COMPARISON OF LIPID AND LIPOPOLYSACCHARIDE OF WILD TYPE *PSEUDOMONAS AERUGINOSA* AND ANTIBIOTIC ADAPTED STRAINS

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PREFACE

The mechanism of action of antibiotic resistance inherent in the readily extractable lipids (REL) and lipopolysaccharides (LPS) in Pseudomonas aeruginosa was studied. The REL of rifamycin resistant strains was compared to the REL of polymyxin resistant strains. Selected components of LPS of polymyxin-resistant and susceptible strains were analyzed to determine which component contributes the most in the exclusion mechanism of the outer membrane.

I wish to express my sincere gratitude to all the people who assisted me in this work and during my stay at Oklahoma State University and at the Oklahoma College of Osteopathic Medicine and Surgery. In particular, I am especially indebted to my major adviser, Dr. Mark Sanborn, and to Dr. Stan Conrad for their intelligent guidance, concern, and invaluable help.

The help of Dr. Ritter in applying statistical analysis to my data is appreciated.

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CHAPTER I

INTRODUCTION

Pseudomonas aeruginosa

Pseudomonas aeruginosa is of great concern to the medical community because it is the primary opportunistic pathogen in severely compromised hosts such as burn patients and cystic fibrotic children. *Pseudomonas* species are the primary pathogens associated with chronic progressive pulmonary disease in the victim of cystic fibrosis . Cystic fibrosis is the number one cause of lethal genetic disease in the United States among white children. Between 40-90% of cystic fibrosis patients have chronic respiratory tract colonization with *P. aeruginosa*. Cystic fibrosis is a hereditary disease of the epithelial tissue affecting primarily the secretion of the various exocrine glands, resulting in a generalized dysfunction of the glands. The heterozygous parents of a child with cystic fibrosis are asymptomatic. Genetic studies cannot distinguish between homozygotes, heterozygotes and normals because no single molecular abnormality has yet been identified as being altered by the genetic defect (10,34).

Most cystic fibrosis patients develop varying degrees

of recurrent pulmonary deficiency, digestive and nutritional problems secondary to pancreatic deficiency, and highly elevated sweat electrolytes (18). Much of the pathology of the disease is caused by over-secretion of viscous mucus resulting in obstruction and subsequent fibrosis of the pancreatic ducts, hepatic ducts and bronchioles. The copious mucus secretions interfere with pulmonary clearance of microorganisms (48). Most infants can with proper care now survive these complications and live well into their third decade of life but all eventually succumb to chronic bronchitis and recurrent infection of Pseudomonas in the respiratory tract (36).

Infection in cystic fibrosis patients normally begin with pathogenic strains of Hemophilis or pneumococcus, then switch to Staphylococcus and then invariably to Pseudomonas (33,35). Apparently Pseudomonas finds a favorable environment for colonialization in the abundant static bronchial secretions of the lungs. Pseudomonas infection in the lungs of cystic fibrosis patients cause fibrosis and calcification presenting similar radiographs found in Mycobacterium infections and as a result cystic fibrosis and tuberculosis are often confused (35). P. aeruginosa can injure pulmonary tissue directly with local nonspecific or specific host immune response (10,33,48). P. cepacia infections in cystic fibrosis patients are rare but when they occur they have been associated with septicemia leading to rapid progression

toward respiratory failure and death (34).

Atypical smooth mucoid strains of Pseudomonas aeruginosa are common in cystic fibrosis patients. These patients normally have high antibody titers to the bacteria's mucoid layer (10). Approximately 80% of Pseudomonas from cystic fibrosis patients are mucoid strains compared to the 0.8-1.2% found in the general population (41). Evidence is clear that specific immune systems, including the humoral immunity and cell immunity, in cystic fibrosis patients appears normal (26,27). This suggests that some deficiency exists in the host mechanisms in the respiratory tract, such as the alveolar macrophages. Prophylactic immunization is effective in preventing upper respiratory tract colonization but is of little benefit once the mucoid strain colonize in the tissue because the antigenic-determinants for opsonic antibody are blocked by the mucoid layer (10,41).

P. aeruinosa, P. cepacia and Staphylococcus aureus infections of cystic fibrosis patients are located almost exclusively in pulmonary tissue (10). Cystic fibrotic patients usually deteriorate and succumb to the combined effects of the metabolic defects and the presence of the antibiotic-resistant bacterial strains. The choice of antimicrobials in the treatment is difficult and often arbitrary. The concentration of the antibiotic should be two to four times the minimum inhibitory concentration (MIC) of the infecting bacteria at the site of infection (33).

McLaughlin performed pulmonary function tests of cystic fibrosis patients who had been treated with antibiotics over a period of ten days. Improvement in pulmonary function did not correlate with a change in the sputum bacterial concentration, antibiotic susceptibility of bacteria present in sputum, or antibiotic administered. At the end of one month after treatment, the concentration of bacteria measured in the sputum returned to pretreatment levels. Neither antibiotics or antipseudomonal antibody protect the pulmonary system against Pseudomonas infection (10,36). Pseudomonas is rarely eradicated from the sputum (3). Several aminoglycosides, such as tobramycin and gentamicin have demonstrated efficiency against Pseudomonas. They have the disadvantage of vestibuloauditory and renal toxicities at high concentratons. Poor clinical results may reflect lack of compliance, incorrect recogniton of the infecting bacteria or failure to achieve adequate concentration of active drug in sputum or bronchial tissue (33).

Polymyxin

In this study I examined a wild type Pseudomonas strain which was stepwise adapted to polymyxin, giving us the strain IA, and a deadapted strain IA-Reverted (IAR). The polymyxins are one of the few groups of antibiotics that can be used successfully against pseudomonal infections (6). Polymyxin is a basic peptide antibiotic with a molecular weight of approximately 1200, having amphiphilic properties

with a charged hydrophilic headgroup and a hydrophobic side chain (Figure 1) (24).

It is usually used in atopic ointments, being administered to cystic fibrosis patients only as a last resort. Polymyxin is a broad spectrum antibiotic effective against gram-negative bacteria, yeast and protozoa (45). It can be useful clinically as it has lower toxicity for eucaryotic cells. It is active against most P. aeruginosa isolates with an MIC of <2μg/ml. Polymyxins have been compared to a cationic detergent. However, polymyxins exhibit bactericidal activities at lower concentration and slight modifications in the antibiotic results in significant changes in biological activities (45).

This study also examined several Pseudomonas strains and their stepwise adaptation to rifamycin. Rifamycin is an amphiphilic antibiotic having an aromatic chromophore spanned by a long aliphatic bridge and as a result is more nonpolar than polymyxin (Figure 2) (8). It is bactericidal and functions by blocking transcription at the initiaton step, specifically, RNA polymerase. Rifampin is a semi-synthetic derivative of the naturally occurring rifamycins and is widely used clinically. It is selective for acid-fast and gram-negative procaryotes and therefore useful in selective antibacterial chemotherapy.

The permeability charateristics of the outer membrane (OM) of the polymyxin-resistant cells used in this study appear to be altered for a number of anibiotics (17). As shown by

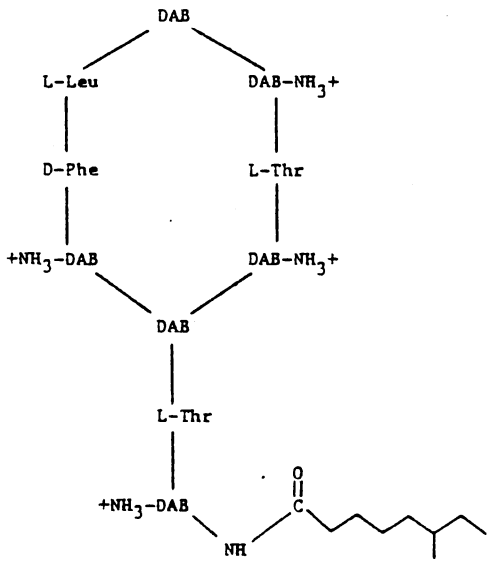

Figure 1. Polymyxin

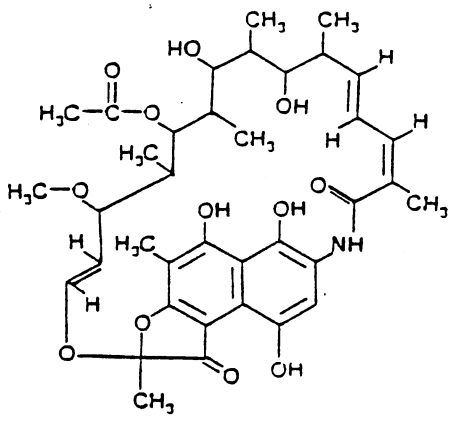

Figure 2. Rifamycin

Gilleland and Farley, the MIC changes for polymyxin coincide with MIC changes in rifamycin (17). As the wild type strain, PAO1, was adapted to polymyxin B it become more sensitive to rifamycin which is more nonpolar than polymyxin B (19). In this same study, the MIC changes for polar antibiotics were unaffected thereby giving credence to the hypothesis that hydrophilic antibiotics diffuse through acqueous porins, whereas hydrophobic antibiotics utilize the hydrophobic hydrocarbon inner leaf of the OM.

Exclusion Mechanism

The OM of gram-negative bacteria plays an important role as an ionophore size-dependent permeability barrier and in resistant cells fuction to exclude antibiotics, such as polymyxin B and rifamycin (Figure 3) (33,44,50). It is permeable to saccharides of molecular weight between 3000-9000 Daltons (22). Outer membrane permeability decreases as antibiotic resistance increases (1). The primary sites of interaction on the OM surface are the phospholipids and the lipopolysaccharides (LPS) (38). Storm and Rosenthal hypothesized polymyxin B disrupts the membrane structure by electrostatic interactions with phospholipids (45). This change is sufficient to increase permeability of the lipid barrier with respect to charged or polar molecules. The fatty acid chain on the polymyxin B molecule is shorter (C_8 - C_{11}) than the fatty acid chains of the phospholipids (C_{14} - C_{18}) (21). The insertion of the

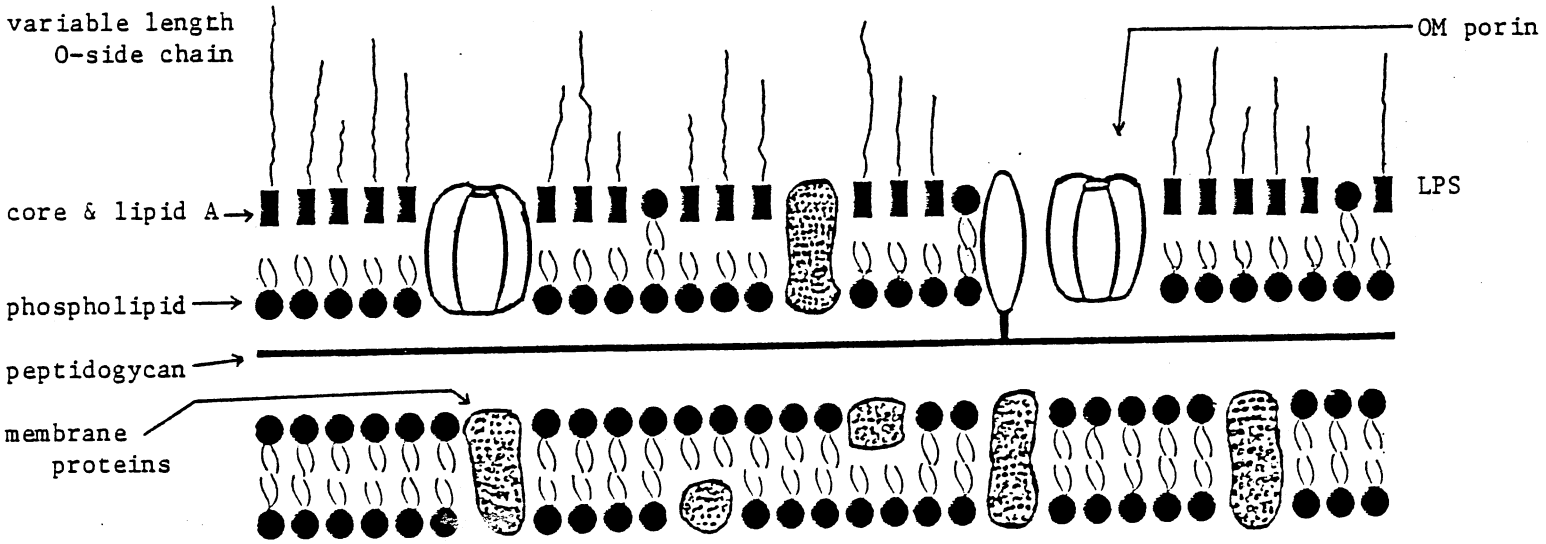

Figure 3. Outer Membrane of Gram-Negative Bacteria

shorter chains into the hydrophobic interior causes a disruption of the lattice structure. The interaction of the positively charged peptide head with the negatively charged phosphates causes a widening of the structure and consequently an 11% increase in the area. Few demonstrated increased surface pressure on phospholipid monolayers by external polymyxin B. The pressure could be caused by the insertion of the fatty acid tails into the hydrophobic core of the OM or by electrostatic force due to interactions between peptide amino groups and phospholipid phosphate groups (45).

Binding of polymyxin to the OM can be inhibited by divalent cations. Magnesium and calcium ions act as metal-ion bridges between the phosphate groups of the phospholipids and the LPS to preserve the OM structural integrity (45).

Gilleland and Murray suggested that exclusion of nonpolar antibiotics by the OM might be the mechanism of resistance. They subjected cells to cold shock and osmotic cold shock and found no evidence for a polymyxin-inactivating enzyme in osmotic shock fluid from the polymyxin-resistant strains (19). This is further supported by electron microscopy studies of isolated cytoplasmic membrane (CM) and OM from resistant and susceptible strains (17). Polymyxin disrupted the OM and CM of susceptible cells and the CM of resistant cells, leaving the OM of the resistant cells unaffected.

The exclusion mechanism for the resistance of polymyxin on the OM of P. aeruginosa may be explained by way of at least two possible hypotheses, or some combination thereof. The first hypotheses encompasses two model systems. In the first model it is assumed that the hydrophobic paraffin tail of the polymyxin molecule slides in-between the lipid bilayer of the OM, leaving the hydrophilic, charged headgroup lying flat against the outer surface. The result would be an expansion of the lipid lattice and a tilting of the lipid chains which would lead to a disruption in the fluidity and permeability of the OM thereby allowing the antibiotic to enter. The second model requires the entire polymyxin molecule to enter the lipid bilayer causing a disruption of the lipid lattice and resulting in a complete disorganization of the membrane structure. In both models it should be clear that if the lipid content and composition were to be altered, through a loss of receptor phospholipid, antibiotic resistance might result since polymyxin could no longer bind and interact with the OM layer.

The second theory for polymyxin resistance suggests that the antibiotic penetrated the OM via an OM porin after first being bound to LPS or OM phospholipid (12). Here, the exclusion mechanism works through a loss of porin protein which can no longer facilitate passage of the antibiotic.

Once through the OM, polymyxin is free to reach its target sites on the cytoplasmic membrane (CM). The bactericidal action of polymyxin on gram-negative bacteria

results from the interaction of the antibiotic with the phospholipids in the CM causing leakage of the cell contents (2). The CM and OM of sensitive cells both bind polymyxin B in equivalent amounts per mole of lipid phosphate (45). A specific protein receptor seems unlikely in view of the fact that binding of polymyxin B to the membrane shows saturation at concentrations of one to two orders of magnitude higher than MIC or minimum inhibitory bacteriocidal (MIB) concentrations. Based on an experiment with agarose beads, Teuber hypothesized that polymyxin B did not have to enter the cell to inhibit growth, only surface contact was necessary (45). Biosynthesis, for instance, depends on the energy charge of the cell. Polymyxin B binding to the OM surface disrupts the energy charge thus causing general inhibition by inhibiting electron transport and oxidative phosphorylation. As polymyxin concentration increases, the efflux of cytoplamic components into the media occurs and permeability for small molecular weight compounds increases. Divalent cations inhibit this effect (45).

The initial interaction of the polymyxin molecules with the gram-negative bacterial OM involves binding to LPS and phospholipids (2,25,38,39). It has been reported by a number of workers that polymyxin-resistant strains of P. aeruginosa have alterations in their lipid content and composition in the OM (2,3,5,12,18). Dorrer and Teuber reported that lipids on the polymyxin-resistant strains are altered when grown on various carbon sources (24). Conrad reported polymyxin resistant strains had a

significant increase in the readily extractable lipid (REL) content and a significant decrease in selected phospholipids (4). The reduction of overall phospholipid content, specifically phosphatidylglycerol (PG), and phosphatidylethanolamine (PE), could reduce the ability of the OM to bind to polymyxin B. There was a significant increase observed also in the unsaturated straight chain fatty acids accompanied by a significant decrease in the cyclopropyl fatty acids in polymyxin-resistant strains as compared with PAO and the deadapted strain, IAR (4).

The polymyxin B adapted Pseudomonas strain used in our study has been previously shown to have reduced amounts of three major OM porin proteins (19), having approximate molecular weights of 24,000, 36,500 and 47,000 (2). The loss of major porin proteins of polymyxin-resistant strains was found to be dependent on the carbon sources (6,16). It should be noted that in Conrad and Gilleland's polymyxin-resistant strain, the loss of OM porin protein was accompanied by lipid alterations (4).

Polymyxin resistance could be explained by a combined effect of the exclusion mechanism by the OM with an antibiotic deactivating enzyme in the periplasmic space, loss of sensitivity of the active site in the CM, and a repair mechanism that repairs damage to the CM (1,17). There is, however, no evidence to support these mechanisms.

Lipopolysaccharide

The lipopolysaccharide (LPS) component of the OM of gram-negative bacteria consists of three regions, Lipid A, core oligosaccharides and O-antigenic side chains (31). The three regions are covalently attached to one another. Lipid A, the hydrophobic component, consists of a diglucosamine phosphate with five or six attached fatty acid chains and is inserted into the outer leaflet of the OM (32). A significant portion of the fatty acid chains are 2- or 3-hydroxyl fatty acids, which are unique to LPS in gram-negative bacteria (28,42). The core consists of about ten to twelve sugars and contains most of the cellular octose and heptose present as 3-keto-D-mannooctulosonic acid (KDO) and L-glycero-D-mannoheptose or D-glycero-L-mannoheptose respectively, as well as a number of hexoses. The side chain consists of a variable number of repeating saccharide units and is the portion of the LPS which usually determines antigenic specificity (8,36) and heterogeniety (20,25).

The LPS is a permeability barrier to antibiotics, an obstacle to the functioning of phagocytes and antibodies, and functions as a receptor site for the adsorption of some bacteriophage (36). It is important to note that the cell controls the LPS to maintain a fixed surface charge (9). Polymyxin B disrupts this surface charge by crossing the OM of Pseudomonas via a pathway in which the cationic antibiotic interacts with a magnesium ion binding site on

the OM LPS. The phospholipids and LPS are forced further apart altering the surface charge consequently making the OM more permeable to other antibiotics (40).

Polymyxin B has been hypothesized to bind primarily with the KDO portion of the LPS molecule according to a study in which the peptide-lipid portion contained approximately three moles of antibiotic per one mole of LPS (45). In another study, it was reported that the level of LPS was decreased in the polymyxin-resistant strains as compared to the wild type PAO1 strain (18).

Since cystic fibrosis patients are susceptible to P. aeruginosa and Pseudomonas quickly acquires resistance to most antibiotics, antimicrobial agents and physio-therapy, it would be of value to determine the mechanism of action of antibiotic resistance inherent in the OM of Pseudomonas. Results from our rifamycin experiments will allow us to determine whether the conclusions derived from the polymyxin studies which analyzed REL were unique to polymyxin or can be applied to nonpolar antibiotics in general. This will give a clue to the mechanism of action of resistance to rifamycin-resistant cells. Both mechanisms of polymyxin penetration through the OM and consequent interaction on the CM are hypothesized to function together. The question remains as to whether lipids or proteins or both play a crucial role in bringing about polymyxin resistance. Our working hypothesis in this study was that acquisition of polymyxin resistance results from alterations in the LPS component, the component most likely to have the

greatest effect on the OM permeability. Here we are concerned with the qualitation and quantitation of LPS in resistant and susceptible cells to determine which component was responsible for the exclusion of polymyxin B in the OM of resistant cells.

CHAPTER II

METHODS AND MATERIALS

Bacterial Strains

The polymyxin-resistant strains of *P. aeruginosa* used in this study have been previously described by Conrad and Gilleland (4). The rifamycin-resistant strains (RIF G and RIF H) were derived during the present study and included RIF G and RIF H resistant strains. They were derived from the wild type PAO1 strain. They are not stable mutants but represent an adaptive response to the presence of 6000 U of rifamycin per milliliter (ml) of growth medium. Adaptive cells grown in the absence of rifamycin reverted to rifamycin susceptibility. The polymyxin system included polymyxin-susceptible PAO1 parent strain, polymyxin-resistant strain, isolate A (IA), which was adapted to 6000 U of polymyxin per ml of growth medium, and polymyxin-susceptible revertant, isolate A revertant (IAR).

P. aeruginosa PAO1 was grown at 30°C in a basal salts medium (BM) described below. Resistant strains to either polymyxin or rifamycin were obtained using the following method. All cultures were grown at 30°C in an Environ-shaker (LABLINE Instruments, Inc., type 3597-2), rotations at 180 rpm. PAO1 was grown in BM and incubated

in 125 ml Erlenmyer screw-capped flasks containing twenty mls of BM or BM plus appropriate concentrations of either polymyxin B or rifamycin SV. PAO1 was adapted to resistance by repeated subcultures in the presence of increasingly greater antibiotic concentrations. The rifamycin-resistant strains were adapted by increasing the concentration from 100 μg/ml after 42 hours to 250 μg/ml, then at 24 hour intervals increased to 500, 1000 then finally 2000 μg/ml. Using this method of transferring the cells from low concentrations to higher concentrations of antibiotics, cells were obtained which could grow in the presence of 6000 U of polymyxin or 6000 U of rifamycin. Stock cultures of the isolates were maintained on BM or BM plus the appropriate antibiotic at 4°C and on agar plates at room temperature until needed. All strains were subcultured bimonthly.

Media

All cells were grown in a previously described basal medium of Gilleland and Murray (18). This medium contained the following ingrediants at a final pH of 7.0: 0.03 M glucose, 0.04 M K_2HPO_4, 0.022 M KH_2PO_4, 0.007 M $(NH_4)_2SO_4$ and 0.0005 M $MgSO_4$. All media minus glucose and antibiotics, which were added separately, were sterilized in an autoclave at 121°C at 15 psi for 15 minutes. D-glucose was sterilized separately for twelve minutes and the antibiotics were filter sterilized by millipore filter (.45 μm) in a type S Nalgene filtration unit. Rifamycin and

polymyxin-containing media consisted of BM plus the appropriate concentration of antibiotic per ml. The polymyxin B sulfate was purchased from Sigma Chemical Co., and rifamycin SV was purchased from Applied Science Labs.

The cells were routinely grown in two- and four-liter Erlenmyer flasks containing one- and two-liters respectively of medium with either vigorous stirring using a magnetic stirring bar at 30°C or in a shaker at 180 rpm at 30°C. A starter culture was used to innoculate the fresh flask of medium to give an initial absorbance of 0.05 at 660 nm. The growing cells were monitored by measuring a 20 ml aliquot in a Bausch & Lomb Spectronic 20. The cells were harvested at the mid- to late-logarithmic phase by centrifugation at 6500 rpm, or 8000 x g, at 0-4°C in a Sorvall RC-5 Superspeed Refrigerated Centrifuge (Du Pont Instruments), and washed twice with a 50 m<u>M</u> phosphate buffer at pH 7.0. The cells were stored frozen at 0°C and then freeze-dried in a LABCONCO Freeze-dryer-5 lyophilizer.

LPS Isolation

Freeze-dried cells were used in the isolation of LPS in a method that extracted both smooth and rough LPS as described by Darveau and Hancock (7). Each extraction utilized either 0.5 to 2.5 g of cells. The freeze-dried cells were suspended in 10 m<u>M</u> Tris-hydrochloride (Tris-HC) buffer (pH 8.0), 2 <u>M</u> $MgCl_2$, 100 µg of pancreatic DNase I (DN100; Sigma Chem. Co.) per ml, and 25

µg of pancreatic RNase A (R-4875 Sigma) per ml. This suspension was passed twice through a French pressure cell (American Instrument Co.) at 15,000 lb/in^2. The cell lysate was sonicated (Lab-Line Ultra-tip Labsonic System) for two 30 second bursts at a probe intensity of 75. DNase and RNase were added to final concentrations of 200 and 50 µg/ml respectively, and the suspension was incubated at 37°C for 2 hours. After the incubation period, 0.5 M tetrasodium ethylenediaminetetra acetic acid (EDTA) (Sigma) dissolved in 10 mM Tris-HC (pH 8.0), 20% sodium dodecyl sulfate (SDS) dissolved in 10 mM Tris-HC (pH 8.0) and 10 mM Tris-HC (pH 8.0) were added to give a final concentration of 0.1 M EDTA, 2% SDS and 10 mM Tris-HC at a pH of ca. 9.5. The sample was vortexed and centrifuged in a Beckman Model L5-65 Ultracentrifuge (Beckman Instrument Inc.), using a preparative TYPE 65 rotor) at 50,000 x g for 30 minutes at 20°C. The supernatent was decanted, and pronase (Sigma) was added to give a final concentration of 200 µg/ml, and the sample was incubated overnight in a shaker at 180 rpm at 30°C. Two volumes of 0.375 M $MgCl_2$ in 95% ethanol were added and the mixture cooled to 0°C in a preset refrigerator. After centrifugation at 12,000 x g for 15 minutes at 0-4°C, the pellet obtained was suspended in 9% SDS-0.1 M EDTA, dissolved in 10 mM Tris-HCl (pH 8.0) and sonicated. The pH was lowered to 7.0 by drop-wise additon of 6N HCl and incubated at 80°C for 20 minutes. The pH was then raised by drop-wise

addition of 4 N NaOH to 9.5, pronase added to 25 µg/ml and the sample was then incubated overnight at 37°C as before. Again, 0.375 M $MgCl_2$ was added, the sample cooled to 0°C and centrifuged as before. The pellet obtained was suspended in 10 mM Tris-HCl (pH 8.0), sonicated as before and centifuged at 1000 rpm for 5 minutes. This step was repeated twice, decanting the supernatent each time, which was then centrifuged at 200,000 x g for 2 hours at 15° in the presence of 25 mM $MgCl_2$. The pellet obtained was resuspended in 1 ml of distilled water and freeze-dried.

LPS Analysis

Total hexose and heptose were measured for by the sulfuric-acid method of Wright and Rebers (49). LPS was resuspended in 0.1 N H_2SO_4 by sonification at a probe intensity of 75 for 30 seconds before starting the assays. D-glucose was used as the standard for the hexose assay. For the heptose assay, the sample was read at 505 and 545 nm and the difference in these two ODs were taken. The proportionality was such that 1.0 change in the optical density was equal to 230 µg of heptose. KDO was quantitated by the colorimetric assay described by Weissback and Hurwitz (47). Total carbohydrate was assayed by the phenol-sulfuric acid methods of Dubois and Gilles (11). Dextran was used as the standard. The phosphate content of LPS was measured by the Ames total phosphate method. For each assay, from 10-100 µg of lyophilized LPS was measured. The percent of

LPS in the whole cells was determined gravimetrically after lyophilization.

Lipid Analysis

The REL fraction of the rifamycin strains was obtained by chloroform-methanol (2:1) extraction of 500 mg freeze-dried whole cells by a previously described modification (6) of the method of Folch et. al. (14). The percent of REL in whole cells were determined gravimetrically by evaporating 5 mls of extract to dryness at room temperature for 48 hours in tared aluminum weigh pans. Qualitative resolution of major membrane phospholipids and separation from neutral lipids were performed on thin layer chromatography (TLC) plates. This was used for REL samples and as a check for LPS purity. A sample aliquot was dried under flowing N_2 gas at 40°C and redissolved into a 0.1 volume of chloroform-methanol (2:1) or hexanes solvent. A 5 µl deposition of concentrated sample was applied to the plate with a Pasteur pipet. The plates were first developed 10 cm in chloroform:methanol:water (65:25:4), dried at room temperature and then developed 14 cm in n-hexane:diethylether (4:1). The TLC chamber (Analytical Technology) for both systems contained 125 ml of solvent. The glass plate was 20 x 20 cm and had a silica gel G (Sigma) suspension spread to a 250 µm thickness. Tentative identifications were made by comparision with reference

standard lipids (Supelco and Applied Science Lab.). For quantitative analysis, the plates were sprayed with 50% sulfuric acid in saturated KCr_2O_4 and charred 45 minutes at 150°C in an oven. Relative densites were read in a densitometer (E-C Apparatus Corp.). The phosphate content of the freeze-dried LPS was measured by the Ames total phosphate method (5). The Lowrey protein assay was used as a check for LPS purity with bovine serum albumin as a standard (7).

Fatty acids from whole cells, REL and LPS were quantitated and qualitated by gas-liquid chromatography (GLC). Methyl esters were prepared by the boron trifluoride-methanol method of Metcalfe and Schmitz (37). Methyl esters were separated from non-esterfied fatty acids by using petroleum ether (30-60°):diethyl ether:glacial acetic acid (90:10:1). The characteristic yellow fluorescence of methyl esters was detected by ultraviolet light after the plate was sprayed with Rhodamine 6G (6 mg/100 ml water). Methylated fatty acids were scraped into scintered glass filters and extracted by n-hexane. Whole cell and LPS fatty acids were saponified and methylated by the boron trichloride method as described in the Supelco bulletin #767A, used specifically for the identification of hydroxy-fatty acids. The methylated fatty acids were identified using a Hewlett-Packard 5840A GLC equipped with a flame ionization detector, glass column (6 ft. x 4 mm) packed with two support systems. The first column was packed with 3% SP-2100 DOH on 100/120 Supelcoport with an

injection port temperature of 250°C and a detector temperature of 250°C. Column temperature was progammed to run for four minutes at 150°C then increased to 225°C at a rate of 4°C per minute. The second support system used for REL methyl esters consisted of a glass column (6 ft x 4 mm) packed with 10% SP-2330 on 100/120 Chromosorb, wih an injection port temperature of 200°C and a detector temperature of 225°C. Column temperature was programmed to run four minutes at 100°C then increased to 200°C at a rate of 10°C per minute. The carrier gas flow for both systems was N_2 at a rate of 40 ml per minute. Methyl esters were stored in hexane under N_2 gas at -20°C.

Statistics

The statistical significance of the values obtained in the chemical analyses was determined by performing the Student *t* test on the mean, employing the two-tailed *t* table (37).

CHAPTER III

RESULTS

REL

The percent readily extractable lipids (REL) in PAO1 and the adapted rifamycin strains, RIF G and RIF H, were determined gravimetrically. Analysis of the percent REL demonstrated significant quantitative changes in RIF G, as compared to PAO1. The percent REL in RIF H remained unchanged. PAO1, RIF G, and RIF H percent REL was 12.43%, 11.02% and 11.69% respectively (TABLE I). Percent phosphate was determined by the Ames total phosphate assay (14). RIF H REL was significantly changed in the phosphate content while the percent phosphate of RIF G remained unaltered. PAO1 had a value of 1.28 μmol PO_4/mg REL, RIF had 1.18 μmol PO_4/mg REL and RIF H had a significant change with a value of 1.56 μmol PO_4/mg REL. PAO1 REL was 12.8% PO_4 and 3.95% Pi. By comparison RIF G REL was 11.76% PO_4 and 3.66% Pi, and RIF H REL was 15.29% PO_4 and 4.79% Pi.

The REL fractions of susceptible and resistant cells were analyzed by thin layer chromatography (TLC) in a multisolvent system which separated the neutral lipids (nonpolar) and phospholipids (polar) (6). Analysis of these chromatograms demonstrated significant qualitative (Figure 4)

TABLE I

COMPARISON OF REL AND PO_4 AND RIFAMYCIN ADAPTED STRAINS

	PA01	RIF G	P	RIF H	P
% REL	12.43(±1.67)	11.02(±0.57)	<.025	11.69(±1.23)	>.20
μmol PO_4/ mg REL	1.28(±0.18)	1.18(±0.28)	>.10	1.56(±0.27)	<.001
%PO_4	12.80(±2.03)	11.76(±2.74)	>.10	15.29(±2.63)	<.005
%Pi	3.95(±0.54)	3.66(±0.83)	>.10	4.79(±0.08)	<.001

Values represent the means of seven to niney-two determinations with standard deviations given in parentheses.

Values determined by performing the Student *t* test on the means, employing the two-tailed *t* table. P values of 0.05 and lower were considered statistically significant. Comparison is with the PA01 strain.

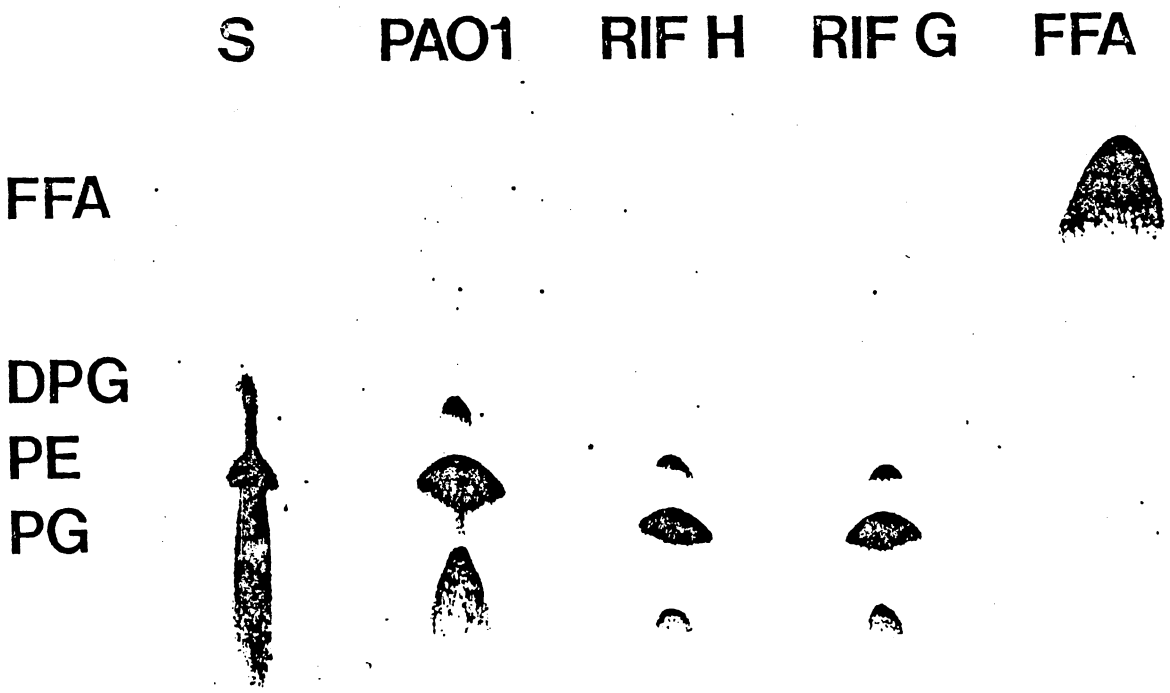

Figure 4. Relative Concentration of Major Phospholipids in REL in PAO1 and Rifamycin Adapted Strains. FFA = Free Fatty Acids, DPG = Diphosphatidyl glycerol, PE = Phosphatidyl ethanolamine, PG = Phosphatidyl gycerol, S = Phospholipid Standards, PAO1 = wild type Pseudomonas aeruginosa, RIF G, RIF H = Rifamycin Adapted Strains.

and quantitative changes (TABLE II). These changes occurred as a function of adapted rifamycin resistance. The lipid profile of RIF G and RIF H were significantly different in diphosphatidylglycerol (DPG) relative to the susceptible parent strain PAO1. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and free fatty acids (FFA) in the three strains, however, were unchanged. Comparitive analysis of percent REL phospholipids indicated that the percent FFA of PAO1, RIF G, and RIF H were 4.89%, 3.60% and 3.79% respectively. PAO1 REL percent DPG was 5.90%, RIF G was 7.91% and RIF H was 9.61%. Both RIF G and RIF H percent DPG were considered significantly different. PAO1 REL percent PE was 65.58% , RIF G was 66.82% and RIF H was 68.28%. The results for REL percent PG for PAO1, RIF G and RIFH was 23.62%, 21.67%, 18.84% respectively. There were no significant changes in either PE or PG for RIF G and RIF H.

Fatty Acid Composition

The fatty acid composition of the REL fraction from PAO1 and rifamycin adapted strains were analyzed by gas liquid chromatography (GLC) (37). Lipid analysis demonstrated significant qualitative and quantitative changes occurring in rifamycin adapted cells (TABLE III). The fatty acid profile of RIF G and RIF H were almost identical. In both RIF G and RIF H there occurred an almost two-fold increase in C_{14} and a three-fold increase in C_{18}. A two-fold decrease in $C_{16:1}$ and $C_{18:1}$ was observed in both strains. A

TABLE II

COMPARISON OF MAJOR PHOSPHOLIPIDS IN REL IN PAO1 AND RIFAMYCIN ADAPTED STRAINS

%	PA01	RIF G	P	RIF H	P
FFA	4.89(±2.30)	3.60(±1.29)	>.05	3.79(±1.03)	>.05
DPG	5.90(±1.26)	7.91(±2.70)	<.03	9.61(±2.85)	<.001
PE	65.58(±7.06)	66.82(±6.06)	>.05	68.28(±4.68)	>.20
PG	23.62(±7.39)	21.67(±6.27)	>.40	18.84(±4.00)	>.05

Values represent the means of fifteen to eighteen determinations with standard deviations given in parentheses.

Values determined by performing the Student *t* test on the means, employing the two-tailed *t* table. P values of 0.05 and lower were considered statistically significant. Comparison is with the PA01 strain.

TABLE III

FATTY ACID COMPOSITION OF REL FRACTION FROM PAO1 AND RIFAMYCIN ADAPTED STRAINS

Methyl Esters of Fatty Acids	Area % for strain/rifamycin (6000 U/ml) in growth medium				
	PA01/0	RIF G/6000	P	RIF H/6000	P
Saturated					
C_{14}	0.48(±0.02)	0.74(±0.03)	<.001	0.75(±0.07)	<.025
C_{16}	37.12(±0.67)	47.38(±2.93)	<.005	47.05(±2.10)	<.001
C_{18}	6.80(±0.28)	20.11(±1.77)	<.001	19.82(±0.98)	<.001
Unsaturated					
$C_{16:1}$	1.88(±0.40)	0.44(±0.37)	<.005	0.85(±0.14)	<.005
$C_{18:1}$	44.47(±1.91)	27.86(±1.77)	<.001	29.23(±0.45)	<.001
Cyclopropane					
C_{17cy}	2.23(±0.02)	0.65(±0.28)	<.001	0.62(±0.12)	<.001
C_{19cy}	5.02(±0.35)	0.66(±0.10)	<.001	0.84(±0.39)	<.001
Cyclopropane/ Unsaturated	0.17	0.05		0.05	

Values represent the means of three to seven determinations with standard deviations given in parentheses.

Values determined by performing the Student *t* test on the means, employing the two-tailed *t* table. P values of 0.05 and lower were considered statistically significant. Comparison is with the PA01 strain.

Values approximate 100% due to rounding and ignoring of unidentified peaks.

four-fold decrease was found in cyclopropyl C_{17} and cyclopropyl C_{19} for both strains. PAO1 REL was 0.48% C_{14}, as compared to RIF G, 0.74% and RIF H, 0.75%. For C_{18}, PAO1 REL was 6.80%, RIF G was 20.11% and RIF H was 19.82%. For C_{16}, PAO1 REL was 37.12%, RIF G was 47.38% and RIF H was 47.15%. All the saturated straight chain values were significant. PAO1 REL was 44.47% C_{18}, while RIF G was 27.86% and RIF H was 29.23%. PAO1 REL was 1.88% C_{16}, RIF G was 0.44% and RIF H was 0.85%. All the differences between the parent strain and the adapted strains for the unsaturated fatty acids were statistically significant. The same was true for the cyclopropyl fatty acids. PAO1 REL was 2.23% cyclopropyl C_{17}, RIF G was 0.65% and RIF H was 0.62%. PAO1 REL was 5.02% cyclopropyl C_{19}, RIF G was 0.66% and RIF H was 0.84%. The ratio for cyclopropyl fatty acids to unsaturated fatty acids were 0.17% for PAO1, 0.05% for RIF G and 0.05% for RIF H.

LPS

The LPS from PAO1 and polymyxin adapted and reverted strains were isolated by the Darveau and Hancock method which isolated both smooth and rough forms of LPS (7). The percent LPS was determined gravimetrically (TABLE IV). PAO1 percent LPS was 9.15%, IA was 10.79% and IAR was 10.29%. The resistant and reverted strains both had significant changes in the percent LPS. The percent phosphate was determined and both strains showed significant changes. PAO1 LPS was 12.53%

TABLE IV

COMPARISON OF LPS AND PO_4 AND POLY B ADAPTED STRAINS

	PA01	IA	P	IAR	P
% LPS	9.15(±0.75)	10.79(±1.39)	<.025	10.29(±1.69)	>.10
umol PO_4/ mg LPS	1.29(±0.06)	1.57(±0.13)	<.001	1.05(±0.09)	<.001
u%PO_4	12.53(±0.57)	15.31(±1.17)	<.001	10.09(±0.99)	<.001
%Pi	4.04(±0.29)	4.88(±0.34)	<.001	3.20(±0.29)	<.001

Values represent the means of seven to twenty-one determinations with standard deviations given in parentheses.

Values determined by performing the Student *t* test on the means, employing the two-tailed *t* table. P values of 0.05 and lower were considered statistically significant. Comparison is with the PA01 strain.

PO_4, IA was 15.31% and IAR was 10.09%.

The carbohydrates in LPS in PAO1 and polymyxin adapted strains were determined quantitatively. Both the IA and IAR percent hexose were significantly changed as compared to PAO1, with IAR approximating those of PAO1 (TABLE V). PAO1 LPS was 3.60% hexose, IA was 1.94% hexose and IAR was 2.42% hexose. IAR showed significant decrease in the percent KDO present while IA did not change. PAO1 LPS was 4.04% KDO, IAR was 4.17% and IAR was 3.64%. The percent heptose of IA was significantly higher than the PAO1 wild type. The percent heptose of IAR was unchanged from that of PAO1. PAO1 LPS was 8.94% heptose, IA was 9.44% and IAR was 9.02%. Both revertant and resistant strains demonstrated a significant change in the total carbohydrates. IA was significantly lower and IAR was significantly higher than the PAO1 wild type strain. PAO1 LPS was 10.15% total carbohydrates, IA was 7.18% and IAR was 11.83%.

The OM LPS of PAO1 and polymyxin resistant and revertant strains along with the PAO1 whole cell lipids were extracted by the saponification and esterfication method in Supelco bulletin #767A. The fatty acids esterfied to PAO1 LPS are quite different from PAO1 whole cell fatty acids (TABLE VI). PAO1 LPS had a significantly greater percentage of 3-hydroxydecanoate (3-OH C_{10}), 2- and 3-hydroxydodecanoate (2- and 3-OH C_{12}), dodecanoate (C_{12}) and hexadecanoate (C_{16}). IA and IAR approximated the fatty acid content of wild type PAO1 in

TABLE V

COMPARISON OF CARBOHYDRATES IN LPS IN PAO1 AND POLY B ADAPTED STRAINS

	PAO1	IA	P	IAR	P
Hexose	3.60(±0.26)	1.94(±0.13)	<.001	2.42(±0.20)	<.001
KDO	4.04(±0.21)	4.17(±0.32)	>.20	3.64(±0.32)	<.005
Heptose	8.94(±0.20)	9.44(±0.27)	<.001	9.02(±0.27)	>.40
Total Carbohydrate	10.15(±0.79)	7.18(±0.89)	<.001	11.83(±1.50)	<.005

Values represent the means of eight to twelve determinations with standard deviations given in parentheses.

Values determined by performing the Student *t* test on the means, employing the two-tailed *t* table. P values of 0.05 and lower were considered statistically significant. Comparison is with the PAO1 strain.

TABLE VI

FATTY ACID COMPOSITION OF LPS FRACTION FROM PAO1 AND POLY B ADAPTED STRAINS

Methyl Esters of Fatty Acids	Area % for strain/poly B (6000 u/ml) in growth medium						
Hydroxy	PA01 (WC)*/0	PA01/0	P	IA/6000	P	IAR/0	P
3-OH C_{10}	4.65 (±0.77)	9.87 (±0.50)	<.001	11.57 (±1.52)	<.025	13.95 (±2.01)	<.001
2-OH C_{12}	0.00 (±0.00)	0.53 (±0.27)	<.005	0.86 (±0.26)	<.001	0.13 (±0.02)	<.001
3-OH C_{12}	0.00 (±0.00)	6.04 (±1.45)	<.005	0.86 (±2.09)	<.001	0.00 (±0.00)	>.05
Saturated							
C_{12}	3.45 (±0.65)	5.58 (±0.49)	<.001	3.40 (±1.83)	>.05	0.13 (±0.02)	<.001
C_{14}	5.99 (±1.00)	4.16 (±1.34)	<.05	0.00 (±0.00)	<.001	0.66 (±0.11)	<.001
C_{16}	18.66 (±2.21)	25.92 (±1.67)	<.001	19.89 (±2.36)	>.05	36.40 (±4.35)	<.001
C_{18}	2.27 (±0.41)	2.83 (±0.84)	>.05	2.64 (±0.60)	>.05	2.49 (±0.48)	>.05
Unsaturated							
$C_{16:1}$	7.71 (±0.44)	4.18 (±0.87)	<.001	6.60 (±0.70)	>.05	5.80 (±0.37)	<.001
$C_{18:1}$	27.62 (±0.41)	23.55 (±1.44)	<.005	28.18 (±2.76)	>.05	30.19 (±1.09)	>.05
Cyclopropane							
C_{17cy}	6.70 (±1.57)	1.91 (±0.40)	<.001	2.49 (±1.18)	<.001	1.65 (±1.92)	<.01
C_{19cy}	9.01 (±0.42)	3.67 (±0.91)	<.001	3.69 (±0.44)	<.001	4.64 (±1.98)	<.005

Values represent the means of three to nine determinations with standard deviations given in parentheses.

Values determined by performing the Student *t* test on the means, employing the two-tailed *t* table. P values of 0.05 and lower were considered statistically significant. Comparison is with the PA01 strain.

*WC = Whole Cells

Values approximate 100% due to rounding and ignoring of unidentified peaks.

$C_{18:1}$ and C_{18}. PAO1 fatty acids derived from whole cells had no measurable 2- or 3-OH C_{12}, but had a significantly reduced 3-OH C_{10} in comparison with IA and IAR. IA LPS had no C_{12} and IAR had a significant decrease in C_{12}. IA LPS and IAR LPS had little cyclopropyl fatty acids.

In TABLE VII, IA LPS and IAR LPS are compared to PAO1 LPS. The fatty acid compositions are significantly altered in the following fatty acids: 3-OH C_{10}, 3-OH C_{12}, C_{12}, C_{14}, C_{16}, C_{18}, $C_{16:1}$. IA had significant increases in the percentage of 3-OH C_{10}, 3-OH C_{12}, $C_{16:1}$ and $C_{18:1}$. IAR LPS was significantly increased in the percentage of 3-OH C_{10}, $C_{16:1}$, C_{16} and C_{18}. Both strains were reduced significantly in C_{12} and C_{14}. The four most prominant fatty acid in IA were 3-OH C_{10} (11.57%), 3-OH C_{12} (12.54%), C_{16} (19.98%) and $C_{18:1}$ (28.18%). The four most prominant fatty acids in IAR were 3-OH C_{10} (13.95%), $C_{16:1}$ (5.80%), C_{16} (36.40%), and $C_{18:1}$ (30.19%). These were compared to the PAO1 LPS which was 9.57% 3-OH C_{10}, 6.04% 3-OH C_{12}, 4.18% $C_{16:1}$, 25.92% C_{16}, and 23.55% $C_{18:1}$.

Percent REL of RIF G was decreased significantly as compared to PAO1. The percent PO_4 of RIF G REL remained unaltered while RIF H REL percent PO_4 was significantly increased. REL percent DPG was significantly increased for both RIF G and RIF H, as were all the fatty acids in the REL. The percent LPS in the polymyxin strains were all significantly increased. The IA LPS percent

TABLE VII

FATTY ACID COMPOSITION OF LPS FRACTION FROM PAO1 AND POLY B ADAPTED STRAINS

Methyl Esters of Fatty Acids	Area % for strain/poly B (6000 U/ml) in growth medium				
	PA01/0	IA/6000	P	IAR/0	P
Hydroxy					
3-OH C_{10}	9.87(±0.50)	11.57(±1.52)	<.025	13.95(±0.01)	<.005
2-OH C_{12}	0.53(±0.27)	0.86(±0.26)	<.05	0.00(±0.02)	<.05
3-OH C_{12}	6.04(±1.45)	12.54(±2.09)	<.001	0.00(±0.00)	<.005
Saturated					
C_{12}	5.58(±0.49)	3.40(±1.83)	<.025	0.13(±0.02)	<.001
C_{14}	4.16(±1.34)	0.00(±0.00)	<.005	0.66(±0.11)	<.005
C_{16}	25.92(±1.67)	19.89(±2.36)	<.001	36.40(±4.35)	<.001
C_{18}	2.83(±0.84)	2.64(±0.60)	<.05	2.49(±0.48)	<.05
Unsaturated					
$C_{16:1}$	4.18(±0.87)	6.60(±0.70)	<.001	5.80(±0.37)	<.025
$C_{18:1}$	23.55(±1.44)	28.18(±2.76)	<.005	30.19(±1.09)	<.001
Cyclopropane					
C_{17cy}	1.91(±0.40)	2.49(±1.18)	>.05	1.65(±1.92)	>.05
C_{19cy}	3.67(±0.91)	3.69(±0.44)	>.05	4.64(±1.98)	<.05

Values represent the means of three to nine determinations with standard deviations given in parentheses.

Values determined by performing the Student *t* test on the means, employing the two-tailed *t* table. P values of 0.05 and lower were considered statistically significant. Comparison is with the PA01 strain.

Values approximate 100% due to rounding and ignoring of unidentified peaks.

PO_4 was significantly increased while the IAR LPS was significantly decreased. The hydroxy fatty acids results of the poly B strains indicate that the hydroxy fatty acids increase in response to antibiotic resistance.

CHAPTER IV

DISCUSSION

Pseudomonas aeruginosa is resistant to a wide variety of antibacterial agents. It has the intrinsic mechanism to adapt to 6000 U/ml polymyxin B (poly B) and 6000 U/ml rifamycin and can revert back to its original wild type susceptibility. Therefore, the resistant strains used in this study are adapted to the antibiotic, and are not true genetic mutant strains. Pseudomonas resistance to antibiotics such as poly B is due to low permeability in the outer membrane (OM). The favored sites for resistance are the phospholipids and lipopolysaccharide (LPS) which compromise the outer layer of the OM structure. Since antibiotic resistance to poly B in the pseudomonads is due in part as a function of alterations in the readily extractable lipids (REL), we studied the more non-polar rifamycin antibiotic to determine what changes there are in rifamycin-resistant REL. LPS, has been identified as a binding site for poly B, and was therefore studied in order to determine what role it plays in the resistance mechanism. We determined the percentage of total carbohydrates, phosphate and fatty acids of the LPS in wild type (PAO1), poly B-resistant strain (IA), and the

revertant strain (IAR).

The rifamycin resistant strains, RIF G and RIF H, differed biochemically from the poly B-resistant strain, IA, as shown in the REL alterations. The percent REL of both RIF H and RIF G decreased as compared to the wild type PAO1, whereas the change in the percent REL for IA resulted in a significant increase. RIF G had a significant decrease in REL, whereas RIF H was similar to that of PAO1. RIF H and IA both had significant changes in the percent phosphate (μmol/mg of REL) with RIF H increasing its percent phosphate and IA decreasing its percent phosphate. The percent REL and percent phosphate changes clearly are not identical for IA, RIF H and RIF G strains. Since poly B and rifamycin compete for binding space with phosphate, magnesium ions and calcium ions, the increase in phosphate prevents rifamycin from finding an adequate number of compatable binding sites on the OM surface.

Thin layer chromatography revealed no differences in phospholipid content of the REL of RIF H and RIF G. The only significant change in the major phospholipids in the REL of the rifamycin-resistant strains was diphosphatidyl glycerol (DPG), whereby both strains had significant increases. The poly B resistant strain (IA) has significantly decreased phosphatidyl ethanolamine (PE) and phosphatidyl glycerol (PG), and significantly increased in its DPG. In the rifamycin-resistant strains, no significant loss of receptor phospholipids was found as was

detected in the poly B-resistant strain, (IA). In the poly B-resistant system, there was a decreased number of phospholipid receptor sites, indicating an exclusion mechanism at the OM. The rifamycin-resistant system prevents the rifamycin molecule from binding due to a competition mechanism, whereby the phosphate molecule competes for space with the rifamycin molecule.

In poly B-resistant cells, the loss of the negatively charged phospholipids PG and PE could reduce the ability somewhat of the OM to bind polymyxin. The decreased percent of phosphate and the loss of acidic phospholipids, would make the OM of resistant cells more resistant to poly B binding and give resistance, especially to low concentration of antibiotic, to the cell. The phospholipids have mainly a structural function in the OM due to the phospholipid's unusual physiochemical properties. Yet, the rifamycin-resistant cells did not demonstrate a significant loss of PE or PG. The concentration of phosphate did significantly increase in RIF H, however.

The fatty acid composition of the RIF G and RIF H were almost identical results. However, the fatty acid content of rifamycin-resistant strains were different from the poly B resistant strain. The IA strain had decreased percent saturated straight chain fatty acids in comparison to PAO1, while RIF H and RIF G had increased percent saturated straight chain. The results were also opposite for the unsaturated straight chain fatty acids. IA had a

significantly increased amount of unsaturated fatty acids and the rifamycin strains had a significantly decreased amount. These diverges in fatty acid content could be due to the fact that rifamycin is much more non-polar than poly B. Decreasing the amount of unsaturated straight chain fatty acids facilitates the cell to become less amenable to penetration by a hydrophobic molecule. Peptide antibiotics, such as poly B, form complexes with metal ions and disrupts the control of ion permeablitiy in the OM. Rifamyin, being a polyene antibiotic, interferes with proton and ion transport (8).

The ratio of cyclopropane to unsaturated fatty acids decreased in the rifamycin strains. This was identical to changes noted in IA as compared to the wild type strain. These alterations in unsaturated/cyclopropyl ratio might be necessary in IA to bring about the proper OM fluidity characteristics in the presence of decreased OM LPS and protein; however, this is not true for the rifamycin strains. The amount of unsaturated fatty acid in the rifamycin strains actually decreases overall. The ratio demonstrates that the cyclopropyl fatty acids decrease more dramatically than the unsaturated fatty acids. Lowering the cyclopropane fatty acid content results in a fatty acid composition similar to that of mammalian cell membranes, which are more resistant to polymyxin than are bacterial cell membranes.

The REL, phosphate and fatty acid alterations in the poly B-resistant strains strongly suggested the existence

of an exclusion mechanism previously discussed. However, the REL, phosphate and fatty acid changes in the rifamycin strains are not as dramatic, suggesting a different mechanism of resistance. Perhaps the main thrust of the cell's resistance lies in the cytoplasm. In a resistant mutant the RNA polymerase beta subunit binding site has a decreased affinity for the antibiotic (8). Therefore a partial exclusion mechanism whereby the OM calcium and magnesium ions compete for binding sites with the antibiiotic on the OM surface and a decreased affinity of the binding site in the cytoplasm would both seem to operate in the resistance mechanism.

These experiments does not allow us to conclusively determine the mechanisms of resistance in the rifamycin strains. The OM is a dynamic structure and a change in one of its chemical components may be the cell mechanism for compensating for a change in another chemical component. For example, the decrease in percent REL may be compensated for by an increase in porin proteins which could act to exclude the non-polar antibiotic. The increase in saturated fatty acids could be a compensation for the decrease in unsaturated fatty acids. This decrease in unsaturated fatty acids could make the OM less fluid and thus less responsive to the rifamycin molecule. Therefore, unless all components are monitored simultaneously, interpretations will be difficult, especially in a system such as the development of antibiotic resistance.

In the second part of this study the role of LPS in

the polymyxin B strains and its possible contribution to the mechanism of resistance was investigated. LPS constitutes a major role of the OM in terms of a permeability barrier. The data indicated that LPS also plays a major role in the mechanism of resistance to antibiotics. Kropinski and his colleagues found that the altered LPS of antibiotic-supersusceptible cells resulted in a higher OM permeability (30). Tsang and his colleagues examined electron micrographs and poly-acrylamide gel electrophoresis of poly B-resistant and susceptible cells of Serratia marcescens and reported the formation of complexes between poly B and LPS in the susceptible cells (46).

The smooth LPS (minus the O antigen) in Pseudomonas aeruginosa varies from 0.2 to 13.7 mol% of the total LPS (24). For this reason, in our study the LPS isolation precedure of Darveau and Hancock was used as it isolates both smooth and rough pseudomonal types (7).

The resistant strain IA showed a slight significant increase in the percent LPS, while the percent LPS of IAR was similar to PAO1. The rich phosphate content is found within the inner region of the LPS core, mostly in the heptose residues. The percent phosphate in IA increased in proportion to the increase in percent LPS, as did the percent heptose. The changes in the level of heptose is known to reflect the compositional variation in LPS (9). Our resistant cells produced only slight changes in the percent LPS, even though LPS composition changes in

accordance with environmental factors and that the cells respond by an increase or a decrease in the amount of LPS. Therefore, it is likely that the changes in LPS due to antibiotic resistance is in the LPS composition and not in the level of LPS. The percent total carbohydrate significantly increased and at the same time the percent KDO decreased. This could be of a compensatory nature.

The low recovery of LPS could be due to the presence of acid-labile or other unusual components (32), possibly as a result of a lack of chemical modification during treatment (30). These calculations do not take into consideration the probability of incomplete release during hydrolysis and destruction following release.

LPS directly influences the number of functional open porin protein pores. The porin protein F is usually associated with LPS. Removal of LPS from porin protein renders the protein inactive in black lipid bilayer studies (31). LPS may interact with porin protein through charged phosphate groups or via hydrophobic interactions with acyl chains on lipid residues, in such a way as to determine their orientation or surface exposure. Several OM porin proteins were significantly reduced in one study using a poly B-resistant mutant strain (15). In several other studies, pseudomonal strains adapted to poly B resistance showed no such significant reduction (7).

The largest difference between PAO1 LPS and IA LPS was the change in fatty acid content. In comparing a pseudomonad wild type and a supersusceptible mutant

Kropinski found no significant alterations in the overall fatty acid content (30). Their study found several differences in the fatty acids of resistant and susceptible strains. The poly B-resistant strain, IA, had the five fatty acids most commonly found in the LPS of pseudomonad strains to include: 3-hydroxy decanoate (3-OH C_{10}), dodecanoate (C_{12}), 2- and 3-hydroxy dodecanoate (2- and 3-OH C_{12}), and hexadecanoate (C_{16}). All the hydroxy fatty acids significantly increased in the IA strain, while 2- and 3- OH fatty acids significantly decreased in the IAR strain. There was a significant decrease in the saturated fatty acid of IA with a corresponding increase in the unsaturated fatty acids. The cyclopropyl fatty acids slightly increased in the IA strain. The low 3-OH C_{12} level was due in part to the fact that it is released only after acid hydrolysis. We elected not to perform acid hydrolysis on the LPS as this is a harsh procedure and is responsible for the loss of a large percentage of hydroxy fatty aids (22).

The REL, phosphate and fatty acid alterations in the rifamycin-resistant strains were not as extensive as the alterations in the REL of poly B-resistant strain, IA. Therefore the exclusion mechanism on the OM may not be the primary mechanism of antibiotic resistance. The fatty acid alterations for LPS and REL were very similar. There was a greater percent decrease in the IA LPS saturated fatty acids and a greater percent increase in the unsaturated fatty acids. Both REL and LPS fatty acids undergo similar

alterations in antibiotic-resistant strains. These findings stongly suggest that the fatty acids play a major role in the mechanism of antibiotic resistance in the pseudomonads.

CHAPTER V

SUMMARY AND CONCLUSIONS

This study looked at Pseudomonas aeruginosa, a primary opportunistic pathogen which has an intrinsic ability to adapt to 6000 U/ml of polymyxin B and 6000 U/ml rifamycin. I examined the differences in the readily extractable lipids (REL) between the wild type (PAO1) strain and the rifamycin adapted (RIF G and RIF H) strains, and the differences in the lipopolysaccharide (LPS) between PAO1, the polymyxin adapted (IA) strain, and the polymyxin deadapted (IAR) strain. The REL of rifamycin resistant strains was compared to the REL of polymyxin resistant strain.

This study indicated that the alterations in the REL, phosphate and fatty acids of the rifamycin strains as compared to the wild type strain were less statistically significant than those of the polymyxin strain. The major change in the phospholipids in the REL of the rifamycin resistant strains was diphosphatidyl glycerol (DPG), whereby both strains had significant increases. Both rifamycin resistant strains had a significant increase in the saturated fatty acid chains and a significant decrease in the unsaturated chains. It would be of value to monitor both the outer membrane (OM) fatty acids and the target

site in the cytoplasm, the RNA polymerase.

The major alterations in the OM LPS of polymyxin resistant strain IA were in the fatty acid content. All the hydroxy fatty acids in the IA strain showed significant increases. There was a significant decrease in the saturated fatty acids in the IA strain with a corresponding increase in the unsaturated fatty acids. The cyclopropyl fatty acids significantly increased in the IA strains. These alterations were similar to those of the REL of polymyxin resistant strains. These findings suggest that the OM fatty acids share a major role in the exclusion mechanism of polymyxin resistance.

LITERATURE CITED

1. Angus, Barbara, Alice M. Carey, Dania A. Caron, Andrew M. B. Kropinski and R. E. W. Hancock. 1982. Outer membrane permeability in Pseudomonas aeruginosa: Comparison of a Wild-type with an Antibiotic-supersensitive Mutant. Antimicrob. Agents and Chemother. 21: 229-309.

2. Brown, M. R. W. 1973. The Role of the Cell Envelope in Resistance. In Resistance of Pseudomonas aeruginosa. Ed. by M. R. W. Brown. John Wiley and Sons, Inc. New York, pp. 71-107.

3. Brown, M. R. W. and Sandra M. Wood. 1972. Relation Between Cation and Lipid Content of Cell Walls of Pseudomonas aeruginosa, Proteus vulgaris and Klebsiella aerogenes and Their Sensitivity to Polymyxin B and Other Antibacterial Agents. J. Pharm. Pharmoc. 24: 215-218.

4. Conrad, Robert S., and H. E. Gilleland Jr. 1981. Lipid Alterations in Cell Envelopes of Polymyxin-Resistant Pseudomonas aeruginosa Isolates. J. Bacteriol. 148: 487-489.

5. Conrad, Robert S., R. Gary Wulf and Kathy A. Ankrom. 1981. Fatty Acids of Pseudomonas aeruginosa Grown on Media Affecting Polymyxin Susceptibility. Current Microbiol. 5: 231-234.

6. Conrad, Robert S., R. Gary Wulf and Damonda L. Clay. 1979. Effects of Carbon Sources on Antibiotic Resistance in Pseudomonas aeruginosa. Antimicro. Agents and Chemother. 5: 59-66.

7. Darveau, R. P. and R. E. W. Hancock. 1983. Procedure for Isolation of Bacterial LPS from both Smooth and Rough Pseudomonas aeruginosa and Salmonella typhimurium strains. J. Bacteriol. 155: 831-838.

8. Davis, Benard D., Renato Dulbecco, Herman N. Eisen, and Harold S. Ginsberg. Microbiology. 3rd. Ed. Harper & Row Pub. 1980. pp. 91-92.

9. Day, Donal F., and M. Lorraine Marceau-Day. 1982. Lipopolysaccharide Variability in Pseudomonas aeruginosa. Current Microb. 7: 93-98.

10. Doggett, R. G. and G. M. Harrison. 1972. Pseudomonas aeruginosa: Immune Status in Patients with Cystic Fibrosis. Infect. and Immun. 6: 628-635.

11. Dubois, M., K. A. Gilles, J. K. Hamilton, P.A. Rebers and F. Smith. 1956. Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 28: 350-356.

12. Dunnick, J. K. and W. M. O'Leary. 1970. Correlation of Bacterial Lipid Composition with Antibiotic Resistance. J. Bacteriol. 101: 892-900.

13. El Mashak, E. M. and J. F. Tocanne. 1980. Polymyxin B - Phosphatidylglycerol Interaction, a Monolayer (v) Study. Biochem. Biophys. Acta. 596: 165-179.

14. Floch, J. M. Lees and G. H. S. Stanley. 1957. A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissue. J. Biol. Chem. 226: 497-509.

15. Gilleland, Harry E., Jr., and M. W. Beckham. 1982. Protein Alterations in Isolated Outer Membrane of Polymyxin-Resistant Pseudomonas aeruginosa Isolates. Curr. Microbiol. 7: 235-240.

16. Gilleland, H. E., Jr., and Robert S. Conrad. 1980. Effects of Carbon Sources on Chemical Composition of Cell Envelopes of Pseudomonas aeruginosa in Association with Polymyxin Resistance. Antimicrob. Agents Chemother. 17: 623-281.

17. Gilleland, H. E., Jr., and Linda B. Farley. 1982. Adaptive Resistance to Polymyxin in Pseudomonas aeruginosa Due to an Outer Membrane Impermeability Mechanism. Canadian J. Microbiol. 28: 830-840.

18. Gilleland, H. E., Jr., and R. D. Lyle. 1979. Chemical Alterations in Cell Envelopes of Polymyxin-Resistant Pseudomonas aeruginosa Isolates. J. Bacteriol. 138: 839-845.

19. Gilleland, H. E., Jr., and R. G. E. Murray. 1976. Ultrastructural Study of Polymyxin-Resistant Isolates of Pseudomonas aeruginosa. J. Bacteriol. 125: 267-281.

20. Goldman, Robert C., and Loretta Leive. 1980. Heterogeneity of Antigenic-Side-Chain Length In Lipopolysaccharide from Escherichia coli 0111 and Salmonella typhimurium LT2. Eur. J. Biochem. 107: 145-153.

21. Hancock, I. C. and Pauline Meadow. 1969. The Extractable Lipids of Pseudomonas aeruginosa. Biochem. Biophys. Acta. 187: 366-379.

22. Hancock, R. E. W., Gary M. Decad and Hiroshi Nikaido. 1979. Identification of the Protein Producing Transmembrane Diffusion Pores in the Outer Membrane of Pseudomonas aeruginosa PAO1. Biochem. Biophys. Acta. 554: 323-331.

23. Hancock, R. E. W. et. al. 1983. Pseudomonas aeruginosa Isolates from Patients with Cystic Fibrosis: A Class of Serum Sensitive, Nontypable Strains Deficient in Lipopolysaccharide O-Side Chains. Infec. Immun. 42: 170-177.

24. Hartman, W., H. J. Galla and E. Sackmann. 1978. Polymyxin Binding to Charged Lipid Membranes, an Example of Cooperative Lipid-Protein Interaction. Biochem. Biophys. Acta. 510: 124-139.

25. Hitchcock, Penny J. and Teresa M. Brown. 1983. Morphological Heterogeneity Among Salmonella Lipopolysaccharide Chemotypes in Silver-Stained Polyacrylamide Gels. J. Bacteriol. 154: 269-277.

26. Hoiby, Niels, Vagn Anderson, and Gunnar Bendixen. 1975. Pseudomonas aeruginosa Infection in Cystic Fibrosis. Acta. Path. Microbiol. Scand. Sect. C. 83: 459-468.

27. Hoiby, Niels, et. al. 1974. Pseudomonas aeruginosa Infection in Cystic Fibrosis. Acta. Paediatr. Scand. 63: 843-848.

28. Humphreys, G. O., I. C. Hancock and Pauline Meadow. 1972. Synthesis of the Hydroxy Acids in Lipid A of Pseudomonas aeruginosa. J. of Gen. Microbiol. 71: 221-230.

29. Kropinski, Andrew M., L. C. Chan and F. H. Milazzo. 1979. The Extraction and Analysis of Lipopolysaccharide from Pseudomonas aeruginosa Strain PAO, and the three Rough Mutants. Canad. J. Microbiol. 25: 390-398.

30. Kropinski, Andrew M. John Kuzio, B. L. Angus, and R. E. W. Hancock. 1982. Chemical and Chromatographic Analysis of Lipopolysaccharide from an Antibiotic-Supersusceptible Mutant of Pseudomonas aeruginosa. Antimicrob. Agents and Chemother. 21: 310-319.

31. Luderitz, Otto. 1970. Recent Results on the Biochemistry of the Cell Wall Lipopolysaccharide of Salmonella Bacteria. Angew. Chem. Internat. Edit. 9: 649-663.

32. Luderitz, Otto et. al. 1973. Lipid A: Chemical Structure and Biological Activity. J. Infect. Disease. 128: S17-S29.

33. Marks, Meliun I. 1981. The Pathogenesis and Treatment of Pulmonary Infections in Patients with Cystic Fibrosis. J. Ped. 98: 173-179.

34. McKevitt, Aideen I., and Donald E. Woods. 1984. Characterization of Pseudomonas cepacia Isolates from Patients with Cystic Fibrosis. J. Clinical Microb. 19: 291-293.

35. McKey, Robert M., Jr. Cystic Fibrosis in Private Practice. Harper & Row Pub. 1980. pp.30-45.

36. McLaughlin, F. J., W. J. Matthews, et. al. 1983. Clinical and Bacterilogical Responses to three Antibiotic Regiments for Acute Exacerbations of Cystic Fibrosis: Ticarcillin-Tobramycin, Azlocillin-Tobramycin, and Azlocillin-Placebo. J. Infec. Diseas. 147: 559-567.

37. Metcalfe, L. D., and A. A. Schmitz. 1961. The Rapid Preparation of Fatty Acid Esters for Gas Chromatographic Analysis. Anal. Chem. 33: 363-364.

38. Miller, I. R., D. Bach and M. Teuber. 1978. Effect of Polymyxin B on the Structure and the Stability of Lipid Layers. J. Membr. Biol. 39: 49-56.

39. Morrison, D. C. and D. M. Jacobs. 1976. Binding of Polymyxin B to the Lipid A Portion of Bacterial Lipopolysaccharide. Immunochemistry. 13: 813-818.

40. Nicas, Thalia I. and R. E. W. Hancock. 1983. Pseudomonas aeruginosa Outer Membrane Permeability Isolation of a Porin Protein F-Deficient Mutant. J. Bacteriol. 153: 281-285.

41. Ohman, Dennis E., and A. M. Chakrabarty. 1982. Utilization of Human Respiratory Secretions by Mucoid Pseudomonas aeruginosa of Cystic Fibrosis Origin. Infect. & Immun. 37: 662-669.

42. Rietschel, Ernst Th., H. Gottert Otto Luderitz and Otto Westphal. 1972. Nature and Linkages of the Fatty Acids Present in the Lipid A Component of Salmonella Lipopolysaccharides. Eur. J. Biochem. 28: 166-173.

43. Roberts, N. A., G. W. Gray and S.G. Wilkinson. 1967. Release of Lipopolysaccharide During the Preparation of Cell Walls of Pseudomonas aeruginosa. Biochem. Biophys. Acta. 135: 1068-1071.

44. Sixl, F. and H. J. Gallo. 1979. Cooperative Lipid-Protein Interaction. Effect of pH and Ionic Strength of Polymyxin Binding to Phosphatidic Acid Membrane. Biochem. Biophys. Acta. 557: 320-330.

45. Storm, Daniel, R. Kenneth S. Rosenthal, and Paul E. Swanson. 1977. Polymyxin and Related Peptide Antibiotics. Ann. Rev. Biochem. 46: 723-763.

46. Tsang, Joseph C., Douglas A. Weber, and David A. Brown. 1976. Evidences for Complex Formation Between Polymyxin B and Lipopolysaccharide from Serratia marcescens. J. Antibiot. 29: 735-742.

47. Weissbach, A. and J. Hurwitz. 1959. The Formation of 2-Keto-3-Deoxy-Heptonic Acid in Extracts of Escherichia coli B. I. Identification. J. Biol. Chem. 234: 705-709.

48. Woods, Donald E., Stanley J. Cryz, R. L. Friedman and Barbara H. Iglewski. 1982. Contribution of Toxin A and Elastase to Virulance of Pseudomonas aeruginosa in Chronic Lung Infections of Rats. Infect. and Immun. 36: 1123-1128.

49. Wright, B. G., and P. A. Rebers. 1972. Procedure for Determining Heptose and Hexose in Lipopolysaccharide Modification of the Cysteine-Sulfuric Acid Method. Anal. Biochem. 49: 307-319.

50. Yoshimura, Fuminobu and Hiroshi Nikaido. 1982. Permeability of Pseudomonas aeruginosa Outer Membrane to Hydrophilic Solutes. J. Bacteriol. 152: 636-642.

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