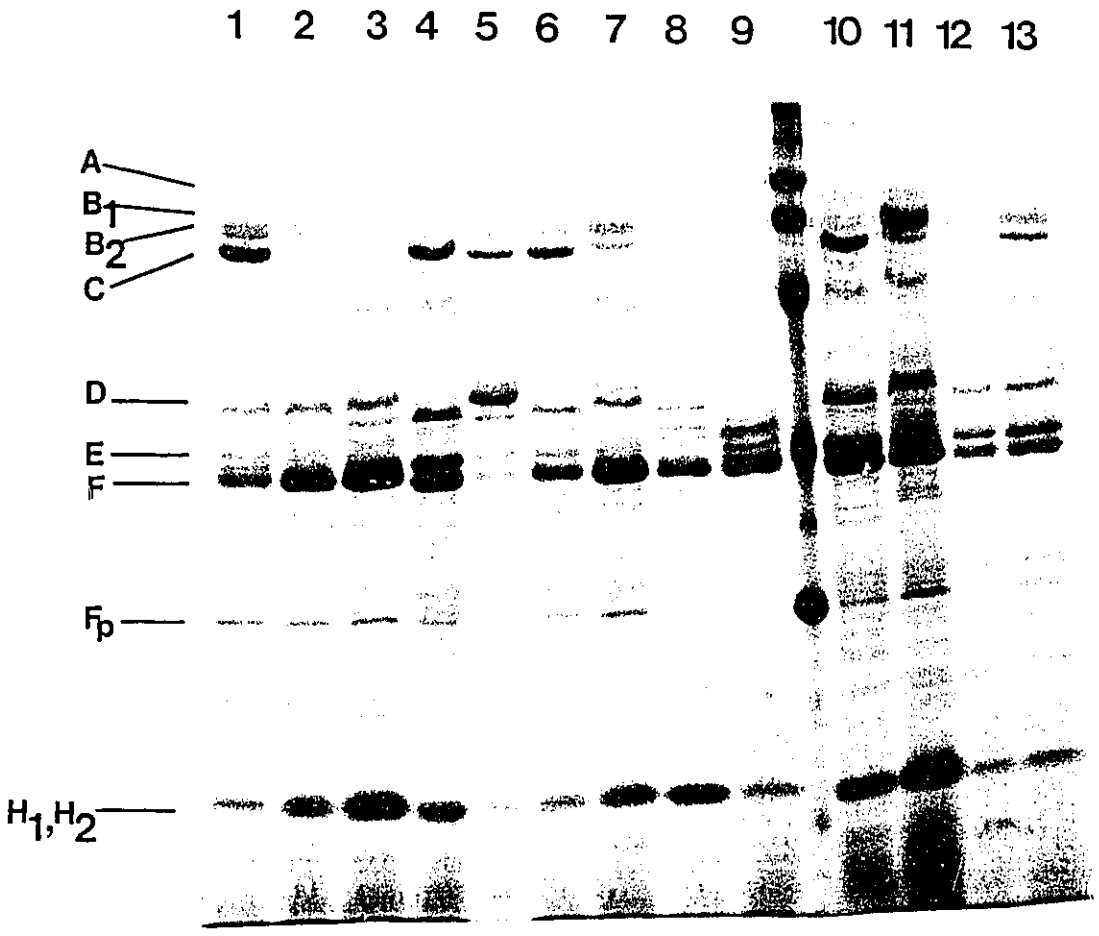


Table 6

Induction (+) or Repression (-) of Band E by Growth of *P. putida* on Various Carbon Sources.

Carbon Source	Glucose Innoculum	Succinate Innoculum
Glucose	+ (4,10)	+/-
Lactose	+ (12)	nd
Maltose	+ (13)	nd
Gluconate	- (11)	nd
Citrate	- (8)	- (3)
Succinate	- (6)	- (1)
Malate	- (7)	- (2)
Proteose peptone	+/- (9)	nd
Glucose + Succinate	-	nd

(+) Band E induced, (-) Band E repressed, nd - not determined, numbers in parentheses refer to respective lane in figure 12.

incubation with 4FG the outer membrane suspension was examined microscopically with the Gram stain to determine if microscopic growth had occurred. The preparation was highly contaminated with cells which included both gram negative and gram positive cells. This was very surprising since precautions such as sterile buffer, glassware and filter sterilized 4FG were employed.

A number of protein synthesis inhibitors were chosen to prohibit cell growth but have minimal effect on the membrane, these included chloramphenicol (which was shown by D'Amore to inhibit defluorintion in whole cells), kanamycin and gentamycin. These antibiotics were included in the defluorination assays to determine their effect on fluoride release and cellular contamination. Each antibiotic eliminated or drastically reduced the cellular contamination (not shown) in the outer membrane suspensions after incubations of 24-36 h and as summarized in Table 7, inhibited defluorination concomitantly in both density gradient and lyzozyme digestion prepared outer membranes. The inhibition of F- release with protein synthesis inhibitors suggested that defluorination was caused by whole cell contamination of the outer membrane preparation. Succinate was added to test this hypothesis since it is an repressor of glucose metabolism. Succinate also resulted in significant inhibition of defluorination in the contaminated outer membrane preparations (Table 7) further suggesting that metabolically active whole cells were responsible for the fluoride ion release.

Finally, defluorination of 4FG in whole cells of *P. putida* has been shown by D'Amore (164) and others (165) in this laboratory to be induced not only on glucose, but on gluconate and 2-ketogluconate. The rate and

Table 7

Effect of Various Compounds on the Defluorination of 4FG by Outer Membrane Preparations of P. putida.

Compound		% Inhibition of Defluorination
Sucrose Density Gradient Prep.		
Chloramphenicol	(0.01%)	100
	(0.05%)	100
Gentamycin	(0.01%)	80
	(0.05%)	83.5
Kanamycin	(0.01%)	60
	(0.05%)	82
Succinate	(1.0%)	44
Sphaeroplast Prep.		
Chloramphenicol	(0.01%)	75

4FG defluorination activity deterined as described in Materials and Methods.

extent of defluorination by gluconate grown cells was almost identical to that of glucose grown cells (164,165). However, growth on gluconate represses the glucose inducible outer membrane protein in *P. putida* (Figure 10) and in *P. aeruginosa* (78). This demonstrates that Band E alone is not responsible for the defluorinating activity observed in whole cells. These results taken together indicate that 4FG defluorinating activity is not located in the outer membrane of *P. putida*.

Hancock and Carey (78) reported that protein D1 in *P. aeruginosa* was induced under conditions where high affinity glucose uptake (Km 7uM) and the periplasmic glucose binding protein were induced. They suggested that D1 may be involved in the high affinity uptake of glucose. Presumably, binding of glucose to this protein would be the first step in the uptake process. Purified, reconstituted D1 does not significantly bind glucose but does form glucose permeable pores which appear to be selective for D-glucose (81,125). Direct binding studies for maltoporin in *E. coli* have also failed to demonstrate specific binding of maltose (167). In preliminary studies we have attempted to determine if glucose binding could occur in isolated outer membranes from glucose grown *P. putida*. To do this both a dialysis technique and an ultracentrifugation technique were attempted (Materials and Methods). ^{3}H-Glucose binding was not observed by either technique (data not shown).

If band E was involved in the glucose uptake process it would be expressed, at least partially on the surface of the cell. If that were the case, then some sort of affinity probe or label for glucose binding on the exterior of the cell might be employed to inhibit glucose binding

or uptake. Surface expression of the glucose inducible outer membrane protein was tested by labeling cell surface proteins with I^{125} using the surface labeling agent, Pierce Iodobeads (Materials and Methods) and observing the labeled proteins by SDS-PAGE followed by autoradiography. The labeled cell surface proteins are shown in Figure 13 (lanes 1 and 4). The position of I^{125} labelled band E is marked by a sample of purified, labeled band E run in an adjacent lane. The autoradiograph shows that the major outer membrane proteins (cf. Figures 10-12) including glucose inducible band E and thiol modifiable band F are expressed at the cell surface. Surface expression of the major outer membrane proteins of *P. aeruginosa* by surface iodination was reported by Sokol and Woods (173).

Since the glucose inducible protein is surface expressed, it should be physically possible for a non-permeant affinity probe to bind to the protein on the surface of the intact cell. For this reason we decided to use polyclonal antibodies to probe band E and its possible requirement in glucose uptake. Antibodies because of their size are impermeant and may be large enough to hinder the passage of the glucose through the protein pore. The use of antibodies to establish a role for an outer membrane protein in solute uptake was demonstrated by Sokol and Woods (168) who were able to inhibit both binding and uptake of ^{59}Fe-Ferripyochellin in *P. aeruginosa* and by Benz et al (126) who used specific polyclonal antisera to inhibit LamB mediated black lipid membrane conductance. In order to produce antibodies against band E it would have to be first purified from the outer membrane. Hancock and Carey (78) described a method for the purification of D1 in *P. aeruginosa* where Triton X-100-EDTA extracted D1 was purified by DEAE Sephadex ion-exchange

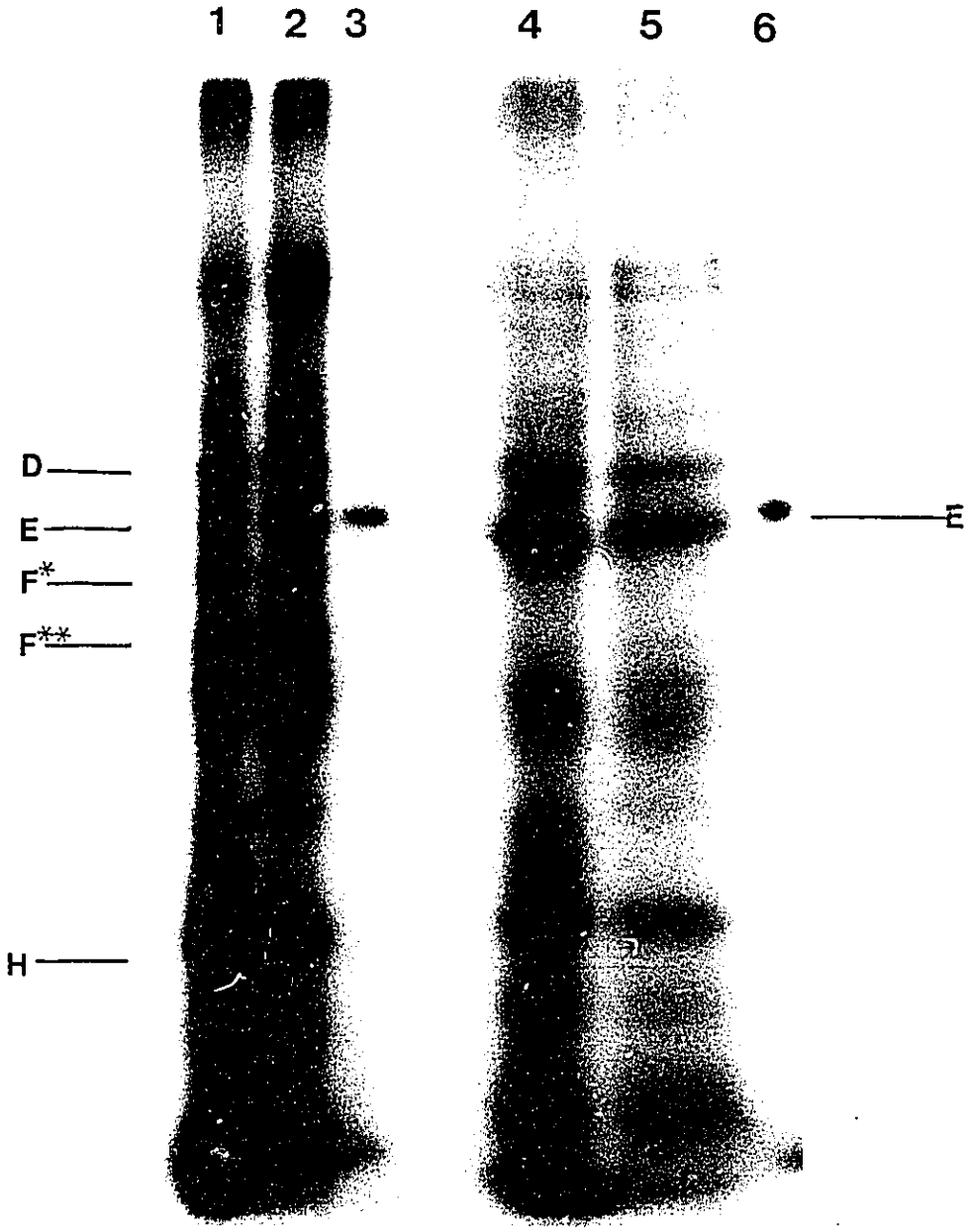
Figure 13

SDS-PAGE Autoradiography of ^{125}I-Surface Labelled Proteins from Whole Cells of *P. Putida*

Whole cells and purified band E were labelled as described in Materials and Methods.

Lane 1: Non-reduced succinate grown *P. putida*
Lane 2: Non-reduced glucose grown *P. putida*
Lane 3: Non- reduced band E
Lane 4: Dithiothreitol treated succinate grown *P. putida*
Lane 5: Dithiothreitol treated glucose grown *P. putida*
Lane 6: Non-reduced band E

Figure 13



chromatography. However Triton X-100 contains an aromatic ring which absorbs strongly at 280 nm, a wavelength very convenient to test for the presence of protein, especially during elution from chromatography columns.

A survey of the ability of a number of ionic and non-ionic detergents to extract band E from isolated outer membranes was performed and the extraction of band E was determined by inspection of electrophoretic gels. The results of the extraction survey are summarized in Table 8. Ionic detergents such as sodium dodecylsulfate, and sodium N-laurylsarkosinate (sarkosyl) were capable of extracting band E (and other proteins) from the outer membrane effectively without the use of EDTA. However, the use of these ionic detergents would add excess negative charges to the protein which would preclude further purification of band E by the use of ion exchange chromatography. Of the ionic detergents surveyed, sarkosyl was the most selective for band E extraction. Even so, we were unsuccessful in using gel filtration (75) to purify sarkosyl extracted band E as it was particularly difficult to resolve band E from contaminating band F. Typically, most proteins were not significantly extracted from the outer membrane with non-ionic detergents in he absence of EDTA. Also, incubation with EDTA alone did not result in the loss of protein from the outer membrane. Usually, extensive extraction of several proteins was observed when high concentrations of detergent (ionic or non-ionic) were combined with EDTA (Table 8, Figure 14). This observation is consistent with results obtained by Hancock and others (26,58,59) who suggested that divalent cations Ca^{++} and Mg^{++} link the negative charges on LPS to confer stability to the outer membrane.

Table 8

Band E Detergent Extraction Studies

Detergent	Detergent Concentration				
	2%	1%	0.5%	0.25%	0.1%
SDS	+	+	+	+	-
Sarkosyl	+	+	+	+	-
Cholate	-	-	-	n.d.	n.d.
Cholate + 5 mM EDTA	-	-	-	"	"
Deoxycholate	+/-	-	-	"	"
Deoxycholate + 5 mM EDTA	+	+	+/-	"	"
Triton X-100	-	-	-	"	"
Triton X-100 + 5 mM EDTA	+	+	+	"	"
Tween 20	-	-	-	"	"
Tween 20 + 5 mM EDTA	+/-	+/-	+/-	"	"
Lubrol PX	-	-	-	"	"
Lubrol PX + 5 mM EDTA	+	+	+/-	"	"
B-octylglucoside	+	+	-	"	"
B-octylglucoside + 5 mM EDTA	+	+	+	n.d.	n.d.

(+) Band E extracted, (-) Band E not extracted, (+/-) partially extracted, n.d. indicates not determined. Detergent extraction performed as described in Materials and Methods.

The most selective of the non-ionic, non-aromatic detergents for the extraction of band E was Lubrol PX at a concentration of 2 % in the presence of 10 mM EDTA. This detergent is a mixture of fatty alcohol condensates of polyoxyethylene which has the advantage of not absorbing at 280 mm but the disadvantage of being poorly chemically and physically defined. The extraction and purification of band E from the outer membrane of *P. putida* is shown in Figure 14. In addition to the extraction of band E virtually all of the lipopolysaccharide (as KDO) was extracted from the outer membrane.

The 2% lubrol-10 mM EDTA extract contained small amounts of band B, D, F and H1. Yoshimura *et al* (78) used ammonium sulfate precipitation to remove residual contaminants from a porin F preparation and so ammonium sulfate precipitation was used to remove residual contaminating proteins in the lubrol-EDTA extract. The extract was precipitated with 30%, 45% and 90% ammonium sulfate and the residual salt was removed from the precipitated protein by elution through a Sephadex G-25 column. The 90% precipitate contained the purest fraction of band E (Figure 14, lane 6) and was only contaminated slightly with H1. This fraction was applied to a DEAE Sephacel column equilibrated with 0.2 % lubrol-EDTA containing 20 mM Tris and 50 mM NaCl. Washing the column with this buffer removed the residual H1 (Figure 14, lane 7). Pure band E (lane 8) could be eluted using a 50-400 mM linear gradient of NaCl (Figure 15). The same procedure was used to isolate protein D1 from the outer membrane of *P. aeruginosa*. In this case, contaminating bands C and G persisted throughout the purification (Figure 16 and 17).

Both bands E and D1 eluted relatively late from the DEAE Sephacel

Figure 14

SDS-PAGE of Non-Reduced Samples from the Extraction and Purification of Band E from the Outer Membrane of Glucose Grown *P. putida*.

The extraction and purification of band E is described in Materials and Methods. The Lubrol PX/EDTA extracted pellet was resuspended to the original volume of the outer membrane suspension. Aliquots of ammonium sulfate precipitated proteins were obtained from pooled fractions eluted from the Sephadex G-25 desalting column. Samples from DEAE Sephacel ion-exchange chromatography of band E were removed from the fractions indicated below. Molecular weight markers are listed in Appendix V.

Lane 1: Outer Membrane from Glucose grown *P. putida*
Lane 2: Lubrol-EDTA Extracted Pellet
Lane 3: Lubrol-EDTA Extract
Lane 4: 30 % Ammonium sulfate precipitated protein
Lane 5: 40 % Ammonium sulfate precipitated protein
Lane 6: 90 % Ammonium sulfate precipitated protein
Lane 7: Fraction 15 DEAE Sephacel ion-exchange chromatography of Band E
Lane 8: Fraction 39 DEAE Sephacel ion-exchange chromatography of Band E

Figure 14

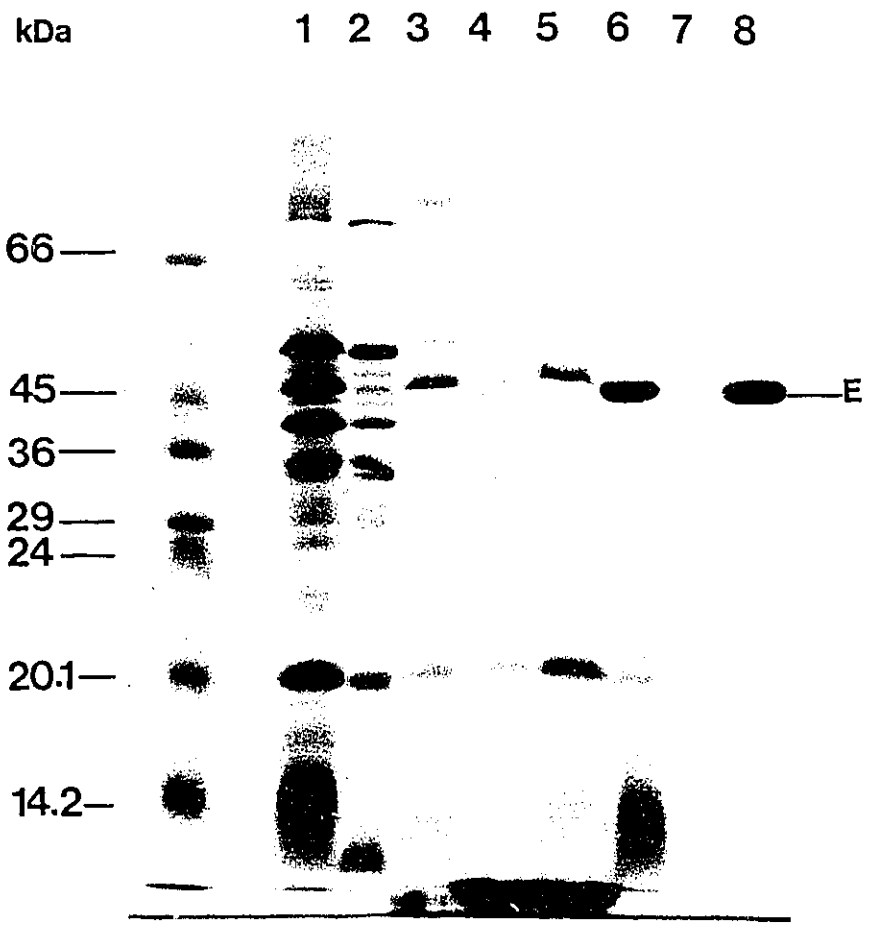
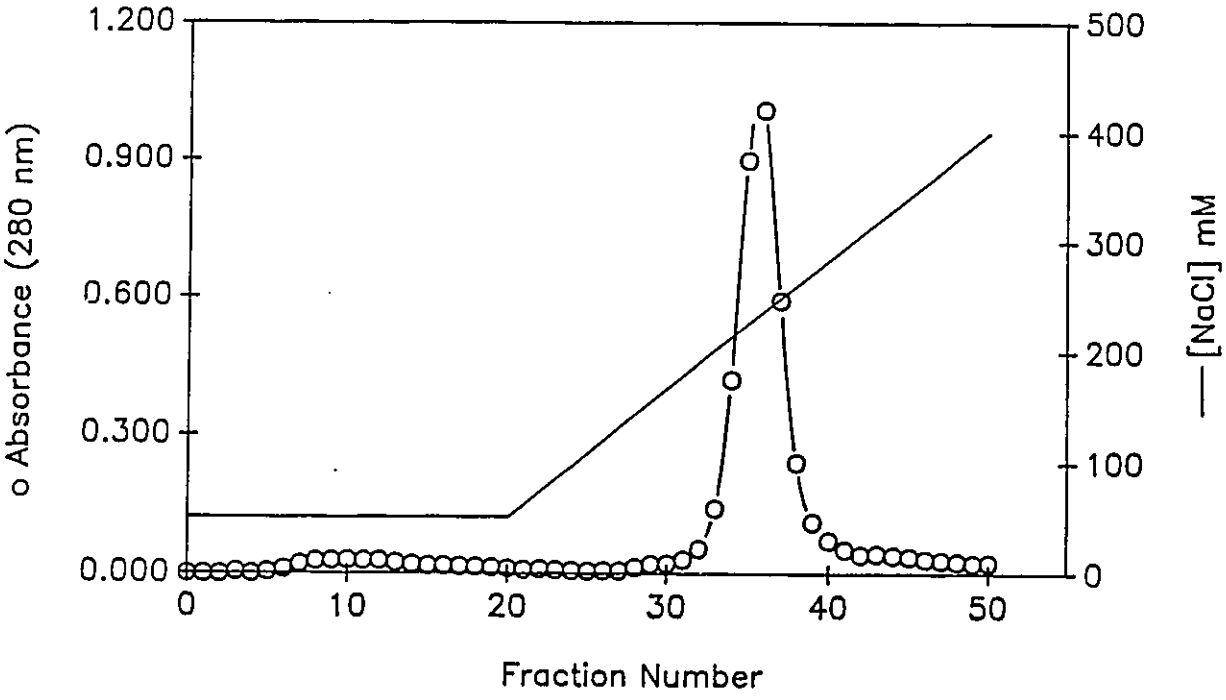


Figure 15

DEAE Sephacel Ion-Exchange Chromatography of Lubrol-EDTA Extracted Glucose Inducible Protein (Band E) from *P. putida*.



The 90% ammonium sulfate fraction was loaded onto the column and eluted as described in Materials and Methods. Solid line - NaCl concentration, Closed circles - 280 nm absorbance.

Figure 16

SDS-PAGE of Non-Reduced Samples from the Lubrol-EDTA Extraction and Purification of Protein D1 from *P. aeruginosa*.

The extraction and purification of protein D1 is described in Materials and Methods. Samples were prepared for SDS-Page as described in Figure 14.

Lane 1: Outer membrane from glucose grown *P. aeruginosa*
Lane 2: Lubrol-EDTA extracted pellet
Lane 3: Lubrol-EDTA extract
Lane 4: 30 % Ammonium sulfate precipitated protein
Lane 5: 40 % Ammonium sulfate precipitated protein
Lane 6: 90 % Ammonium sulfate precipitated protein
Lane 7: Fraction 7 Deae Sephacel ion-exchange chromatography of Protein D1
Lane 8: Fraction 41 DEAE Sephacel ion-exchange chromatography of Protein D1

Figure 16

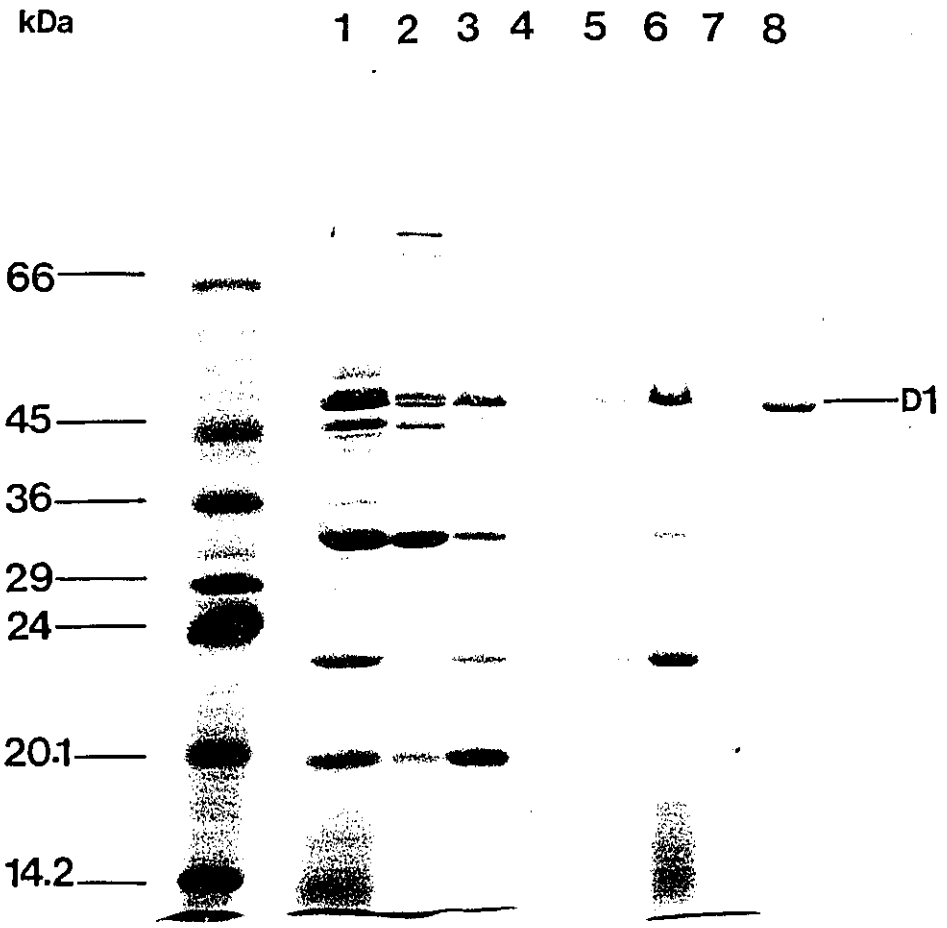
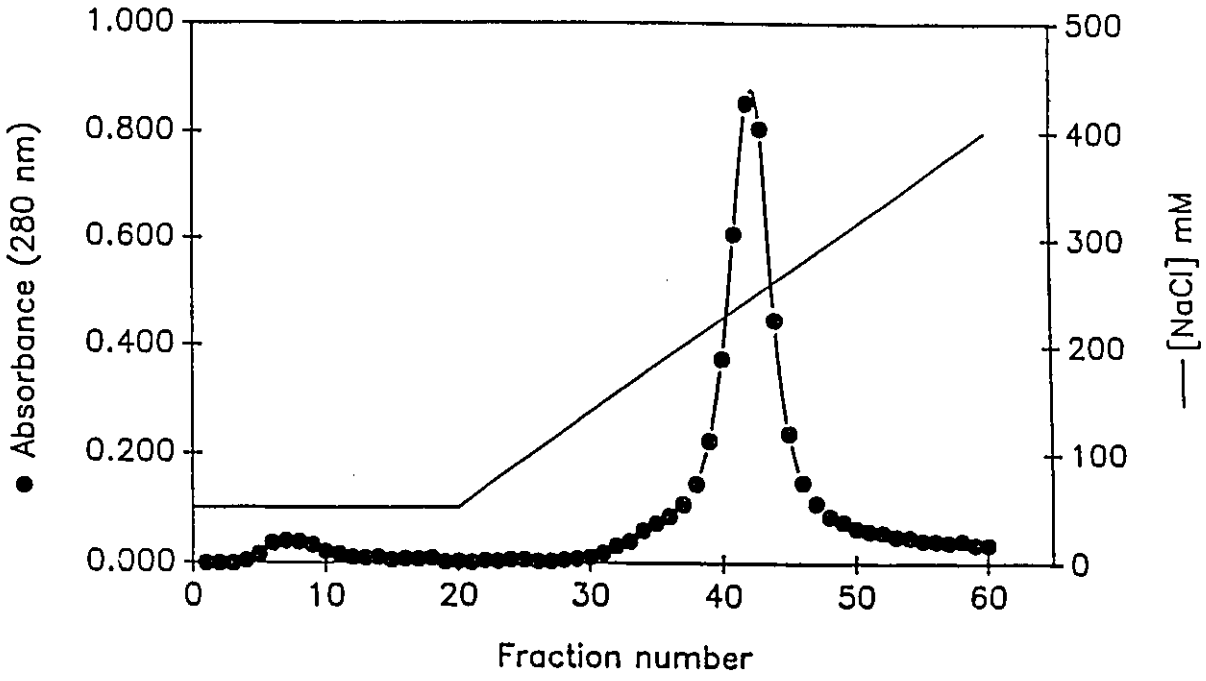


Figure 17

DEAE Sephacel Ion-Exchange Chromatography of Lubrol-EDTA Extracted Glucose Inducible Protein (D1) of P. aeruginosa.



The 90 % ammonium sulfate fraction of Protien D1 was loaded and eluted onto the column as described in Materials and Methods. Solid line - NaCl concentration, Closed circles - 280 nm absorbance.

column and precipitated primarily at high ammonium sulfate concentrations. Preliminary studies with iso-electric focusing gels have indicated that band E has a very acidic pI as it ran off of a gel with ampholytes at a pH range of 4-9. This is consistent with reported low pI values for other outer membrane proteins (115). These characteristics indicate that the proteins are highly polar. Both proteins co-purify with lipopolysaccharide and band E is normally associated with 1.9 to 22% (w/w) LPS measured as KDO. The extent of the LPS contamination is usually greatest in preparations with lower yields of protein. By some estimates (47) LPS in *P. aeruginosa* is about 4.3 % KDO, thus using the above data band E may be contaminated with 2 to 50 times its own weight with LPS. Since LPS contains a high percentage of sugars, amino sugars and sugar-acids, it is not surprising then that band E behaves as a highly polar, acidic protein.

Both purified band E and D1 show heat modifiable mobility on SDS-PAGE (Figure 18). When heated to 95°C for 5-20 min Band E and D1 have apparent molecular weights of 42.8 kDa and 47.5 kDa respectively. But when solubilized in sample buffer at 30°C for 20 min both proteins run at 35.5 kDa. This is identical to the molecular weight of unheated, SDS-treated protein D1 reported by Hancock and Carey (65). The heat modifiable mobility of protein D1 and porin F in *P. aeruginosa* has been attributed to the high β-sheet content of these porin proteins (74,81). To determine if band E also had a high β-sheet consistent with its heat modifiable mobility, a circular dichroism spectra for the purified protein was obtained (Figure 19). A protein (or synthetic peptide) with high β-sheet content has a single characteristic CD spectra minimum at

Figure 18

Heat and DSP Modifiable Mobility of Glucose Inducible Proteins from *P. putida* and *P. aeruginosa*

Purified Bands E and D1 were prepared for SDS-PAGE as described in Materials and Methods. Unheated proteins were incubated in sample buffer at room temperature for 20 minutes. Heated proteins were incubated in sample buffer at 95°C for 20 minutes. Band E was treated with DSP as described in Materials and Methods.

Lane 1: Protein D1 not heated
Lane 2: Band E not heated
Lane 3: Band E heated
Lane 4: Protein D1 heated
Lane 5: DSP treated Band E
Lane 6: DSP treated Band E reduced with dithiothreitol

Figure 18

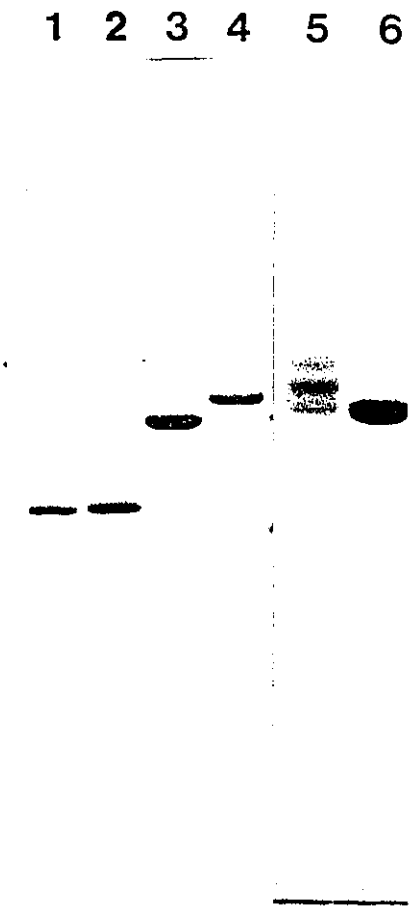
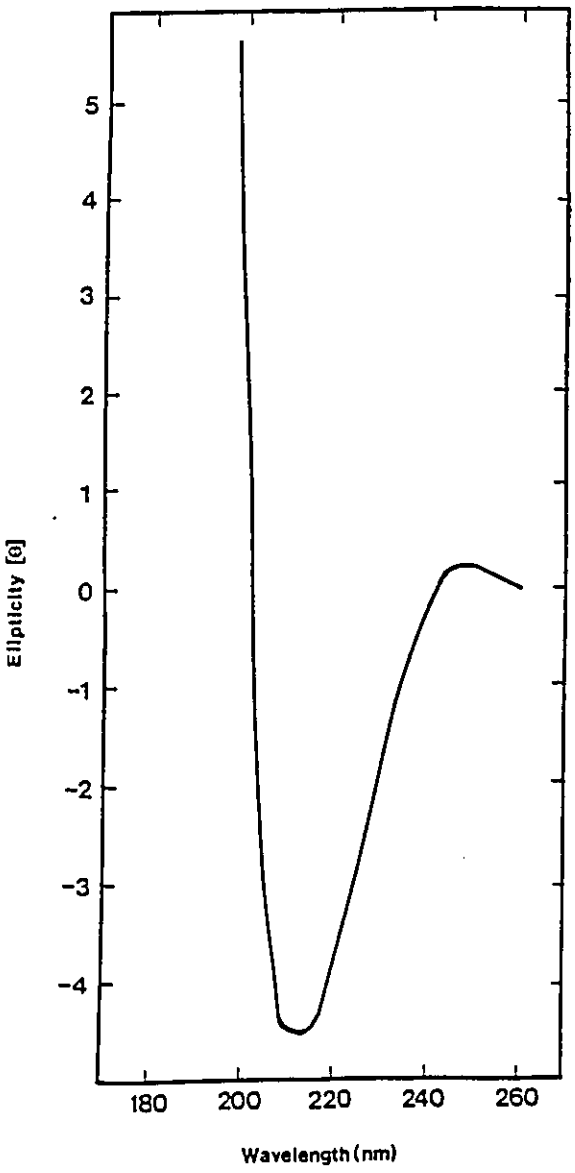


Figure 19

Circular Dichroism Spectrum of Purified Band E.



Spectra was recorded as described in Materials and Methods. Elipticity [Θ] is in arbitrary units.

217-219 nm (172) a minimum at about 214-215 nm was observed for band E. In addition, the spectrum for band E lacks the double minumum at 222 nm and 208-209 nm common to proteins containing significant -helix content (172). Thus when compared to standard spectra for ß-sheet, -helix and random coil (Appendix VII)(171,172) the CD spectra of band E appears to be consistent with a high ß-sheet content. This compares favorably with results from CD spectra for porins F and P which have been calculated to have 62% and 65% ß-sheet content respectively (74).

Angus and Hancock (76) reported that by cross-linking purified outer membrane protein D1 with DSP, oligomeric complexes, dimers and trimers could be detected on SDS-PAGE as well as other higher molecular weight complexes which did not enter the gel. Purified band E was treated with DSP and run on an 11% gel. After this treatment 3-4 bands were usually detected (Figure 18 lane 6) by staining with coomassie blue which were centered around the monomer molecular weight but no bands were observed in areas corresponding to possible dimer (95 kDa) or trimer (130 kDa) molecular weights. This cross-linked products could be cleaved by the addition of DTT. The observed bands were most likely due to cross-linking to contaminating LPS which is possible because DSP could react with amino groups (i.e. ethanolamine) (Appendix VIII). This has been suggested for cross-linking results with porin F in *P. aeruginosa* (76).

Using equilibrium dialysis no ^{3}H-glucose binding activity could be detected for purified band E. Similarly no binding activity could be detected for Triton X100-extracted, purified protein D1 (78). Hancock and Carey (65,78) suggested that there may be some relationship between protein D1 and the glucose binding protein isolated by Stinson et al (21)

since both were induced under conditions where high affinity glucose transport was induced. Hancock and Carey (65) isolated a 53,000 Dalton periplasmic protein using the method of Stinson et al (21) but did not test it for glucose binding activity. In other work, Hancock and Carey (78) detected a glucose binding activity in crude shock extracts of *P. aeruginosa* but not from purified D1. In this case the shock extract control was not similarly detergent treated. Thus in no positive way was protein D1 distinguished from the glucose binding protein. Stinson et al (21) performed amino acid analysis on the purified glucose binding protein. In this study, amino acid analysis was performed on purified proteins E and D1 in order to compare the amino acid content between them and with that reported for the glucose binding protein of *P. aeruginosa* (Table 9). It is remarkable that the amino acid content of D1 and E are very similar suggesting that there is potential for a high degree of homology between the proteins. This is not unlikely since the proteins are regulated in an identical manner in two closely related pseudomonad species. The amino acid content of the glucose binding protein is similar in some respects but not nearly identical to that reported for D1 (21). This provides a positive distinction between the outer membrane and periplasmic proteins.

Band E was prepared for immunization of rabbits by removing the highly antigenic LPS from the protein. To accomplish this the protein was phenol extracted using the method of Sokol and Woods (166). The assay for KDO revealed that LPS was removed to below the limit of detection for the thiobarbiturate assay (less than 0.2% w/w or 1 nmole per aliquot). The rabbits were injected at 2 week intervals for the first three injections

Table 9

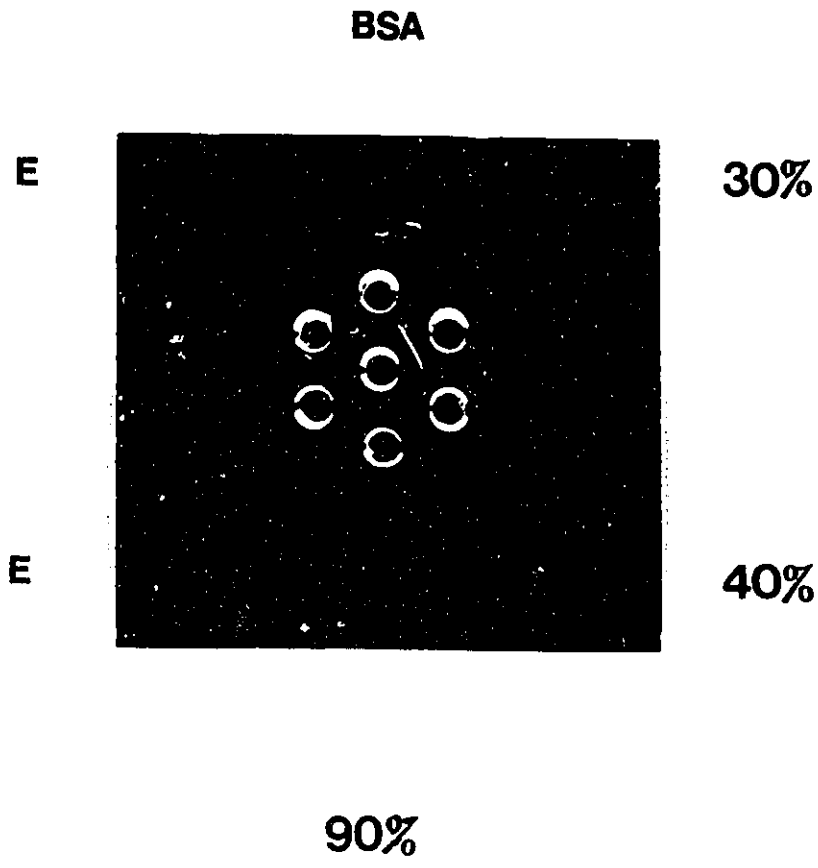
Amino Acid Composition of Purified Band E and Comparison with Outer Membrane Protein D1 and the Glucose Binding Protein of *P. aeruginosa*[a].

Residue	Residues/100 amino acid residues		
	Band E	Protein D1	*P. aeruginosa* GBP[b]
Aspartic acid	11.6 (0.48)	12.0 (0.3)	11.2
Glutamic acid	12.9 (0.32)	12.2 (0.15)	13.0
Serine	6.0 (0.07)	5.7 (0.1)	5.4
Glycine	12.8 (0.04)	11.3 (0.16)	10.1
Histidine	2.1 (0.23)	1.9 (0)	0.9
Alanine	10.0 (0.21)	7.4 (0.05)	14.7
Proline	3.8 (0.04)	4.2 (0.05)	5.2
Tyrosine	3.1 (0.03)	4.2 (0.1)	1.4
Valine	7.5 (0.31)	8.6 (0.24)	6.6
Methionine	1.3 (0.09)	1.0 (0.01)	0.3
Isoleucine	2.9 (0.07)	3.6 (0.22)	2.9
Leucine	7.4 (0.05)	8.6 (0.24)	7.3
Phenylalanine	4.0 (0.61)	3.7 (0.1)	4.5
Lysine	4.1 (0.23)	5.9 (0.13)	7.1
Arginine	4.7 (0.07)	5.3 (0.95)	2.1
Threonine	4.3 (0.09)	4.5 (0.15)	4.1

[a] Amino acids analysis performed by T. Leung in the laboratory of Dr. L. Lee (U. of Windsor) using the Waters Pico Tag system, average of four determinations.
[b] Results taken from Stinson *et al.*
Standard deviation in brackets.

Figure 20

Ouchterlony Double Diffusion Test of Rabbit Polyclonal Antisera Aganst Purified Band E



Ouchterlony gel prepared as described in Materials and Methods.

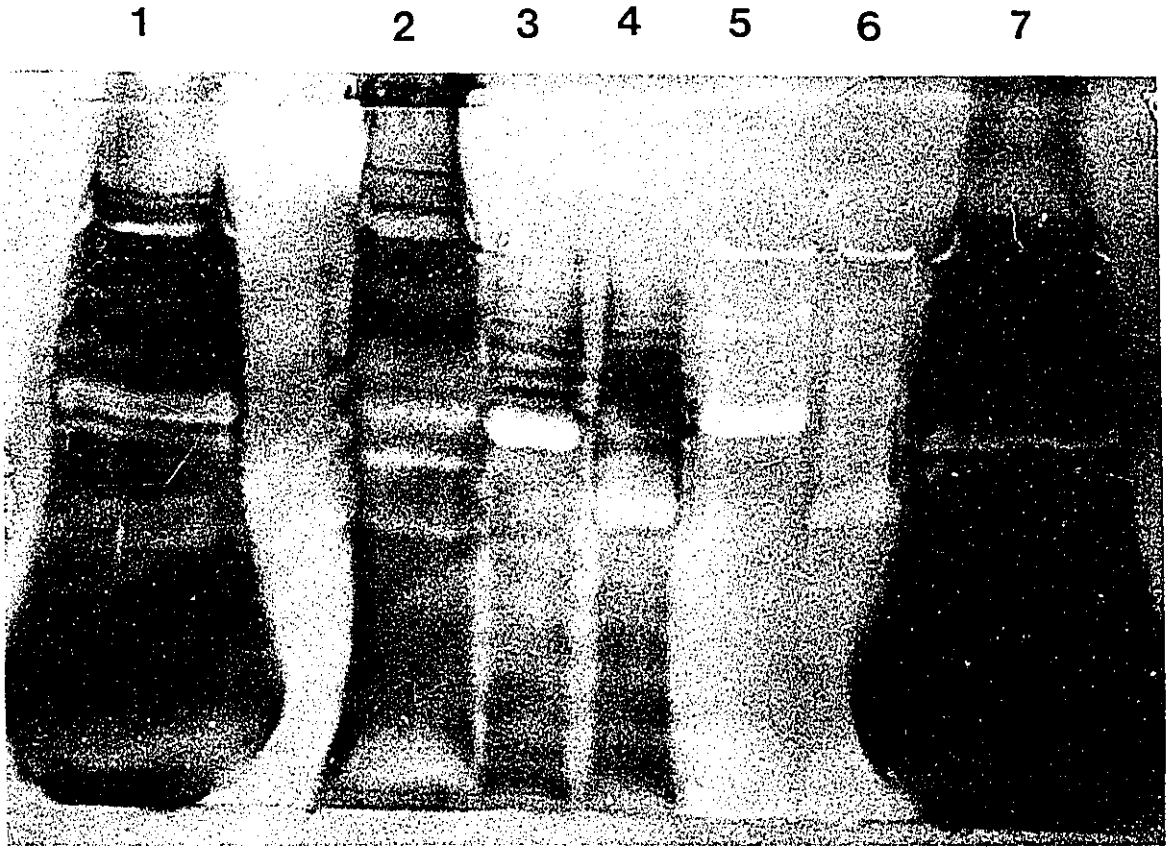
BSA: Bovine serum albumin negative control
30%: Desalted 30% Ammonium sulfate precipitate
40%: Desalted 40% Ammonium sulfate precipitate
90%: Desalted 90% Ammonium sulfate precipitate
E: Purified glucose inducible protein from *P. putida*

and monthly there after. After the first 6 weeks of injection the rabbits were bled every 2-3 weeks and checked for titre using the Ouchterlony double diffusion assay (158). Of six rabbits inoculated with "LPS-free" band E, only one developed antisera which could be detected by Ouchterlony analysis (Figure 20). This antisera reacted with soluble samples containing band E protein obtained from each step of the purification including the crude extract and each ammonium sulfate precipitate. A precipitin line of identity could be traced through each sample but this did not give an indication of antisera specificity because both band E and LPS were detected in each antigen sample by SDS-PAGE and KDO assay respectively. Thus it could not be established that the antisera was specific for band E or LPS. In addition, the Ouchterlony technique is amongst the least sensitive methods for detecting antisera. Non-precipitating sera from the other 5 rabbits may contain antibodies to band E but at a level below the limit of detection by the Oucherlony method (158).

Western blot analysis of the antisera is a highly sensitive method for the detection of antibodies. In this case a Western blot kit was obtained to detect rabbit antisera (primary antibody) bound to antigens immobilized on a charge modified nylon membrane using alkaline phosphatase conjugated goat anti-rabbit IgG (secondary antibody). Samples of outer membrane and purified bands E and D1 were run on 11% Lugtenburg (147) SDS-PAGE mini-gels and blotted onto the nylon membrane. If the antisera were specific for band E the rabbit IgG and hence the AP-conjugated secondary antibody would bind only to the location of the membrane where band E was blotted. Antisera from each of the 6 rabbits

Figure 21

Western Blot Analysis of Rabbit Polyclonal Antisera Against Purified Band E



The Western Blot was performed as described in Materials and Methods. Purified proteins were treated as described in Figure 18.

Lane 1: Dithiothreitol treated outer membrane from glucose grown *P. putida*
Lane 2: Non-reduced outer membrane from glucose grown *P. putida*
Lane 3: Band E heated
Lane 4: Band E unheated
Lane 5: Protein D1 heated
Lane 6: Protein D1 unheated
Lane 7: Dithiothreitol-treated outer membrane from glucose grown *P. putida*

were analyzed with the western blot and each appeared to be specific only for LPS. A representative western blot of the rabbit antisera is shown (Figure 21). The specificity for LPS, and not band E could be observed in a number of ways. First, each showed a "ladder" pattern of bands characteristic of Mw increases of one unit of the o-antigen of LPS (46,47,73,170). Second, the ladder pattern of "LPS" bands was not observed in lanes 5 and 6 containing protein D1 from *P. aeruginosa*. This is typical of antisera to LPS which is usually strain and species specific (99,170). Third, inspection of the blots showed that the background staining was lowest in the location where bands E and D1 were blotted onto the membrane. Thus the blotted proteins provided a blocking action which prevented any non-specific binding of the primary antibody to the membrane.

Both the relatively low molecular weight of the band E protein and the high antigenicity of the trace contaminating LPS likely combined to result in antisera which was not specific for band E. The Western blot technique was invaluable in assessing apparently positive antisera to band E, and preventing erroneous conclusions about the role of band E in the uptake of glucose in *P. putida*. As a result, it was not possible to use the antisera as an affinity agent to block any specific pore function which band E may have in glucose uptake in this organism.

Band E was submitted to the laboratories of Dr. R.E.W. Hancock and Dr. R. Benz for reconstitution into black lipid membranes to determine if the protein has a pore function (Benz, R., Hancock, R.E.W., Saravolac E.G., and Taylor N.F. unpublished results). Band E was tested for both single channel conductance and macroscopic conductance. The conductance

studies were performed to determine if band E forms a water filled channel in lipid bilayer membranes and if so to ascertain whether the pore has any specificity for glucose. In 1M KCl band E forms water filled channels with an average single channel conductance of 34 pS. This is about 5 times lower than that reported for LamB porin and may represent a smaller pore size (126). In addition, preliminary studies have shown that the size distribution of the conductance steps is quite narrow indicating that band E forms pores of a single homogeneous size. Macroscopic conductance studies were performed to determine the specificity or binding of the porin formed by band E. Using high levels of band E reconstituted into the black lipid membrane, the macroscopic conductance (caused by K^+ and Cl^- passing through the porin) was inhibited by titrating with increasing concentrations of glucose. A resulting 50% inhibition constant of 80-100 mM for glucose was determined by this method. Benz *et al* (126) have also demonstrated the binding of maltose to LamB using this same technique. The ability of glucose to inhibit macroscopic conductance indicates that band E most likely forms a glucose-specific pore in the outer membrane of *P. putida*.

SUMMARY AND FUTURE PERSPECTIVES

The outer membrane of *P. putida* was isolated using both a lysozyme digestion procedure and a French press-sucrose density gradient procedure. The latter method gave increased yields of outer membrane and made possible the continued investigation of the putative 4FG defluorinating activity of this sub-cellular fraction. Microscopic inspection of outer membrane suspensions with 4FG showed that they became contaminated with whole cells after incubation at 30°C for 24-36 hours. Protein synthesis specific antibiotics used to inhibit cell growth in outer membrane suspensions concomitantly inhibited defluorination. It was concluded therefore that the outer membrane defluorinating activity observed by D'Amore and Taylor (6) was caused by contaminating cells.

Inspection of the outer membrane preparation by SDS-PAGE showed that it contained 9 major protein bands and a nomenclature for these bands based on that for *P. aeruginosa* was suggested. Like *P. aeruginosa*, the outer membrane of *P. putida* contained both a glucose inducible and a thiol-modifiable protein. The glucose inducible protein, band E, was induced by growth of *P. putida* on glucose, maltose and lactose and repressed by organic acids like gluconate and citric acid cycle intermediates. The glucose inducible protein was extracted from the outer membrane by a variety of ionic detergents and non-ionic detergents in the presence and absence of EDTA. Band E was purified from a 2% Lubrol-EDTA extract of the outer membrane followed by ammonium sulfate precipitation and DEAE Sephacel ion-exchange chromatography. Using this procedure protein D1 was isolated from the outer membrane of *P. aeruginosa*. The mobility of both proteins on SDS-PAGE was heat modifiable and the

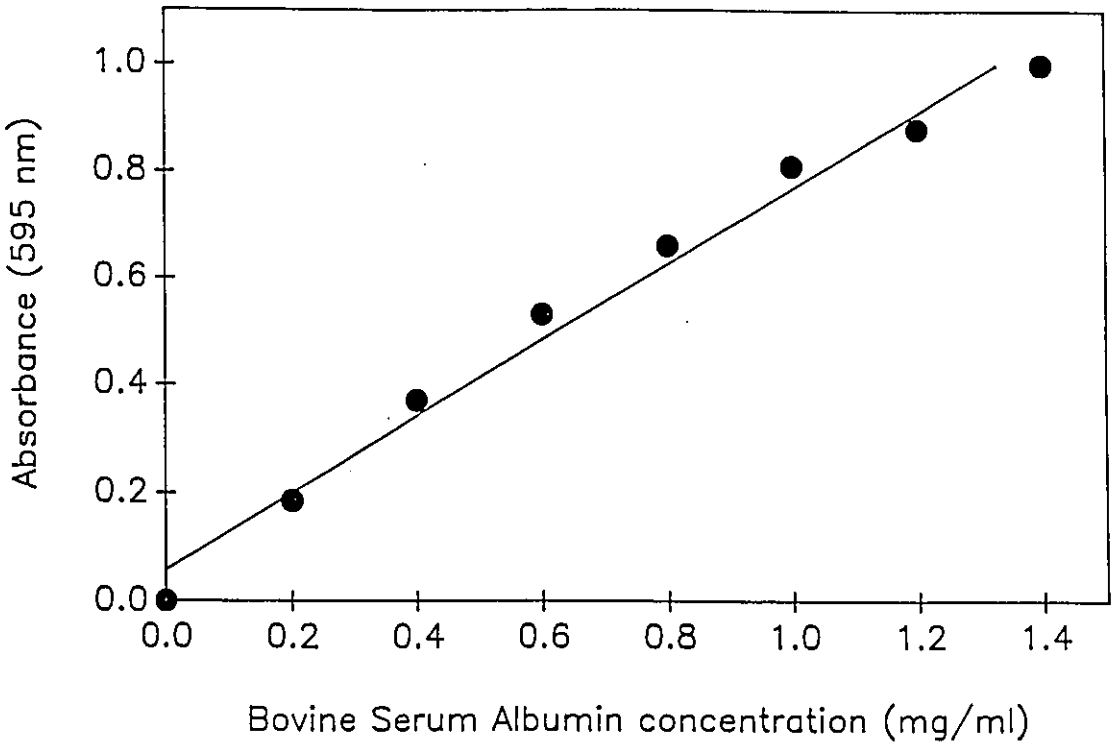
unheated forms of the proteins had identical apparent molecular weight although the heated forms differed in Mw. by approximately 4700 daltons.

We have collaborated with several other laboratories to obtain more information on the structure and function of band E. High β-sheet content has been suggested to be responsible for the heat modifiable mobility observed in outer membrane proteins. To indicate if β-sheet is prevalent in band E a circular dichroism spectra was obtained in conjunction with the laboratory of Dr. R. Reithmeier at the University of Toronto. The high β-sheet type circular dichroism spectra observed for purified band E is consistent with its variable heat modifiable mobility. The amino acid content was performed by collaboration with T. Leung and Dr. L. Lee of the University of Windsor. Amino acid analysis of proteins E and D1 has shown that the amino acid content of each is very similar. These similarities between protein E and D1 are consistent with the identical induction and repression patterns of these proteins and the close taxonomic relationship of the bacteria from which they came. In addition, amino acid analysis as also allowed us to positively distinguish between protein D1 and the glucose binding proteins of *P. aeruginosa*. Attempts to produce antisera specific to band E were performed with Dr. N. Karrupiah at the University of Michigan. One out of five rabbits immunized produced sera positive by Ouchterlony analysis but further testing by Western blotting showed that the antisera from all rabbits immunized was specific to LPS. Further experiments in collaboration with other laboratories await completion. Black lipid membrane conductance studies of band E are presently being performed in the laboratory of Dr. R.E.W. Hancock, University of British Columbia and Dr. R. Benz University of Wurzburg

(F.R.G.). Preliminary results from these studies have shown that band E functions as a porin forming water filled channels with an average single channel conductance of 34 pS. In addition macroscopic conductance was inhibited with D-glucose with a 50% inhibition constant of 80-100 mM indicating that band E forms a glucose-specific channel. Finally, quantitative circular dichroism spectroscopy of reconstited band E is being performed in the laboratory of Dr. C-Y. Jung. State University of New York (Buffalo). Results from these studies will provide a rare opportunity to determine the extent of β-sheet formation of an outer membrane protein in a membrane bilayer.

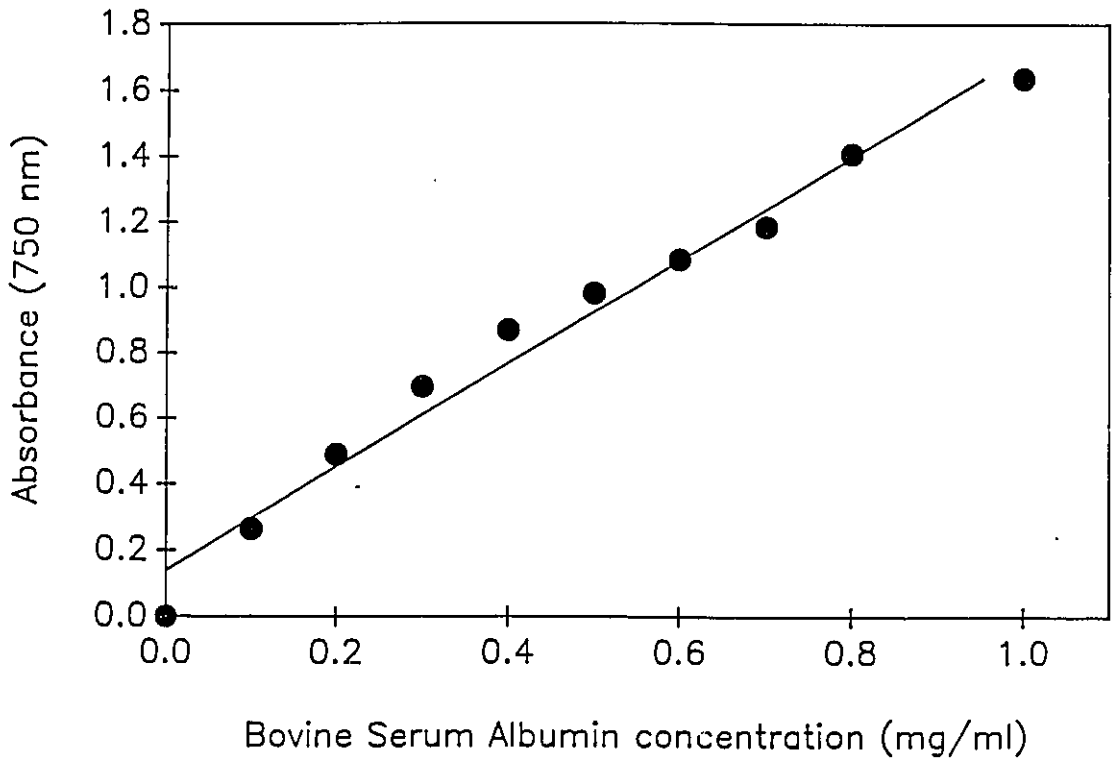
When taken together, induction in the outer membrane, surface expression, glucose-specific pore forming activity and homology with the pore forming protein D1, these results suggest that band E may be involved in some way with the uptake of glucose in *P. putida*. The role of band E in uptake may be in conjunction with a periplasmic binding protein as in the model for maltose uptake in *E. coli* (Figure 7). Further studies into the pore forming activity of band E are required. In addition, efforts to obtain LPS-free protein, perhaps by electroelution from SDS-PAGE or preparative electrophoresis are necessary for band E specific antibodies. Such studies may result in the conformation of glucose transport role for band E in *P. putida*.

Appendix I



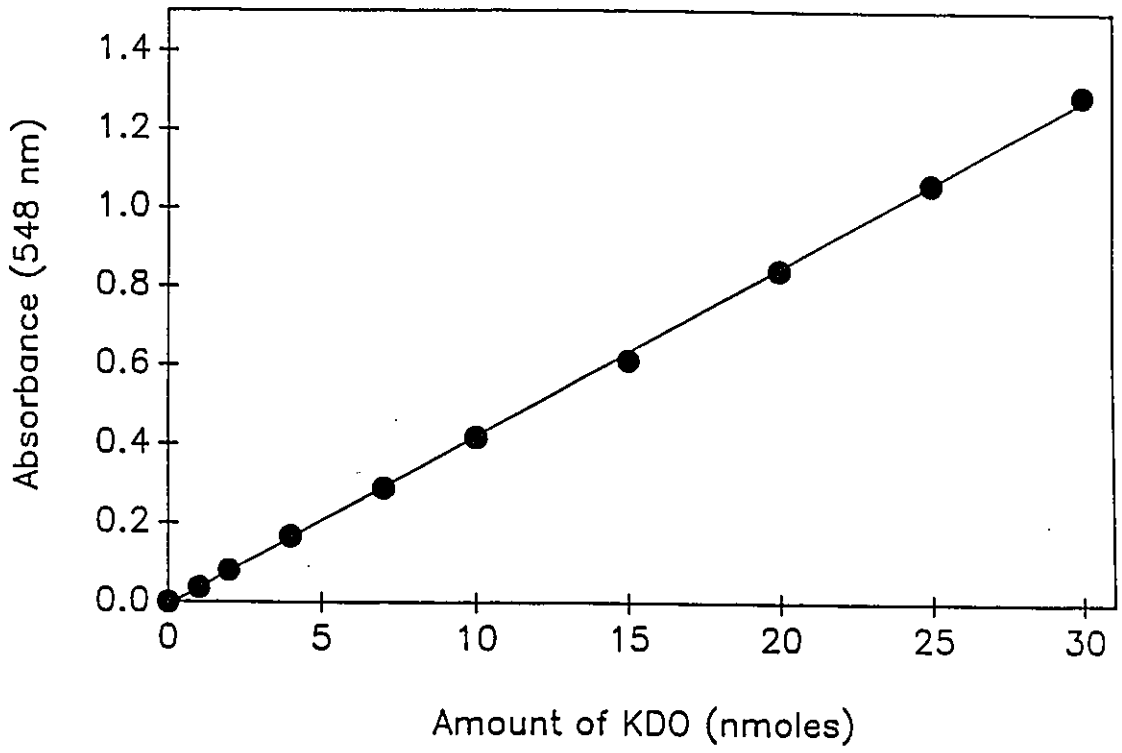
Bradford Protein Assay Standard Curve. Protein was assayed by the method of Bradford (152) in the Bio-Rad kit form using bovine serum albumin as the standard protein in a range of 0.2-1.4 mg/ml. The Bio-Rad reagent was diluted 1:5 before the addition of 0.1 ml aliquots of protien and allowed to incubate 15-20 minutes before reading absorbance at 595 mn. Concentrations were determined using the best-fit 1st order regression line of the standard curve data calculated on the Shimadzu UV-160 recording spectrophotometer.

Appendix II



Lowry Protein Assay Standard Curve. Protein was assayed by the method of Lowry et al (153) as modified by Scopes (154) using bovine serum albumin as the standard protein in the range of 0.1-1.0 mg/ml. Lowry reagent B ($CuSO_4$ in Trisodium citrate) was diluted 1:50 in Lowry reagent A (0.1 M NaOH in 2% $NaHCO_3$) to make Lowry reagent C which was prepared fresh daily. Aliquots (0.2 ml) of protein were incubated in 1.0 ml of reagent C for 10 minutes after which 0.1 ml of 1:2 diluted Sigma Folin and Ciocalteau's reagent was added to each sample which was shaken and incubated for a further 30 minutes before measuring absorbance at 750 nm. Concentrations were determined using the best-fit 1st order linear regression line of the standard curve calculated on the Shimadzu UV-160 recording spectrophotometer.

Appendix III



KDO Assay Standard Curve. KDO was assayed by the method of Karkhanis et al (151) described in Materials and Methods. Concentrations were determined using the best-fit 1st order regression line calculated on the Shimadzu UV-160 recording spectrophotometer.

Appendix IV

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Method of Lugtenburg et al (147)

The following solutions were made with electrophoresis grade reagents.

Acrylamide Solution I

Acrylamide .....44 g
N,N-methylene-bis-acrylamide .....0.8 g
Distilled Deionized H_2O .....100 ml (final volume)
Stored in the dark at 4°C

Acrylamide Solution II

Acrylamide .....30 g
N,N-methylene-bis-acrylamide .....0.8 g
Distilled Deionized H_2O .....100 ml (final volume)
Stored in the dark at 4°C

0.75M Tris-HCl pH 8.8

Tris(hydroxymethyl)aminomethane .....45.43 g (dissolve in 400 ml H_2O)
HCl .....Adjust to pH 8.8
Distilled Deioized H_2O .....500 ml (final volume)
Stored at 4°C.

0.25M Tris-HCl pH 6.8

Tris(hydroxymethyl)aminomethane .....15.14 g (dissolve in 400 ml H_2O)
HCl .....Adjust to pH 6.8
Distilled Deionized H_2O .....500 ml (final volume)
Stored at 4°C.

10% SDS

Sodium dodecyl sulfate 1g
Distilled Deionized H_2O .....10 ml (final volume)
Stored at room temperature.

10% Ammonium persulfate

Ammonium persulfate .....0.1 g
Distilled Deionized H_2O .....1.0 ml
Prepared fresh daily.

Tank Buffer

Glycine .....3.03 g
Tris(hydroxymethyl)aminomethane .....14.4 g
SDS .....1.0 g

Distilled Deionized H_2O .....1 l (final volume)

Sample Buffer

10% SDS .....0.4 ml
Glycerol .....1 g
Bromophenol Blue .....1 mg
0.08M Tris-HCl pH 6.8 .....10 ml (final volume)

For reducing sample buffer (1M DTT) add 1.54 g Dithiothreitol

30 ml Resolving Gel (11%)

Acrylamide I .....7.5 ml
Distilled Deionized H_2O .....6.8 ml
0.75 M Tris-HCl pH 8.8 .....15.0 ml
10% Ammonium persulfate .....0.078 ml

Degas *in vaccuo* for 5 min while stirring.

10% SDS .....0.6 ml
TEMED .....0.045 ml (added last)

Mix 15-30 s more and add immediately to glass plates fitted with spacers. Carefully overlay with 1 ml of butanol, ensure the acrylamide-butanol interface is level. Incubate for 1-1.5 hr at room temperature. When gel has polymerized, remove butanol, rince with distilled deionized water, drain and remove excess water by inverting gel.

15 ml Stacking Gel (5%)

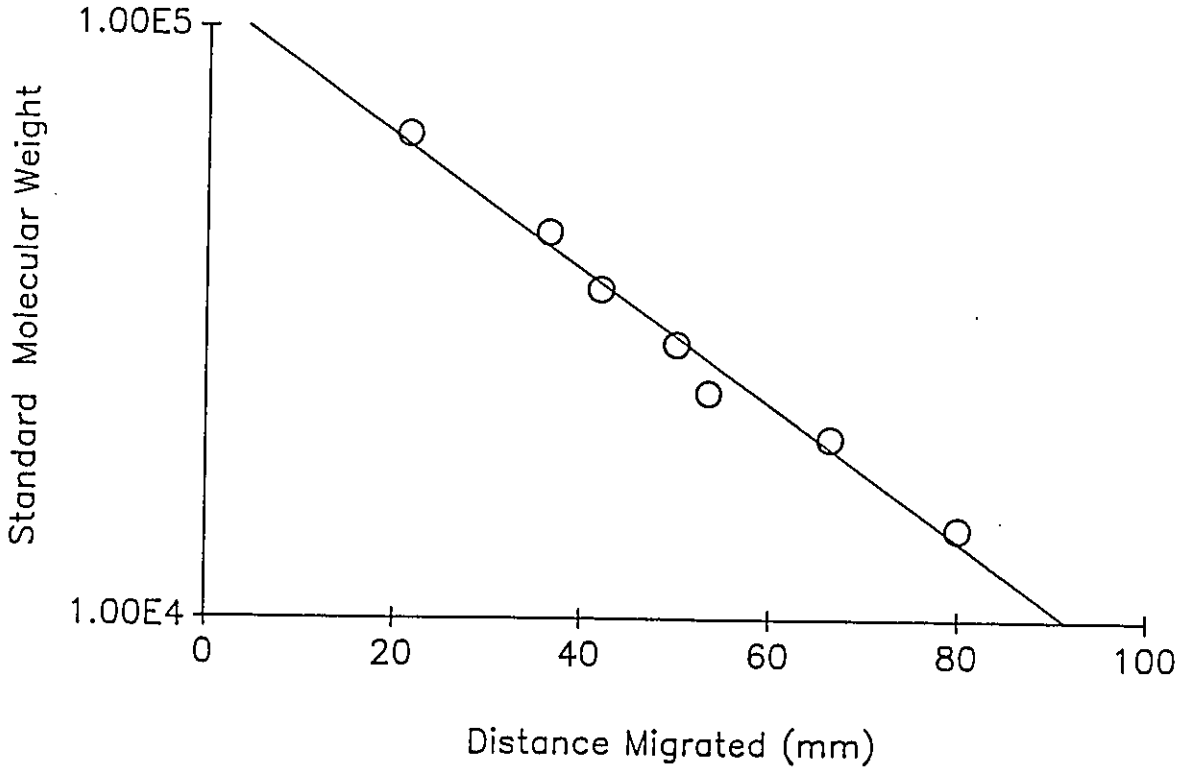
Acrylamide II .....2.5 ml
Distilled Deionized H_2O .....4.2 ml
0.25M Tris-HCl pH 6.8 .....7.5 ml
10% Ammonium persulfate .....0.2 ml
10% SDS .....0.15 ml
TEMED .....0.025 ml (added last)

Mix 15-30 sec and pour over resolving gel to within 0.5 cm from the top of the lower glass plate. Add the comb ensuring to avoid forming bubbles. Incubate for 20-30 minutes at room temp.

Minigel Preparation

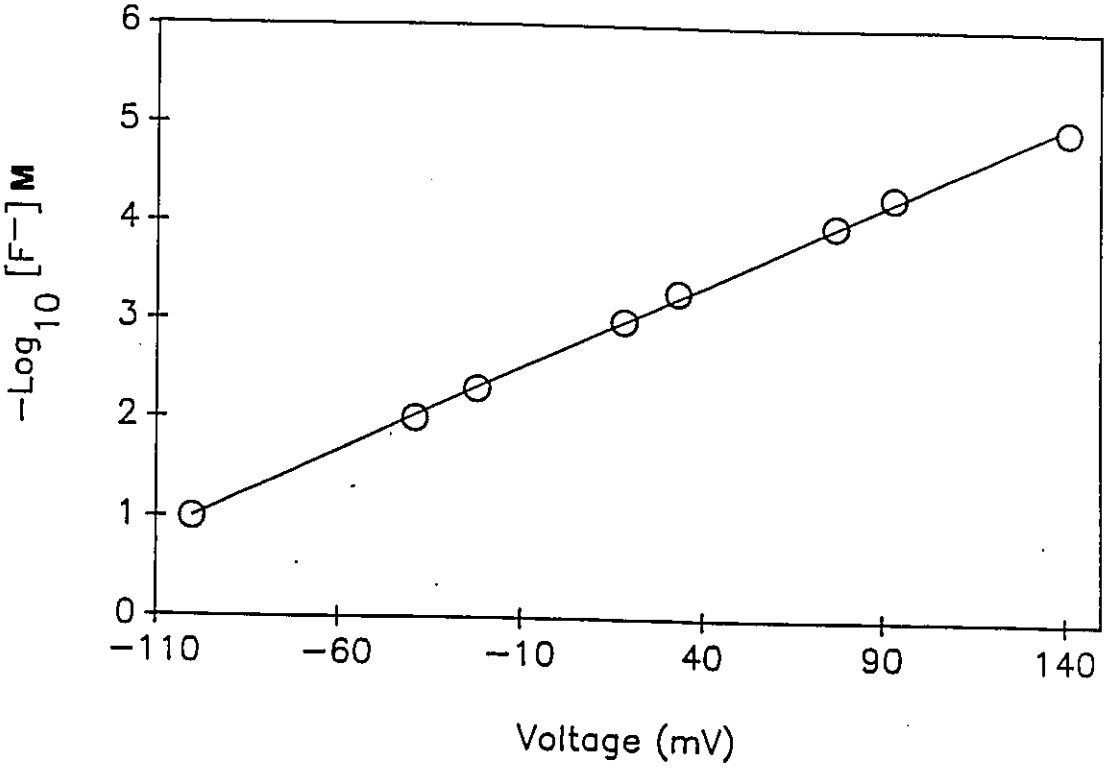
Using Hoefer Scientific type mini-gel apparatus cast 3 minigels simultaniously using above volumes for 1 Lugtenburg gel (45 ml).

Appendix V



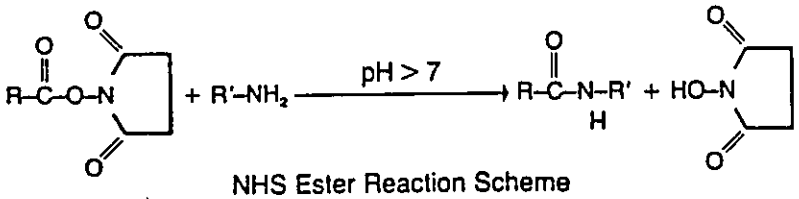
SDS-PAGE Molecular Weight Standard Curve. The molecular weight standards were prepared as described in Materials and Methods. The standards obtained from Sigma Chemical Co. are bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100) and lactalbumin (14,200). Alternatively the BioRad low molecular weight standards were used (not shown). Molecular weights were determined using the DNA gel programe (Materials and Methods) or using the best-fit 1st order regression line calculated on Sigmaplot (Jandel Scientific, Corte Madera, Ca.).

Appendix VI



Fluoride electrode standard curve. Standard concentrations of NaF were prepared in 100 mM potassium phosphate pH 7.1 and the voltage corresponding to each was measured with an Orion Research fluoride specific electrode. Fluoride concentration was determined using the best-fit line 1st order regression line calculated on the Sigmaplot programe (Jandel Scientific, Corte Madere, Ca.)

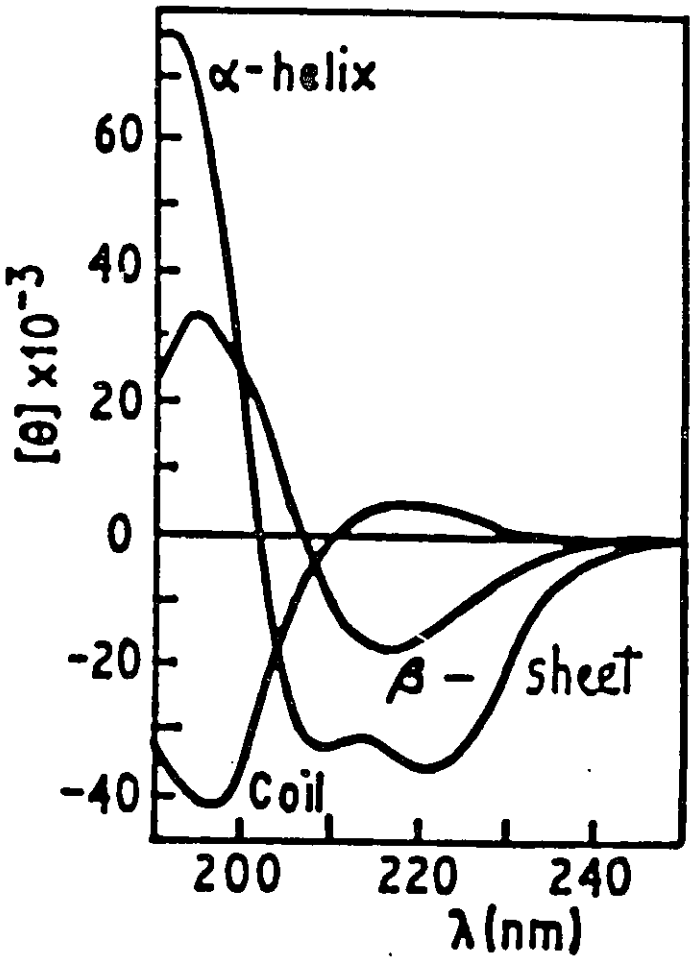
Appendix VII



NHS Ester Reaction Scheme

Mechanism of action of DSP (3,3'-dithio-*bis*-(propionic acid) N-hydroxy-succinimide ester). Reproduced with permission from Pierce Chemical Co. R = dithio-*bis*-(propionic acid) moiety.

Appendix VIII



C.D. Spectra of the α-helix, β-sheet, and random coil forms of a model polypeptide poly-L-lysine. Reproduced with permission (171).

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