CHARACTERIZATION OF A COMMON ANTIGEN LIPOPOLYSACCHARIDE FROM *PSEUDOMONAS AERUGINOSA* AK1401

By

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ABSTRACT

CHARACTERIZATION OF A COMMON ANTIGEN LIPOPOLYSACCHARIDE FROM *PSEUDOMONAS AERUGINOSA* AK1401

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Lipopolysaccharide (LPS) isolated from *Pseudomonas aeruginosa* PAO1 (05 serotype) was separated by gel filtration chromatography into two antigenically distinct populations: the A-band and B-band LPS. The A-band population, containing shorter polysaccharide chains (~30 repeat units), reacted with a monoclonal antibody (MAb) to a *P. aeruginosa* common antigen but did not react with antibodies specific to 05-serotype LPS. In contrast, the LPS population containing long polysaccharide chains (B-band) (>30 repeat units) reacted only with the 05-specific MAbs. Chemical analysis of the A-band or common antigen LPS indicated a lack of reactive amino sugar and phosphate, although low levels of heptose and 2-keto-3-deoxyoctulosonic acid were detected. Also, high levels of rhamnose and stoichiometric amounts of sulfate were detected in this LPS isolate; the fatty acid composition was similar to that of the O-antigen-specific or

B band LPS. These results imply that PAO1 strains synthesize two type of molecules that are antigenically and chemically distinct.

To analyze the effect of various growth conditions on the size heterogeneity of LPS, *P. aeruginosa* PAO1 was grown in various media and at different temperatures. The size distribution of the serotype-specific or B-band LPS and the A-band or common antigen LPS were analyzed by both polyacrylamide gel electrophoresis and immunoblots. Cells grown at high, near growth-limiting temperatures, at low pH, in low concentrations of phosphate, and in high osmotic strength or salt concentrations, produced decreased amounts of very long chain populations of O-antigen LPS molecules. Lower temperature and lower osmotic strength, low sulfate, lower salt concentration, and elevated pH did not affect the level of this LPS population. The size and amount of common antigen LPS was not significantly affected when the cells were grown under the above stress conditions. Cells grown under normal, nonstressed conditions were agglutinated only by serotype-specific MAbs. In contrast, cells grown under stress conditions, in which the long-O-polymer LPS was absent, were agglutinated by both serotype specific and common antigen-specific MAbs. The results indicate that specific growth conditions limit the production of the long-O-polymer, allowing the exposure and reactivity of the common antigen on the cell surface.

To corroborate that sulfur is incorporated into A-band LPS in stoichiometric amounts, *P. aeruginosa* AK1401 was grown in ^{35}S-labelled sulfate. The advantage of using this PAO strain is that it is a convenient source of A-band LPS

since the synthesis of O-antigen is defective. Gel filtration chromatography separated the LPS into two major size populations: the A-band and B-band or short chain (SC)-LPS. The elution profile, as well as the autoradiogram and the inductively coupled plasma spectroscopy data showed that the A-band and B-band LPS contained labelled sulfur. Thus, A-band LPS contains stoichiometric amounts of covalently bound sulfur, perhaps as sulfate.

Finally, to further analyze the core oligosaccharide structure of A-band LPS, a rhamnanase on bacteriophage A7 was used to specifically hydrolyze the rhamnose polysaccharide chain. The chemical composition of the core components of the phage A7-digested A-band (Dig A-band) LPS was similar to that reported previously for undigested A-band LPS. This Dig A-band isolate was also incubated with MAbs against either inner core or outer core epitopes of *P. aeruginosa* LPS, and the results were compared to that of the serotype SC-LPS. The results from the immunoblots indicated that, even though the inner core region of all AK1401 LPS fractions share a common epitope, the outer core region of the A-band LPS is different from that of the B-band or SC-LPS.

To my Lord Jesus Christ,

my daughter Tanya Y. Collazo,

my parents and

my friend Barbara Hamel

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LIST OF ABBREVIATIONS

$C_{10:0}$	Decanoic Acid
$C_{12:0}$	Dodecanoic acid
$C_{14:0}$	Tetradecanoic acid
CF	Cystic fibrosis
^{13}C NMR	Carbon-13 nuclear magnetic resonance
Dig A-band LPS	Phage A7-digested A-band lipopolysaccharide
DNA	Deoxyribonucleic acid
EDTA	(Ethylenedinitrilo) tetraacetic acid
EI	Electron ionization
ELISA	Enzyme-lined immunosorbent assay
FAB-MS	Fast atom bombardment-mass spectrometry
FTIR	Fourier transformed infrared
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
^{1}H NMR	Proton nuclear magnetic resonance

IATS	International Antigen Typing Scheme
KDO	2-keto-3-deoxyoctulosonic acid
LAL	*Limulus* amebocyte lycate
LOS	Lipooligosaccharides
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MBM	Modified basal medium
$NaBD_4$	Sodium d4-borohydride
NB	Nutrient broth
NBA	Nutrient broth agar
3-OH-$C_{10:0}$	3-hydroxydecanoic acid
2-OH-$C_{12:0}$	2-hydroxydodecanoic acid
3-OH-$C_{12:0}$	3-hydroxydodecanoic acid
3-OH-$C_{14:0}$	3-hydroxytetradecanoic acid
p.f.u.	Plaque forming units
Rha	Rhamnose
SC-LPS	Short chain-lipopolysaccharide
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophore

TB	Terrific broth
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
TYE	Tryptone-yeast extract
Uf-LPS	Unfractionated-lipopolysaccharide

INTRODUCTION

The outer membrane of Gram-negative bacteria is very important for resistance to host defense factors and as a strong permeability barrier to many antibiotics. One of the membrane components that appears to be critical in determining permeability is the lipopolysaccharide (LPS). It has been shown that many strains of *Pseudomonas aeruginosa* produce two chemically and immunologically distinct LPS molecules. These LPS isolates are known as A-band and B-band LPS. B-band LPS is the O-antigen containing LPS and determines the O-specificity of the bacterium, while A-band or common antigen LPS contains shorter chains of predominantly neutral polysaccharide. The O-antigen and lipid A region of the O-specific LPS is well characterized. Only the O-polysaccharide chain structure of A-band LPS has been determined, but little is known about the structure of the core-lipid A region of A-band LPS. Therefore, it is necessary to chemically characterize the A-band core-lipid A region to be able to understand the pathophysiological responses as well as any role that this component might have in antibiotic resistance.

Two major goals of this thesis were 1) to isolate and characterize the LPS from *P. aeruginosa* AK1401, defining the chemical differences between the A- and

B-band LPS, and determining the functional groups that might be replacing phosphate in the A-band core-lipid A region; and 2) find a nondestructive method to hydrolyze the O-polysaccharide chain from A-band LPS to further characterize the core-lipid A.

Lipopolysaccharide from *P. aeruginosa* AK1401 and smooth-PAO1 strains were isolated and separated by gel filtration chromatography (Appendix A and Chapter 2). The different LPS fractions were analyzed using chemical, immunological, and gel electrophoretic techniques. Changes in the size distribution of A-band and B-band LPS with variation in the growth conditions including temperature, osmotic strength, and salt concentrations were studied (Chapter 3). The size heterogeneity was characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblots. Incorporation of sulfur into A-band LPS was further corroborated by growing the cells in ^{35}S-labelled sulfate (Chapter 4). The elution profile from a gel filtration column was monitored for total ^{35}S counts, phosphate, and amino sugar. The polyrhamnose chain of A-band LPS was digested with a rhamnanase associated with bacteriophage A7 (Chapter 5). The phage A7-digested A-band LPS was characterized using SDS-PAGE and chemical analysis to quantitate for heptose, 2-keto-3-deoxyoctulosonic acid, amino sugars, and phosphate. The inner core and outer core region of A-band as well as B-band LPS was characterized using monoclonal antibodies.

The first chapter provides general background on LPS from Gram-negative

bacteria. The final chapter summarizes the results found and proposes the physiological importance of these results as they relate to the organism. The chemical differences between the O-serotype specific or B-band and the common antigen or A-band LPS are emphasized. An experimental approach to elucidate the structure of A-band core-lipid A region is also given.

CHAPTER 1

Literature Review

Characteristics, Ecology, and Pathogenicity of *Pseudomonads*.

The family *Pseodomonadaceae* presently includes four genera, namely, *Pseudomonas, Xanthomonas, Frateuria*, and *Zoogloea* (58). The type genus of the family is *Pseudomonas*, one of the most complex groups of Gram-negative bacteria. Members of this genus are characterized by their ability to grow in simple media (58). *Pseudomonads* superficially resemble the enteric bacilli but they differ in several fundamental respects. For example, they have polar flagella, they are strongly oxidase-positive (except for *P. maltophilia* and some strains of *P. cepacia*), and they are strict aerobes (58,77). Some strains produce water soluble pigments; most *P. aeruginosa* strains produce a bluish green phenazine pigment, pyocyanin, as well as fluorescein, a greenish yellow pteridine that fluoresces (58). *Pseudomonads* have a considerably higher G+C content in their DNA than *Enterobacteriaceae*, and most metabolize sugars via the 2-keto-3-deoxygluconate (Entner-Doudoroff) pathway rather than via glycolysis (58,77).

Of all *Pseudomonas* species, by far the best studied genetically are *P. aeruginosa* and *P. putida* (26,27,72). The genetically circular chromosome of *P. aeruginosa* and *P. putida* allows a comparison of this species with the *Enterobacteriaceae* (27,72). The gene arrangement and distribution in pseudomonads are substantially different from that in the *Enterobacteriaceae*. Three features of chromosomal gene arrangement have become apparent (27): (a) the common noncontiguous arrangement of genes of biosynthetic pathways, which contrasts with the contiguous arrangement commonly found in *Enterobacteriaceae*;

(b) the rarity of contiguous functionally related genes; and (c) the clustering of genes with related functions into noncontiguous groups, described as supraoperonic clustering.

Pseudomonads are found primarily in the soil, in water, or on plants, and as a group are able to degrade a variety of organic compounds (58,77). Some pseudomonads and other nonfermenters are found on skin or other body surfaces, and in small numbers in the intestine (77). *Pseudomanad* strains can frequently be isolated from assorted clinical materials, and they can be the cause of nosocomial infections, particularly in patients in which the normal host defenses are depressed (neoplasias, burns, cystic fibrosis, etc.) (16,28).

P. aeruginosa is among the *Pseudomonas* species that can be classified as opportunistic human pathogens (10). Cystic fibrosis (CF) is an inherited disease of children, adolescents, and young adults. Most patients with CF develop lung infections and the dominant pulmonary pathogen is *P. aeruginosa* (3,18,24,64); this organism is responsible for much of the morbidity and mortality associated with chronic pulmonary infections in CF patients (8,15,24). The pathogenicity of *P. aeruginosa* is associated with several virulence factors including extracellular enzymes, lipopolysaccharides (LPS), and the production of exopolysaccharide, known as alginate (7,9,19,39,53,61). Antibiotic therapy for such infections are difficult since resistance in *P. aeruginosa* is comprehensive for many drug classes due to low permeability of the cells' outer membrane (21). Also, LPS appears to be critical in determining permeability (54,74). To understand the nature of

antibiotic interaction with LPS as well as the role that this component has as a virulence factor and in host response, it is important to characterize the chemical structure and composition of the LPS molecule.

Bacterial Cell Wall of Gram-negatives.

The *P. aeruginosa* cell envelope is typical for gram-negative bacteria. It includes an inner cytoplasmic membrane, a peptidoglycan layer, and an outer membrane (Figure 1). The cytoplasmic membrane is involved in cell division, in the active transport of materials across the bilayer, in synthesis of cell envelope components, in the electron transport chain, and in oxidative phosphorylation (6).

The peptidoglycan layer, located between the inner and outer membranes, is composed of a repeating disaccharide polymer crosslinked by peptides bridges, and is important in maintaining mechanical rigidity (55). This peptidoglycan structure is covalently linked to the outer membrane via a small lipoprotein (55). Also found within the periplasm are a wide variety of enzymes which process compounds into molecules capable of transport across the inner membrane (52).

The outer membrane is composed of proteins (structural proteins and porins), phospholipids, various other amphiphiles, including the capsular-antigens, the lipoproteins, and the endotoxins or lipopolysaccharides (LPS) (22,67). The outer membrane has an asymmetric architecture, i.e., phospholipids (mostly phosphoethanolamine) are present mainly in the inner monolayer; the high molecular weight amphiphiles, including LPS, are located exclusively in the outer

Figure 1 Schematic molecular representation of the Gram-negative envelope. Ovals and rectangles depict sugar residues. Circle represent the polar headgroups of phospholipids. MDO are membrane-derived oligosaccharides, and KDO is 3-deoxy-*D-manno*-octulosonic acid. KDO and heptose make up the inner core of LPS.

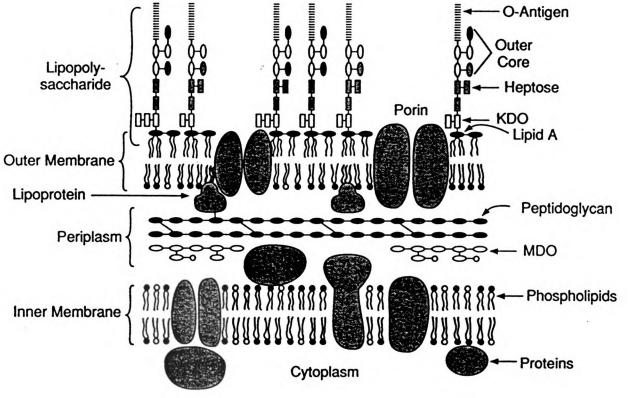

O-Antigen
Outer Core
Heptose
KDO
Lipid A
Porin
Lipopoly-saccharide
Outer Membrane
Lipoprotein
Peptidoglycan
Periplasm
MDO
Inner Membrane
Phospholipids
Proteins
Cytoplasm

Figure 1

leaflet (22). It is through this outer membrane that Gram-negative bacteria communicate with, interact with, and adapt to the natural environment. In this interaction the outer membrane regulates the uptake of nutrients required for growth (attributed to the porin proteins), prevents the penetration of toxic molecules into the bacterial cell, and impedes microbial destruction by serum components and phagocytic cells (22,52,55). As an integral component, LPS participates in many of these active or passive membrane functions and is indispensible for the proper assembly and architecture of the outer membrane. The outer membrane of Gram-negative bacteria forms a well regulated permeability barrier due to the interactions of LPS and porins.

Like many other Gram-negative bacteria, extracellular polysaccharides with the same structures as O-antigens have been found in cultural fluid or in the slime of different *P. aeruginosa* strains. Their production increases after cessation of logarithmic growth. It is unclear whether these antigens are synthesized as pure polysaccharides or if they are attached to a lipid moiety. Some *P. aeruginosa* clinical isolates produce a mucoid alginate-like glycuronan. Such mucoid strains are usually found in association with certain human pathological conditions, especially with respiratory tract infections in CF patients (20,33). Kelly *et al* (32) reported that there is an association between the development of mucoid or alginate-producing variants and the loss of the long-chain or serotype LPS on the cell surface. Alginate is an unbranched polysaccharide composed of 1,4-linked residues of 6-D-mannuronic acid and α-L-guluronic acid which can be arranged

in homopolymeric or heteropolymeric blocks (2,75). Synthesis of alginate by mucoid strains of *P. aeruginosa* is dependent on growth conditions and depends on the temperature of growth (33).

General Characterization of Outer-membrane Lipopolysaccharide.

LPS from *P. aeruginosa* possesses the same general molecular architecture as enterobacterial LPS. The high-molecular-weight LPS (S-form) molecule can be divided into three parts: the hydrophobic lipid A component, and the two hydrophilic parts composed of the O-antigenic polysaccharide attached to a core oligosaccharide (Figure 2) (33). This structure is characteristic of wild-type smooth strains. R-form or rough LPS is characterized by the absence of any O-antigenic side chain and is present in both smooth and rough strains (33,69).

Lipid A consists of a phosphorylated glucosaminyl disaccharide backbone, to which several fatty acid chains are attached via ester and amide linkages (33,81). Lipid A is the part of the molecule that by hydrophobic interaction anchors the LPS in the outer membrane (Figure 1) (67). It plays a significant role in the organization and stability of the outer membrane. This component is responsible for the LPS-induced endotoxic responses in mammals and participates in the initiation of a number of pathophysiological responses to infection (39).

The oligosaccharide core region is directly linked to the lipid A headgroup via an unusual sugar present in most LPS molecules, 2-keto-3-deoxyoctulosonic acid (KDO) (33,81). Within the core and lipid A headgroup region there is a

Figure 2 Proposed structure for the lipopolysaccharide molecule of *Pseudomonas aeruginosa* PAO1 (O5 serotype according to the IATS scheme).

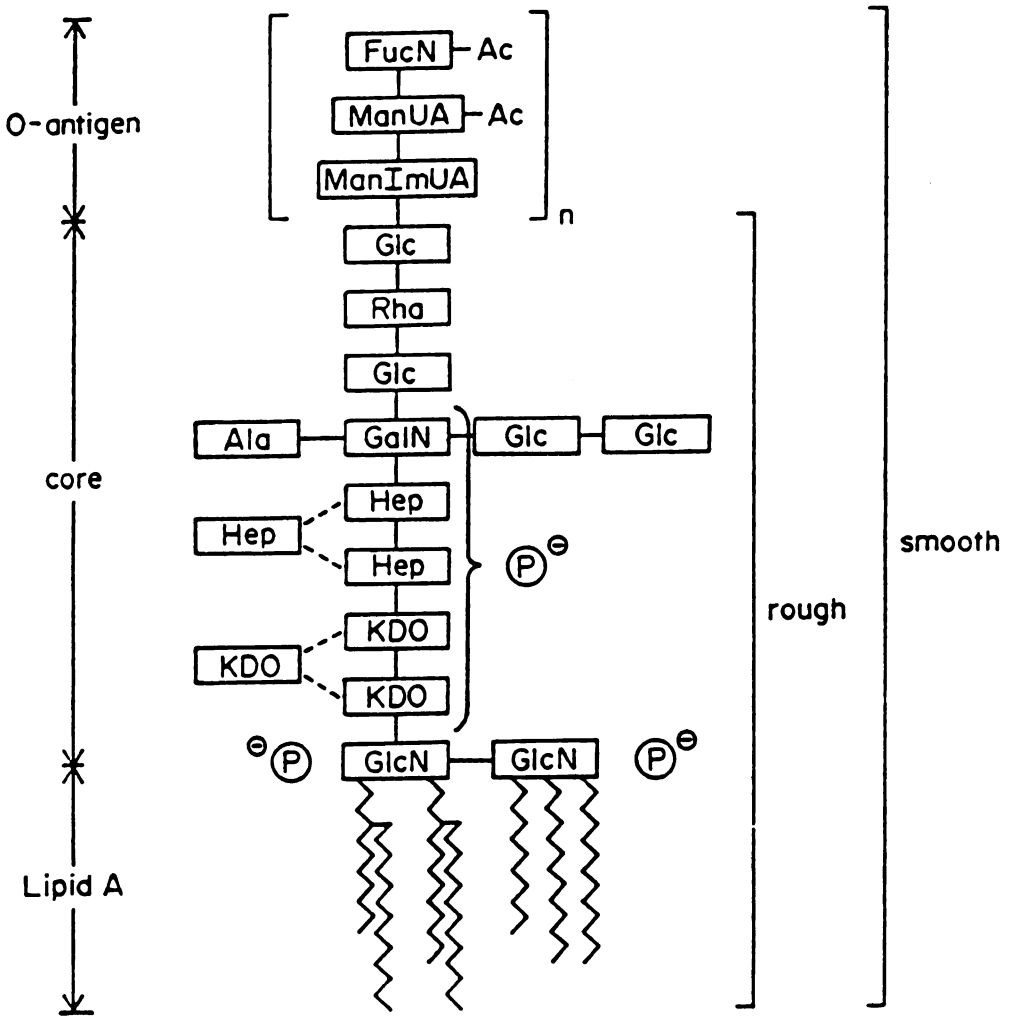

O-antigen
core
Lipid A
FucN
Ac
ManUA
Ac
ManImUA
n
Glc
Rha
Glc
Ala
GalN
Glc
Glc
Hep
Hep
Hep
KDO
KDO
KDO
GlcN
GlcN
rough
smooth

Figure 2

variety of ionic groups such as acidic phosphates and carboxyl moieties (33). Due to the high level of phosphorylation in *P. aeruginosa*, compared to enterobacteria, the core oligosaccharide has an exceptionally high metal-binding capacity (33,54). This makes the outer membrane particularly dependent on divalent cations for stability. Chelation of these cations by EDTA causes disordering of the membrane with release of LPS (48,51). The disruption of outer membrane integrity and increase in permeability induced by polycationic antibiotics results from binding of these compounds to the anionic groups of LPS perhaps displacing the stabilizing divalent cations (54,60). Therefore, the combination of the LPS's negative charge and the divalent cation cross-bridging of LPS provides the gram-negative cell surface with a tight barrier, important for the cells' resistance to hydrophobic antibiotics, bile salts, detergents, protease, lipases, and lysozymes (54,55).

In smooth strains, O-polysaccharides are attached to the core region of LPS and are made up of repeating units of identical oligosaccharides (33,81). The portion of LPS molecules that have an attached polysaccharide is low for *P. aeruginosa* isolates, usually less than 15% (69,81). The remainder of the molecules contains only core and lipid A components. The structure of the polysaccharide repeat unit defines the serotype of the strain (33,81). The O-side chains from *P. aeruginosa* are rich in amino sugars, some of which are unique among natural products (35,54). As mentioned previously, each of the at least 20 serologically distinguishable strains of *P. aeruginosa* produces a unique O-antigen with a specific composition and structure (43,44). The presence and the amount

of O-antigen as well as the length of O-antigen chain influences various other *P. aeruginosa* cell surface phenomena, including antibiotic susceptibility (1), bacteriophage recognition (40), virulence and sensitivity to bactericidal action of serum (7,11), and the capacity to induce protective antibodies (45).

Lipopolysaccharides of most bacteria exhibit structural heterogeneity in all three regions of the molecule (56). In enterobacterial S-forms, as well as in *Pseudomonads*, a collection of LPS species is present which differ in the number of repeating units, i.e., the length of the O-specific chain (56,69). As mentioned before, a certain portion of molecules lacks the O-specific chain. In addition, some of the charged substituents of the inner core and lipid A and the acyl groups of lipid A, are not present in molar amounts (33,54,67,81).

The O-polymers from various *P. aeruginosa* strains have been resolved into two chemically distinct sets, an amino sugar-rich fraction and a neutral sugar-rich fraction (69,73,82,84). The shorter, neutral sugar-rich fraction has been shown to contain a polysaccharide region composed of a three rhamnose repeat unit (4,84). Rivera *et al* (69) have demonstrated that the *P. aeruginosa* strain PAO1 is capable of synthesizing more than one type of LPS. Analysis by gel filtration chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the PAO1 LPS consisted of two antigenically and chemically distinct molecules termed A- and B-band LPS (69). The B band, usually composed of LPS with long O-antigen chain, is the LPS specie responsible for the O-specificity of the organism, while a second LPS, the A band fraction,

is composed of molecules that only have intermediate size polysaccharide chains (approx. 30 repeat units) (42; M. Rivera, T.J. Chivers, J.S. Lam, and E.J. McGroarty, J. Bacteriol., submmited).

The isolation of *P. aeruginosa* rough strains from sputum in CF patients is correlated with chronic and severe infection (12,29,47). The lack of O-antigen expression accounts for the unusually high percentage (60-80%) of nontypeable strains of *P. aeruginosa* encountered in many studies of CF patients (13,14,23,41,57,65). Lam and coworkers (41) have produced seven MAbs to the A-band isolate. Using Western immunoblot analysis, they have shown that A band molecules are present as a common antigen on many but not all serotypes of *P. aeruginosa*. Furthermore, they found that A bands are present in a high percentage of clinical isolates and appeared to be the main antigen on nontypeable strains deficient in high-molecular-weight B band-type LPS.

Chemical Structure of Lipopolysaccharide.

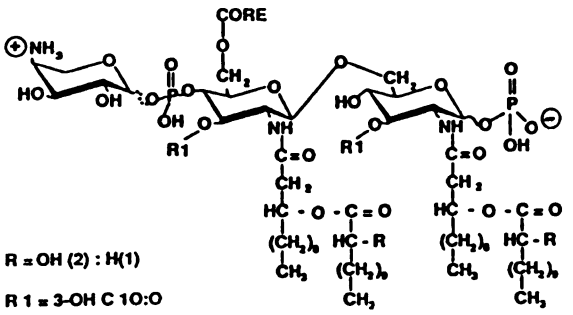
The primary structure of enterobacterial lipid A, as well as *P. aeruginosa*, has been elucidated in great detail. In Figure 3 lipid A structures of *Escherichia coli* (67,68), *Salmonella minnesota* (68), and *P. aeruginosa* (17,33) are shown. In all of these cases, lipid A is composed of a 6-*D*-glucosaminyl-(1->6)-α-*D*-glucosamine disaccharide phosphorylated at positions 1 and 4′. This hydrophilic lipid A head group is acylated by four residues of (R)-3-hydroxy fatty acids at positions 2, 3, 2′, and 3′. Structural heterogeneity in the lipid A (Figure 3)

includes variation in the fatty acid chain length and the location of ester-bound acyl groups. For example, in *E. coli* the (R)-3-hydroxytetradecanoic acid (3-OH-C14:0) is present in ester and amide linkage. The hydroxyl groups of the two 3-OH-C14:0 residues bound to the nonreducing glucosaminyl residue at position 2′ and 3′ carry dodecanoic (C12:0) and tetradecanoic acid (C14:0), respectively. The 3-OH-C14:0 residues bound to the reducing glucosaminyl residue are not 3-O-acylated. In *P.aeruginosa* lipid A, (R)-3-hydroxydodecanoic acid (3-OH-C12:0) is amide-bound and (R)-3-hydroxydecanoic acid (3-OH-C10:0) is ester-linked to the lipid A backbone. The latter are not substituted at their 3-hydroxyl groups while the 3-OH-C12:0 residues at positions 2 and 2′ carry C12:0 and/or (S)-2-hydroxydodecanoic acid (2-OH-C12:0). As indicated in Figure 3, the hydroxyl groups in positions 1 and 4′ may be substituted by phosphate or pyrophosphate, phospho-*D*-glucosamine, and phospho-4-amino-4-deoxy-*L*-arabinopyranose (17,68). It is important to mention that the pathway for biosynthesis of lipid A in *P. aeruginosa* is similar to, but not identical with that of enterobacterial lipid A (17,66). The major precursor of the latter includes only amide-bound fatty acids (66), while that of the former contains all fatty acids present in lipid A of the mature LPS before addition of KDO (17).

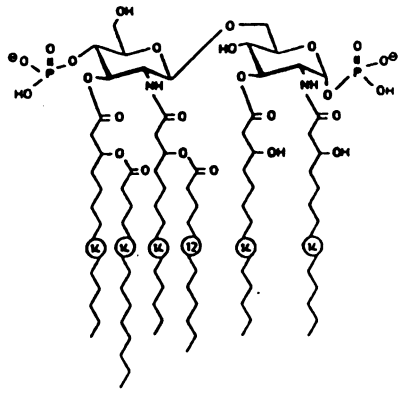
Thus, while lipid A's of different bacterial families share certain chemical features they differ in others. The tetraacyl backbone is ubiquitous and highly conserved and exhibits very low structural variability (66-68). However, individual lipid A's may differ from each other by the presence and nature of polar

Figure 3 Chemical structure of the lipid A component of (A) *Pseudomonas aeruginosa* PAO1, (B) *Escherichia coli*, and (C) *Salmonella minnesota* lipopolysaccharides. Dotted lines indicate incomplete substitution. KDO is linked to the primary hydroxyl group in position 6′. Numbers in circles indicate the number of carbon atoms in the acyl chains.

A



B



C

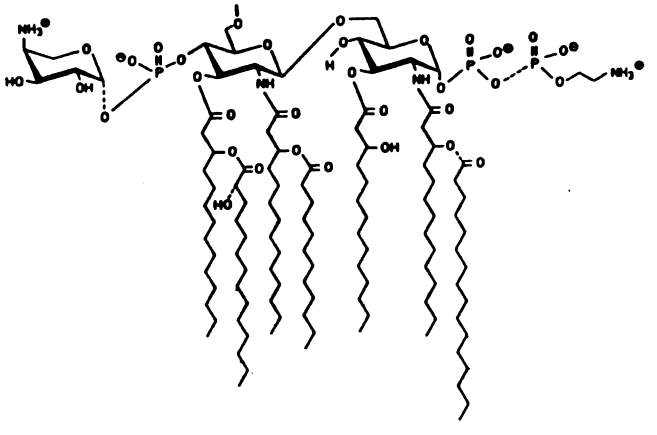


Figure 3

and ionic head groups substituents, and the nature and chain lengths of fatty acids acylating the 3-hydroxy fatty acids. Variations of these parameters create structural diversity which is responsible for intrinsic heterogeneity of lipid A (17,56,66).

It should be noted that certain Gram-negative bacteria synthesize LPS with a lipid A structure that is radically different in architecture from that of enterobacterial lipid A structure (25,49). As an example, lipid A of *Rhodopseudomonas viridis* has been found to be devoided of glucosamine and phosphate but to contain the rare sugar 2,3-diamino-2,3-dideoxy-*D*-glucose monomer which carrys 3-OH-C14:0 residues (71). Hollingsworth and Lill-Elghanian (25) isolated and characterized two major lipid A components of *Rhizobium trifolii* ANU843. They demonstrated that the free lipid A component contained a novel long-chain carboxylic acid and 2-amino-2-deoxy-*gluco*-hexuronic acid and was totally devoided of phosphate.

The core region of enterobacterial and *P. aeruginosa* LPS consists of a heterooligosaccharide which can be subdivided into the lipid A-proximal inner core and the distal outer core region. The enterobacterial outer core region contains the common sugars *D*-glucose, *D*-galactose, and N-acetyl-*D*-glucosamine (67), whereas in *P.aeruginosa* *D*-glucose, *D*-galactosamine, *L*-rhamnose, and *L*-alanine are the components found in this region (81). The inner core region of *Enterobacteriaceae* as well as *P.aeruginosa* is composed of the unusual sugars heptose, mainly in the *L-glycero-D-manno* and the *D-glycero-D-manno*

configuration, and 2-keto-3-deoxyoctulosonic acid (KDO, also termed 3-deoxy-*D-manno*-2-octulosonic acid, dOclA) (Figure 4) (33,66,67). ^{13}C NMR spectroscopy suggests that KDO is attached to lipid A by an α2-6′ linkage (78,79). In general, these residues are substituted by charged groups such as phosphate, pyrophosphate, phosphoethanolamine, and pyrophosphoethanolamine, often in nonstoichiometric amounts (33,66,67). The structural variability of the core within different bacterial species is limited. Based on the sugar composition of the LPS isolated from various enterobacterial R mutants, the core domain can be classified by chemotypes, namely, Ra, Rb_1, Rb_2, Rc, Rd_1, Rd_2,and Re LPS (46,66). Type Ra represent a complete core while type Rd has defects in adding any sugar unit of the outer core region; type Re comprises only lipid A and the KDO units (probably with branch substituents) of the core oligosaccharide. Chemotype variants of *P.aeruginosa* rough mutants have been isolated which are analogous to those of enterobacteria with the exception that Re mutants have not been reported (5,30,38). Interestingly, a variety of nonenterobacterial wild-type strains of some phototropic and pathogenic gram-negative bacteria such as *Neisseria, Acinetobacter, Campylobacter, Bordetella, Bacteroides,* and *Haemophilus* synthesizes LPS which consist only of core oligosaccharide and lack a long O-specific chain (67). These compounds have been termed lipooligosaccharides (LOS).

The chemical analysis of the inner core is very difficult for a number of reasons. (*i*) The ketosidic linkages of KDO are extremely acid-labile. (*ii*) No

Figure 4 Proposed covalent structure of the inner core of *Escherichia coli* K-12. Putative partial substitutions are indicated with dashed bonds.

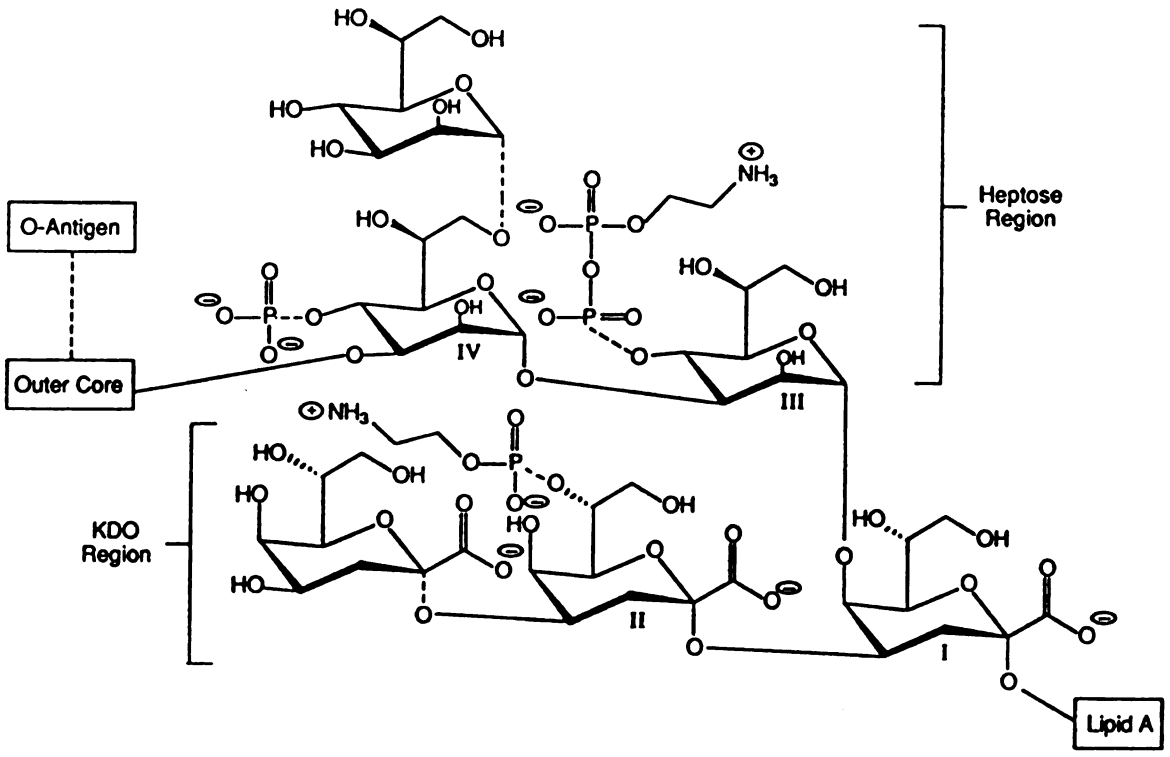

O-Antigen
Outer Core
KDO Region
Heptose Region
Lipid A
IV
III
II
I

Figure 4

satisfactory procedure exists for the quantitative determination of KDO in polysaccharides of unknown structure and substitution pattern. (*iii*) KDO undergoes side reactions under the usual conditions of hydrolysis of polysaccharides, leading to unknown or unstable products (80). Because of the difficulty of analyzing intact LPS under nondestructive conditions, the proposed structure on the enterobacterial inner core (Figure 4) cannot be considered definitive. At least one KDO residue, or KDO-like sugar, has been found in almost all gram-negative bacteria that have been studied (46,67), but in some organisms, subtle modifications of KDO are observed. In *Acinetobacter calcoaceticus*, an octulosonic acid isomer resembling KDO is attached to lipid A which is resistant to acid hydrolysis (31). This isomer differs from KDO by the presence of an additional hydroxyl group at C-3, and this group appears to play a role in the acid stability of its ketosidic linkage. It is not clear whether LPS lacking the KDO residue exists. However, Pask-Hughes and Williams (59) reported that the LPS isolated from extreme thermophiles of the genus *Thermus* lacked heptose, KDO, glucosamine, and phosphorus. Interestingly, hydroxylated fatty acids were not reported. Also, Beconi and Hollingsworth (personal communication) have isolated an LPS fraction from *Rhizobium leguminosarum* viobar *viacea* grown in acidic conditions. The lipid A fraction contains the fatty acids characteristic of this specie but the carbohydrate fraction lacks both heptose and KDO.

The O-antigen is attached to a terminal sugar of the outer core (Figure 4),

and it is composed of a repeating oligosaccharide unit containing up to six sugar residues (33,46,67). The nature, sequence, type of linkage, and type of substitution of the individual monosaccharide residues within a repeating unit is characteristic and unique for a given bacterial strain. Thus, the O-polysaccharide chain is species-specific and determines the O-serological specificity of the molecule and of the parent bacterial strain (33,46,67). The composition of *P.aeruginosa* O-antigens has been found to differ in several ways from that of other bacterial O-antigens studied (34). The *P. aeruginosa* antigens usually are defficient in neutral sugars, and typically contain monoamino and diamino sugars, many of which carry a carboxyl functional group. The structures of some of these O-antigens are given in Table 1. As mention, the O-polysaccharide chain of *P. aeruginosa* exhibits heterogeneity not only in the number of repeating units but also modifications in the repeating unit (33,34). The most common is nonstoichiometric O-acetylation; other variable modifications are nonstoichiometric amidation and epimerization.

The majority of the *P. aeruginosa* strains contain a second LPS specie whose polysaccharide chain differs serologically and structurally from O-antigen chain (37,50,69,70,73). This LPS has been termed A-band or common antigen LPS (Figure 5). In wild type strains, this common polysaccharide antigen is shorter than the O-antigen chain; thus the common antigen is covered and not exposed to the cell surface on smooth strains, but is accessible on the surface of rough-type strains (41,50). The common antigen polysaccharide is a regular

TABLE 1. Structure of O-repeating units in *Pseudomonas aeruginosa* Lipopolysaccharide

Lanyi Type	IATS Type	O-repeating Unit
2a,b	10	4)L-GalNAcUA(α1-3)D-QuiNAc(α1-3)L-2-OAcRha(α1-3)L-2-OAcRha(1-
2a,c		4)L-GalNAcUA(α1-3)D-QuiNAc(α1-3)L-Rha(α1-3)L-Rha(α1-
2a,d	5	4)D-ManImUA(β1-4)D-Man$(NAc)_2$UA(β1-3)D-FucNAc(α1-
3a,b	16	4)D-ManImUA(β1-4)D-Man$(NAc)_2$UA(β1-3)D-FucNAc(β1-
3(a),c	2	4)D-ManImUA(β1-4)L-Gul$(NAc)_2$UA(β1-3)D-FucNAc(β1-
3a,d,e		4)D-ManImUA(β1-4)L-Gul$(NAc)_2$UA(α1-3)D-FucNAc(α1-
3(a),d,f		4)D-ManImUA(β1-?), Hex$(NAc)_2$UA, D-FucNAc
6	1	4)D-GalNAc(α1-4)D-Glc$(NAc)_2$UA(β1-3)D-FucNAc(α1-3)D-QuiNAc(α1-
7ab(ac)	11	3)L-FucNAc(α1-3)D-FucNAc(β1-2)D-Glc(β1-

Nonstandard abbreviations: QuiN, quinovosamine; FucN, fucosamine; $(NAc)_2$UA, 2,3-diacetamido-2,3-dideoxy(hex)uronic acid; and ManImUA, 2-imidadolinomannuronic acid. IATS type referes to the serotype according to the international antigen typing scheme commerically marketed by Difco (taken from ref. 14).

Figure 5 Proposed structure for the O-polysaccharide repeat unit of *Pseudomonas aeruginosa* A-band or common antigen LPS.

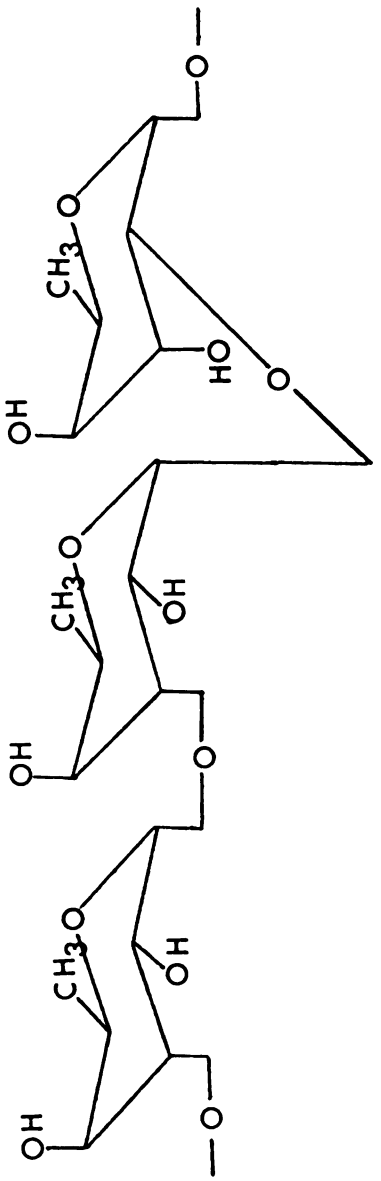


Figure 5

homopolymer of rhamnose. On the basis of NMR and chemical analyses the structure has been shown to consist of the repeating unit [->3)-α-*D*-Rha*p*-(1->3)-α-*D*-Rha*p*-(1->2)-α-*D*-Rha*p*-(1->]$_n$ (4,37,84). Surprisingly, the O-antigens of many phytopathogenic *Pseudomonas* species, such as *P. syringae*, also contain *D*-rhamnose as a main component, and some strains possess the same structure as the *P. aeruginosa* common polysaccharide chain (36,76). Little is known about the structure of the core oligosaccharide of *P. aeruginosa* A-band or common antigen LPS. Yokota *et al* (84) reported that the oligosaccharide core contained 3-*O*-methyl-6-deoxyhexose, xylose, and glucose. On the other hand, Arsenault and coworkers (4) detected minor amounts of 3-*O*-methyl rhamnose, ribose, mannose, glucose, and a 3- or 4-*O*-methylhexose in A-band LPS from *P. aeruginosa* strain. Furthermore, Rivera *et al* (69), and Rivera and McGroarty (70) reported that the A-band LPS isolated from smooth strains of *P. aeruginosa* contained low levels of amino sugars, KDO, and no phosphate but instead stoichiometric amounts of sulfate was detected. In this study, I have initiated a study of the chemical structure of core-lipid A of A-band LPS.

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CHAPTER 2

Analysis of a Common-Antigen Lipopolysaccharide from *Pseudomonas aeruginosa*

ABSTRACT

Lipopolysaccharide isolated from *Pseudomonas aeruginosa* PA01 (05 serotype) was separated into two antigenically distinct fractions. A minor fraction, containing shorter polysaccharide chains, reacted with a monoclonal antibody to a *P. aeruginosa* common antigen but did not react with antibodies specific to 05-serotype lipopolysaccharide. In contrast, fractions containing long polysaccharide chains reacted only with the 05-specific monoclonal antibodies. The shorter, common-antigen fraction lacked phosphate and contained stoichiometric amounts of sulfate, and the fatty acid composition of this fraction was similar to that of the O-antigen-specific fraction. The lipid A derived from the serotype-specific lipopolysaccharide cross-reacted with monoclonal antibodies against lipid A from *Escherichia coli*, while the lipid A derived from the common antigen did not react. We propose that many serotypes of *P. aeruginosa* produce two chemically and antigenically distinct lipopolysaccharide molecules, one of which is a common antigen with a short polysaccharide and a unique core-lipid A structure.

INTRODUCTION

Lipopolysaccharide (LPS) isolated from *Pseudomonas aeruginosa*, like that from enteric bacteria, is a heterogeneous mixture of molecules of different polysaccharide chain lengths (16,26, Appendix A) and with different levels of phosphate substitution (21,31). Structurally, the molecules can be divided into three regions: the lipid A, the core oligosaccharide, and the O-antigen polysaccharide. The lipid A from *P. aeruginosa* is similar to that of many gram negative bacteria; it consists of a 4-phosphoglucosaminyl-(1→6) glucosamine-1′-phosphate head group to which saturated and hydroxy fatty acids are ester and amide linked (21,31). The hydroxy fatty acids present in *P. aeruginosa* LPS are different from that of enteric bacteria, lacking 3-OH-tetradecanoic acid but containing 2-OH- and 3-OH-dodecanoic acids and 3-OH-decanoic acid (9,31,32).

The composition of the core oligosaccharide of *P. aeruginosa* is also somewhat distinct from that of the core oligosaccharide of other gram-negative species, containing D-glucose, D-galactosamine, L-rhamnose, and L-alanine as well as the sugars commonly found in the inner core, L-glycero-D-mannoheptose and 2-keto-3-deoxyoctulosonic acid (KDO) (26,28,31). Also, the core and lipid A regions of *P. aeruginosa* isolates are especially high in phosphate (24,31). In

smooth strains, a long polysaccharide is attached to the core, but usually on only a low proportion of the LPS molecules (26,31). The structure of the polysaccharide repeat unit defines the serotype of the strain (6,15). Generally, O-polysaccharides of the various *P. aeruginosa* serotypes are rich in amino sugars (21,31). Neutral sugars are also found as components of many O-polymers include L-rhamnose and D-glucose (17,31).

Characterization of the structures of various O-specific polysaccharide from different serotypes of *P. aeruginosa* has been complicated in some cases by chemical heterogeneity of the polysaccharide chains (5,6,32). Not only is the length of the O-polysaccharide variable (26), but also the O-polymers have been resolved into an amino-sugar-rich and a neutral-sugar-rich fraction (29,32,34). The shorter, neutral-sugar-rich fraction has been shown to be composed of a three-rhamnose repeat unit (34). Monoclonal antibodies reactive against the polyrhamnose isolate react with many different serotypes of *P. aeruginosa*, suggesting that these molecules compose a common antigen for this organism (29). We have recently reported the isolation and partial purification of an A-band LPS fraction from PA01 strains which is low in phosphate and amino sugars and does not react with serotype-specific monoclonal antibodies (26). In this study we further characterized the structures of these two fractions of LPS.

RESULTS AND DISCUSSION

LPS was isolated from strains PAO1716 (*ade*-136, *leu*-8, *rif*-1) (revertant), PAO1715 (*ade*-136, *leu*-8, *rif*-1, *tol A*12) (an aminoglycoside-supersensitive mutant [20]), and PAZ1 (*met*-28, *trp*-6, *lysA*12, *his*-4, *ile*-226, *absA*), a PAO222 derivative (2), by either the method of Darveau and Hancock (7) as previously described (26) or the hot aqueous phenol method (33). The LPS isolates were fractionated on a Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, NJ) column at room temperature as previously described (23,26). Column fractions were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by using the buffer system of Laemmli (18); the gels were silver-stained by the method of Dubray and Bezard (10). SDS-PAGE of the column fractions revealed two distinct ladder patterns of apparently different sizes (results not shown) which in the unfractionated sample were overlapping and thus superimposed. Previously, we showed that LPS from *P. aeruginosa* PAO1 strains could be resolved into two chemically distinct sets of molecules, the shorter A bands (later-eluting ladder) and the longer B bands (earlier-eluting ladder, [26, Appendix A]). The main serotype-determining antigen, high in amino sugars, was recovered primarily in peaks 1 and 2. The third peak, peak X, contained a

shorter, neutral-sugar polysaccharide. The bands in SDS gels that contained the main O-antigen were termed the B bands, while the bands in peak X were termed the A bands. The last peak recovered from the Sephadex column (peak 3) contained the majority of the molecules, comprising core and lipid A with none or only one or two O-repeat units. The fractions which made up the four peaks were isolated, pooled, and dialyzed extensively (12,000 to 14,000 molecular weight cutoff membranes) against column buffer without detergent at 37°C and then against distilled water at 4°C. The dialyzed fractions were lyophilized and suspended in distilled water to known weight concentrations for further analysis.

In an earlier study we showed that all four fractions contained heptose, a component of the inner core of most LPS isolates (26). The level of this sugar per weight of the four fractions reflected the molecular weight of the molecules, suggesting that the heptose content is similar in all of the isolates (Table 1). In contrast, the sugar 2-keto-3-deoxyoctulosonic acid (KDO), another component common to the inner core of most LPS isolates, appeared to be very low in the peak X isolate (Table 1). However, Caroff and coworkers have shown that this sugar is not detected in the thiobarbiturate assay if the sugar is substituted at specific positions, unless harsher hydrolysis conditions are used (4). When the peak X isolate was assayed after hydrolysis with higher levels of acid and for longer times, the KDO content increased ten-fold. Thus, the fraction contains KDO, but this sugar may be substituted and thus not readily detected. Presumably, the KDO residues in the other three peaks are not substituted; the

TABLE 1. Analysis of Column Fractions of LPS from *P. aeruginosa* PAO1

LPS Sample	Amt (nmol/mg)[a] of: Heptose[b]	KDO[b]	Phosphate[b]	Sulfate	LAL result[c]
Peak 1	39	30	150	13	75
Peak 2	34	28	165	47	72
Peak 3	272	282	1,670	16	408
Peak X	83	2	8	190	57
Unfractionated	ND[d]	162	1,200	37	ND[d]

[a]Average results from two or more isolates.

[b]Data from reference 26.

[c]LAL, *Limulus* amebocyte lysate assay. Results are expressed as endotoxin units per picogram.

[d]ND, Not determined.

level of KDO detected in these fractions was not affected by the hydrolysis conditions

Our earlier studies also indicated that the peak X isolate lacks phosphate groups while the other fractions are highly substituted with phosphate (Table 1). To determine whether another anionic group might replace the phosphate moieties, we assayed the LPS fractions for sulfate by a barium chloranilate assay procedure with K_2SO_4 as a standard (8). Briefly, 5 to 10 mg of LPS were hydrolyzed in 0.5 ml of 6 N HCl at 100°C for 1 h and extracted with 5 ml of chloroform-methanol (2:1 [vol/vol]). The upper phase was washed with chloroform-methanol (17:3) and dried in a boiling-water bath. The samples were then dissolved in 0.5 ml water-methanol (1:1) and dried three times. This hydrolyzed sample was then dissolved in H_2O, diluted into ethanol, and reacted with barium chloranilate. Cross-reaction with KH_2PO_4 was shown to be negligible. The levels of sulfate detected in peaks 1, 2, and 3 were low (Table 1). In contrast, the molar amount of sulfate detected in peak X was over twice the heptose content, indicating sulfate levels greater than stoichiometric levels. The slightly elevated levels of sulfate in peak 2 may reflect a small amount of A-band LPS contaminating this fraction (see below).

Reactivity of the four pooled fractions in the *Limulus* amebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD) was also quantitated (Table 1). Levels were calibrated with the *Escherichia coli* 0111:B4 LPS standard provided in the kit. The reactivity for the high-molecular-weight LPS (peaks 1 and 2) compared

with that of low-molecular-weight LPS (peak 3) expressed on a per-weight basis reflected the difference in sizes of the LPS molecules. The peak X sample did not fit in this pattern, probably because of the chemical differences of the LPS molecules. This A-band-containing fraction did not appear to be as reactive in this assay as the B-band-containing fraction, at least when reactivity is expressed per amount of heptose.

Peaks X and 3 and the unfractionated LPS were analyzed for fatty acid composition (Table 2). LPS fractions (5 to 8 mg) were suspended in a 3-ml solution of HCl-methanol (3:15 [vol/vol]) and hydrolyzed at 85°C for 18 h. The fatty acid methyl esters were extracted into petroleum ether, dried, and suspended into ethyl acetate. Samples of 3 to 4 μl were injected into an HP5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) and separated on a 25-m Ultra II column. The column was run at 150°C for 15 min; the temperature was increased at 2°C/min up to 250°C and then increased at 25°C/min up to 350°C. The retention times were compared to those of a bacteria fatty acid methyl ester mixture (Supelco, Bellefonte, PA) for identification. The fatty acids detected were very similar for all three samples and are similar to what has been reported previously for LPS from *P. aeruginosa* (9,32). All three samples contained high amounts of 2-OH- and 3-OH-dodecanoic acid and lacked 3-OH-tetradecanoic acid. The content of the other fatty acids were similar for all three samples. These results give further evidence that the A-band isolate is an LPS with a fatty acid composition typical of this species.

TABLE 2. Fatty Acid Composition of LPS fractions from *P. aeruginosa* PAO1.

	Recovery from sample[a]		
Fatty Acid	Peak 3	Peak X	Unfractionated
$C_{10:0}$	3	0	4
$C_{12:0}$	9	11	6
$C_{16:0}$	6	6	2
3-OH-$C_{10:0}$	5	5	16
2-OH-$C_{12:0}$	33	32	33
3-OH-$C_{12:0}$	37	43	40
Other	6	3	0

[a]Results are the average of two or more analyses and are expressed as a weight percentage of the total.

To characterize the sugar components in the purified LPS fractions, the four fractions were hydrolyzed in 2 N HCl for 2 h at 100°C and evaporated. The samples were then spotted on cellulose F254 (Merek) thin-layer plates and developed by using the solvent system described by Sawada *et al.* (29). The chromatograms were reacted with alkaline silver nitrate to localize the sugars by previously described procedures (29). This qualitative analysis revealed that rhamnose was present in peak X, but it was not detected in the peak 1 and 2 samples (data not shown). As expected, rhamnose was also present in the peak 3 sample; rhamnose is a known component of the core oligosaccharide (31,32).

Recently Sawada and coworkers reported that acid hydrolysis of *P. aeruginosa* LPS released a rhamnose-containing polysaccharide, shorter than the main amino-sugar-containing polysaccharide, which reacted with a monoclonal antibody E87 (29). We obtained a sample of this monoclonal antibody to see whether it reacted with our A-band fractions. We also tested the fractions for reactivity with the 05-specific monoclonal antibody anti-503 (12). Western immunoblots of SDS-polyacrylamide gels were prepared as described by Towbin *et al.* (30). The gels were electrotransferred with a model TE Transphor Electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, CA) at a constant current of either 150 or 300 mA for 18 h. The nitrocellulose blots were visualized as described by Otten *et al.* (22) with either monoclonal anti-503 antibody specific for 05-serotype LPS (12) or monoclonal antibody E87 specific for a common antigen of *P. aeruginosa* (29). In addition, dot blots were

performed by applying known quantities of LPS directly onto nitrocellulose and reacting the blots with the monoclonal antibodies described above. The blots were washed and visualized by using horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (Sigma Chemical Co., St. Louis, MO). Both the Western immunoblot (Figure 1) as well as the dot blot (data not shown) indicated that the E87 monoclonal antibody reacted with the X peak isolate while the serotype-specific antibody reacted with peak 1 and 2 isolates. There was a weak reactivity of the peak 2 fraction with the E87 antibody on the dot blot, suggesting that this isolate had a minor amount of A-band LPS. Furthermore, the peak X isolate showed some reactivity with the anti-503 antibody. To show that peak X reactivity was due to B-band contamination of this fraction, four peak X isolates were separated on SDS gels, transblotted, and reacted with the two monoclonal antibodies (Figure 2). The results indicate that the E87 antibody reacted exclusively with the more slowly moving bands in this gel while the anti-503 antibody reacted only with the faster-moving B-band molecules that were present in low amounts in this sample. Thus, the A- and B-band LPS molecules appear to be antigenically as well as chemically distinct.

To determine whether the lipid A components of these fractions were also antigenically different, lipid A from peaks 3 and X as well as from nonphosphorylated LPS from *Chromatium vinosium* were isolated and reacted on dot blots with anti-lipid A monoclonal antibody 1D4 or 8A1 (27). The LPS from *C. vinosium* was a gift from R. Hulbert (14). Purified LPS fractions were

Figure 1. Western blots of LPS fractions (peaks 1, 2, 3, and X) from *P. aeruginosa* reacted with monoclonal anti-503 (left) or monoclonal E87 (right) antibody and aligned with a silver-stained SDS-polyacrylamide gel (center; 15% acrylamide) of the same fractions. A 5-μg portion of each sample was applied to each gel. The gels were blotted as described in the text.

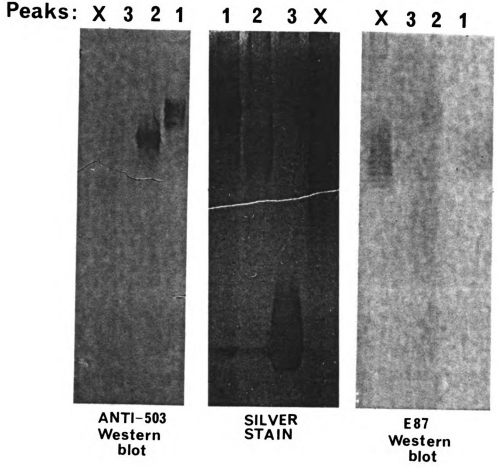
Peaks: X 3 2 1
1 2 3 X
X 3 2 1
ANTI-503
Western
blot
SILVER
STAIN
E87
Western
blot

Figure 1

hydrolyzed to lipid A by treating the samples with 5% acetic acid at 100°C for 2.5 h (14), with 0.1 N HCl at 100°C for 1 h (25), or with 20 mM sodium acetate (pH 4.5) at 100°C for 1 h (1). The samples were neutralized with NaOH, and the polysaccharide was separated from the lipid A either by elution on a Sephadex G-25 column with distilled water, or by sedimenting the lipid A at 10,000 x *g* for 30 min. Hydrolysis in 20 mM sodium acetate (pH 4.5) produced an insoluble lipid A from the peak 3 fraction, but no lipid A could be recovered from peak X or the *C. vinosium* sample by using this hydrolysis protocol. Thus, these two samples, as well as peak 3, were hydrolyzed for 1 h at 100°C in either 0.1 N HCl or 5% acetic acid. The isolated lipid A samples were lyophilized and suspended in distilled water. Samples were spotted onto nitrocellulose at different concentrations and reacted with either 1D4 or 8A1 monoclonal antibody specific for lipid A of *Escherichia coli* (27). On all the blots studied, only lipid A from peak 3 reacted, but all of the peak 3 lipid A isolates reacted with both antibodies (data not shown). Perhaps phosphate on the lipid A is a part of the epitope for these antibodies. The results indicate that the lipid A from peak X is antigenically distinct from that of peak 3 and presumably from that of the other B-band fractions.

In conclusion, the differences between the A- and B-band LPS fractions of *P. aeruginosa* are significant. First, the B-band molecules contain much longer polysaccharide chains, as determined by SDS-PAGE and by separation on Sephadex G-200. Second, the B bands are high in amino sugars and low in

Figure 2. Western blots of four peak X isolates. Isolates I, II and III were from strain 1716, and isolate IV was from strain PAZ1. The blot on the left was reacted with monoclonal antibody E87 and the blot on the right was reacted with monoclonal anti-503 antibody; the gels were aligned with a silver-stained SDS-polyacrylamide gel (center; 15% acrylamide) of the same isolates. A 5-μg sample of each isolate was applied, and after electrophoresis, the gels were blotted or stained as described in the text.

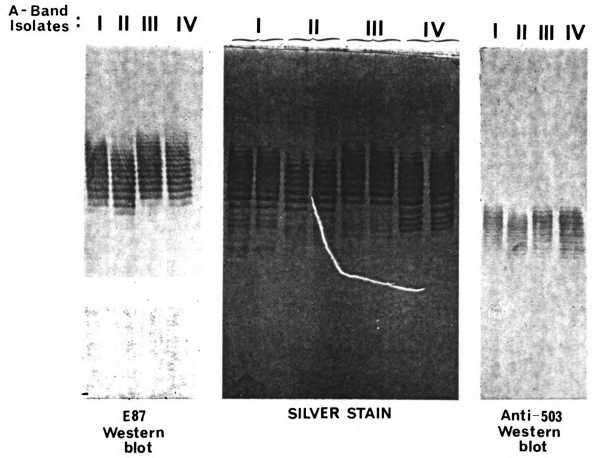
A-Band Isolates : I II III IV
I II III IV
I II III IV
E87 Western blot
SILVER STAIN
Anti-503 Western blot

Figure 2

rhamnose, while the A bands contain rhamnose and are low in amino sugars. These differences in O-polymer structure are also reflected in the reactivities of antibodies to these polymers. The O-specific antibodies reacted only with the amino-sugar-containing polymers, while the A bands reacted only with the polyrhamnose-specific antibody. Third, the B bands are high in phosphate content but low in sulfate, while the A bands lack phosphate but contain sulfate groups. This is the first report that we know of which indicates sulfate as a component of LPS. However, nonphosphorylated lipid As have been reported for a number of bacteria (19); in such isolates, sulfate may replace the phosphate on lipid A. In preliminary studies we have detected stoichiometric levels of sulfate on the lipid A of the nonphosphorylated LPS from *C. vinosium* (14; M. Rivera, A.A. Peterson, R.T. Coughlin, and E.J. McGroarty, manuscript in preparation).

A fourth difference between the A- and B-band LPS is the reactivity of the lipid As with monoclonal antibodies to lipid A from *E. coli*. These antibodies reacted with the B-band lipid A but not with lipid A derived from the A bands. The anti-lipid A antibodies also did not react with lipid A from *C. vinosium*, suggesting that phosphates in the lipid A head group are critical in binding these antibodies; sulfate may not serve as a replacement at the binding site.

A final difference between the A- and B-band LPS isolates is their distribution among the serotypes of *P. aeruginosa*. Each serotype class has a unique B-band type of O-polymer with a different chemical structure (15). In contrast, many of the serotype strains may contain an A-band type of LPS with

similar structure, since reactivity with the E87 monoclonal antibody is found in a large number of serotype strains (29). Recently, Lam and coworkers (M.C. Lam, E.J. McGroarty, and J.S. Lam, unpublished results) have produced seven monoclonal antibodies to the A-band isolate. Using Western immunoblot analysis, they have shown that A-band molecules are present as a common antigen on strains of many, but not all, serotypes. Interestingly, they found that A bands were present in a high percentage of clinical isolates and appeared to be a main antigen on nontypeable strains deficient in high-molecular-weight B-band-type LPS.

We propose that, for strains containing both A- and B-band-type molecules, the longer B-band polymers extend from the surface and constitute the main antigenic structure exposed on the cell. The shorter A bands may be covered and masked by the B-band O-polymers. However, under certain conditions, such as prolonged antibiotic therapy, clinical isolates are found to be nontypeable and appear to lose the O-polymer-containing B-bands (3,11,13). For such clinical isolates the A bands may become exposed and serve as an important antigenic determinant.

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CHAPTER 3

Growth Dependent Alterations in the Production of Serotype and Common Antigen Lipopolysaccharides in *Pseudomonas aeruginosa* PAO1

ABSTRACT

Pseudomonas aeruginosa PAO1 is grown in various media and at different temperatures, and the heterogeneity of the extracted lipopolysaccharide (LPS) was characterized by polyacrylamide gel electrophoresis. The size distributions of the serotype-specific LPS and the common antigen LPS were analyzed on Western blots (immunoblots). Cells grown at high, near-growth-limiting temperatures, at low pH, in low concentrations of phosphate, or in high concentrations of NaCl, $MgCl_2$, glycerol, or sucrose produced decreased amounts of the very long-chain population of O-antigen LPS molecules. Lower temperatures and lowered glycerol, lowered sucrose, low sulfate, lower salt concentrations, and elevated pH did not significantly affect the level of this LPS population. The size and amount of common antigen LPS was either unaffected or increased slightly when the cells were grown under the above stress conditions. Cells grown under normal, nonstressed conditions were agglutinated only by serotype-specific antibodies. In contrast, cells grown under stress conditions, in which the long-O-polymer LPS was absent, were agglutinated by both serotype-specific and common antigen-specific antibodies. The results indicate that the long O polymers cover and mask the shorter common antigen. However, specific growth conditions limit the

production of the long O polymer, allowing the exposure and reactivity of the common antigen on the cell surface.

INTRODUCTION

The lipopolysaccharide (LPS) from *Pseudomonas aeruginosa*, like that from other gram-negative bacteria, is a heterogenous mixture of molecules of different polysaccharide lengths (19,32) and with variable levels of substitutions at specific sites (27,38). In addition, LPS isolates from many serotypes of *P. aeruginosa* contain two chemically and antigenically distinct fractions, a serotype-specific LPS and a common antigen LPS (22,31,32,34). The size heterogeneity of LPS from *P. aeruginosa*, *Serratia marcesens* and *Salmonella anatum* is reported to be altered by growth temperature (1,25,30). These temperature-induced changes in LPS heterogeneity reportedly affected various cell surface properties including bacteriophage-inactivating capacity (25) and efficiency in plasmid transformation (1). Altering growth conditions, such as medium composition, is also reported to modulate the LPS chain length of *Escherichia coli* and to alter the sensitivity of the cells to a neutrophil bactericidal protein (36). Further, growth of *P. aeruginosa* in low magnesium or adaptive growth in polymyxin or aminoglycosides alters the sensitivity of the cells to EDTA and polymyxin (12,17,33). In a similar fashion, polymyxin resistance can be induced in *Pseudomonas fluorescence* by growth in limiting phosphate (8). Such alterations in antibiotic sensitivity may

result from changes in the outer membrane which affect the permeation of these drugs. Alterations in outer membrane structure induced by specific growth conditions may change cation binding sites (12). Since a major site of cation binding in the outer membrane is with LPS, we have initiated an analysis of the influence of various growth conditions on the size heterogeneity of LPS in *P. aeruginosa*. The results reported here indicate that near-growth-limiting conditions including high temperature, high concentration of salt, sucrose, or glycerol, low phosphate concentration, and low pH altered the size heterogeneity of the serotype-specific LPS produced and allowed exposure of the common antigen LPS on the cell surface. In addition, cells grown in limiting phosphate produced LPS that was recovered mainly in the phenol phase, similar to what was observed for LPS from a rough mutant strain.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

Smooth strains of *P. aeruginosa* PAO1 used in this study were H103 (15), PAO1716 (24) and the polymyxin-resistant strain H185 (26). Strain AK1401, an LPS-defective rough mutant of PAO1, was also studied (1). Cultures of 1 liter were grown in 2.8-liter Fernbach flasks on a rotary shaker operating at 230 rpm. Cultures were grown at temperatures between 17 and 42°C in either tryptone (1%, wt/vol)-yeast extract (0.2%, wt/vol) broth (TYE) or in terrific broth (TB) consisting of 24 g of yeast extract, 4 ml of glycerol, and 12 g of tryptone per liter of 0.017 M KH_2PO_4 - 0.072 M K_2HPO_4. Cultures of H103 and AK1401 were also grown in a modified basal medium (MBM) composed of 5 mM NaCl, 5 mM KCl, 40 mM glucose, 30 mM $(NH_4)_2HPO_4$, 30 mM $(NH_4)_2SO_4$, and 0.5 mM $MgCl_2$. For H103 cultures, 0.05 mg/ml of adenine sulfate, and 0.1 mg/ml of L-leucine (pH 7.0) were added; for cultures of AK1401, 20 μg of L-leucine, L-lysine and L-threonine per liter and 5 μg of thiamine per ml were added. For cells grown in low sulfate, MBM was modified; the final $(NH_4)_2SO_4$ concentration was reduced to 0.1 mM and the concentration of $(NH_4)_2HPO_4$ was increased to 60 mM. For cells grown in low phosphate the $(NH_4)_2HPO_4$ in MBM was decreased

to 3 mM and the $(NH_4)_2SO_4$ was increased to 60 mM. To analyze the effect of medium pH, we modified MBM; glucose was replaced with 40 mM citrate and the pH was adjusted with NaOH or HCl. Cells were grown in the various media at defined temperatures for at least two transfers before the culture was harvested late in logarithmic growth phase.

LPS isolation

Cells form 1 liter of medium were washed with distilled water and extracted with hot aqueous phenol (37). The two phases were separated at room temperature by spinning at 5,000 x *g* for 5 min. The phenol and aqueous phases were dialyzed extensively against 5 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), pH 7.5, and then with distilled water. The dialyzed samples were spun at low speeds, and the supernatant solutions were treated with 15 μg/ml DNase I (Sigma Chemical Co., St. Louis, MO) and 5 μg/ml RNase A (Sigma) in 10 mM $MgCl_2$ for 30 min at 4°C. The samples were washed once with 0.1 mM $MgCl_2$ and once with distilled water at 76,000 x *g* for 2 h and then lyophylized. Unless otherwise noted, greater than 85% of the LPS was recovered in the aqueous phase, and only the aqueous phase-fractions were analyzed.

Gel electrophoresis and Western blots (immunoblots).

LPS samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (31,32). The gels were

silver stained by the method of Dubray and Bezard (9). Western immunoblots of SDS-polyacrylamide gels were prepared as described previously (4,31,32,35). The 0-5 serotype-specific LPS was detected with the monoclonal antibody anti-503 (14,31,32) and the common antigen LPS was detected with the monoclonal antibody E87 (31,34). The blots were washed and visualized by using horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (Sigma).

Slide agglutination assays.

Cells grown under specific test conditions were harvested late in the logarithmic growth phase. The cells were sedimented and suspended in saline containing 0, 0.3 or 1.5% Formalin. After the cells were incubated for 30 min at room temperature, the treated cells were washed twice in saline and resuspended in saline at high concentrations (optical density at 560 nm of ~ 20). The cells were mixed with an equal volume of either 0-5 specific monoclonal antibody MF15-4 (21) or common antigen-specific monoclonal antibody E87 (34). Cells were also mixed with saline or with control antiserum. After 5 min, agglutination was assessed by light microscopy. The level of agglutination was quantitated by measuring the relative number of cells which had clumped compared with the number that were dispersed. The agglutination tests were performed at least twice for each culture condition with independently isolated cells. Cells were used in the agglutination studies immediate after isolation and treatment in all cases.

Assays.

Assays for 2-keto-3-deoxyoctulosonic acid (KDO) and protein were done as described previously (32). Phosphate levels were quantitated by inductively coupled plasma emission spectroscopy (6).

RESULTS AND DISCUSSION

LPS recovery and phosphate content of samples from cells grown in different media.

Lipopolysaccharide was isolated from cells grown in several different media by using hot aqueous phenol (37), and the recovery of LPS in the aqueous and phenol phases was quantitated by assaying for 2-keto-3-deoxyoctulosonic acid. Except for the rough mutant AK1401 and the smooth strain H103 grown in low phosphate, greater than 85% of the LPS was recovered in the aqueous phase. For LPS from strain AK1401, between 25 and 80% of the LPS was recovered in the aqueous phase, depending on the growth medium used. Also, for cells of H103 grown in MBM low in phosphate, only 25% of the LPS was recovered in the aqueous phase with the remainder isolated from the phenol phase. The low recovery of LPS in the aqueous phase for these cultures may result from the lack of hydrophilic long O-polymer-containing LPS in the isolates (see below). The amount of LPS recovered in the aqueous phase for both the smooth and rough strains varied depending on the growth medium (Table 1). Generally, the LPS recovered constituted between 2 to 6% of the cell dry weight. This level is similar to that reported for LPS levels from *P. aeruginosa* (7). Modification of the media

TABLE 1. Level of LPS Recovered from Cultures of AK1401 and of PAO1 Smooth Strains H103 and PAO1716 Grown in Different Media.[a]

Medium	PAO1 Smooth Strains[b]	AK1401
TB	2.5 ± 0.6	1.0 ± 0.1
TYE	5.0 ± 1.0	3.9 ± 0.6
MBM	6.1 ± 1.1	4.9 ± 0.4
MBM (low PO_4^{2-})[c]	2.0 ± 1.0	3.1 ± 0.7
MBM-citrate[d]	6.0 ± 0.5	5.3 ± 0.9

[a]The recovery is expressed as a percentage of the dry weight of the cells (mean ± standard deviation). Only recovery from the aqueous phase after phenol extraction is reported.

[b]Recovery for the two smooth strains was not different, and the average recovery is reported.

[c]The MBM medium contained lower levels of $(NH_4)_2PO_4$ as indicated in Materials and Methods.

[d]Glucose was replaced by citrate in the MBM (see Materials and Methods).

or other growth conditions as described below did not significantly alter the level of LPS recovery (data not shown).

The phosphate contents of the various LPS isolates also did not vary appreciably with the growth medium used (Table 2). LPS from the smooth strains grown in TB, which is high in phosphate, had higher phosphate levels, while LPS from cells grown in minimal medium low in phosphate contained lower levels of phosphate. Similar trends were detected in the isolates from strain AK1401. Other modifications of the growth media or growth conditions as described below did not significantly affect the LPS phosphate content (data not shown).

Using these different media we tested the influence of specific growth conditions on the size and heterogeneity of the LPS produced by the smooth and rough strains. The structure of the gram-negative envelope is known to change in response to growth temperature, medium composition, and medium osmolarity (2). Furthermore, the outer membrane of *P. aeruginosa* is exceptionally sensitive to rapid changes in temperature, pH, and toxicity (5). Thus, these growth conditions were examined for their influence on LPS composition.

Growth temperature effects on LPS composition.

Cultures of PAO1716, H103, and AK1401 were grown in TB and TYE at temperatures between 17 and 42°C for two transfers and then harvested. The size heterogeneity of the various isolates from the aqueous-phase fraction was defined by SDS-PAGE (Figure 1; data not shown). When large amounts of the samples

TABLE 2. Phosphate Content of LPS Isolates from Cells Grown in Different Media[a]

Medium	μmol/mg LPS	
	PAO1 Smooth Strains[b]	AK1401
TB	2.1 ± 0.1	1.8 ± 0.1
TYE	1.7 ± 0.1	1.7 ± 0.1
MBM	1.6 ± 0.1	1.4 ± 0.2
MBM (low PO_4^{2-})	1.4 ± 0.1	1.1 ± 0.1

[a]Phosphate contents are given as the weighted average ± standard deviation of the levels recovered from the phenol phase and aqueous phase.

[b]The levels represent the average amount detected in LPS from strain H103, H185 or PAO1716.

Figure 1. Effect of growth temperature on LPS size. Silver-stained gels (A and B) and Western blot (C) of LPS from strains PAO1716 or AK1401 grown in TB at the temperatures indicated. Samples of 10 (A), 0.25 (B), or 30 (C) μg were applied. The Western blot (C) was visualized with the monoclonal antibody E87. Numbers to the left of panel A denote the major size populations.

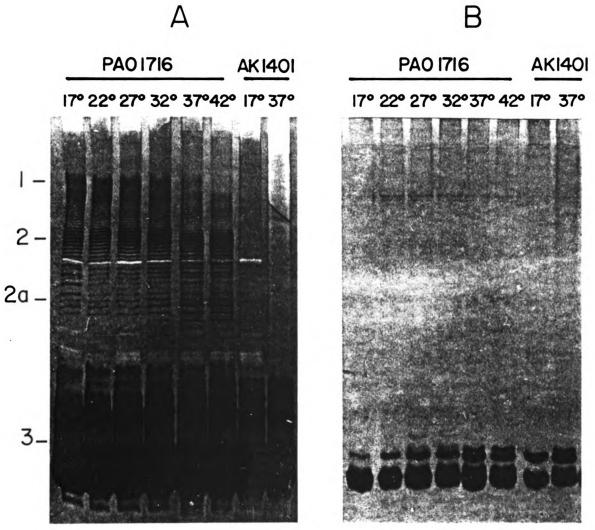
A
B
PAO 1716
AK1401
17° 22° 27° 32° 37° 42° 17° 37°
PAO 1716
AK1401
17° 22° 27° 32° 37° 42° 17° 37°
1
2
2a
3

Figure 1

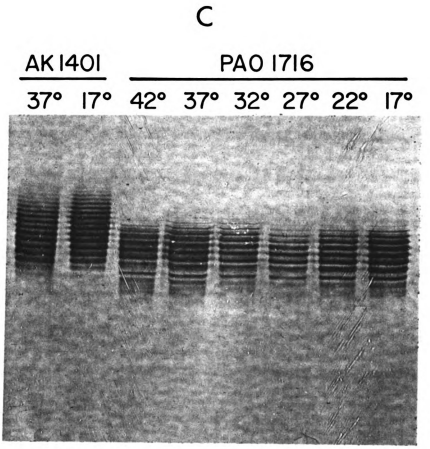

Figure 1

were applied to the gel, the ladder pattern of the high-molecular-weight O-polymer-containing molecules could be detected (Figure 1A). For the isolates from the smooth strain, three to four regions of intense staining could be detected which represented three to four populations of molecules with O-polymers of different lengths. LPS from cells grown at low temperatures had higher levels of the very long O-polymer-containing molecules (population 1) and lacked the shorter-chained population, denoted 2a. As the growth temperatures increased, the level of molecules of the highest molecular weight dropped and the amount of short-chained molecules increased. The level of the intermediate-sized molecules (population 2) showed only marginal changes. These results are similar to those of a previous study which showed a decrease in high-molecular-weight LPS in *P. aeruginosa* with increasing growth temperature (20). The LPS from strain AK1401 grown in TB appeared rough and did not contain very high levels of O-polymer-containing molecules. Only very weak staining in the region of the common antigen bands was detected in the LPS from cells of AK1401 grown in TB at 17 and 37°C (see below). This reflected a general inhibition in common antigen LPS production in all strains grown in TB (data not shown).

When these same samples were applied at low concentrations, the heterogeneity of the predominant, smaller-sized molecules could be resolved (Figure 1B). For the PAO1716 samples, as the growth temperature increased, there was an increase in the band immediately above the fastest-moving band. The band of higher mobility presumably corresponds to molecules containing core-lipid

A, while the slower moving band likely contains molecules capped with one O repeat. This increased capping with increasing growth temperature has been noted by others (20). The LPS from the rough mutant also revealed the same two bands, confirming a previous study which showed that AK1401 is an SR mutant (22). For both strains grown at temperatures above 32°C, the LPS contained a second, slower-migrating band which could be resolved above each of the two fastest-migrating bands; these secondary bands increased in relative amounts with increasing growth temperature.

Since the LPS isolates contain a common antigen LPS in addition to the main, serotype-specific LPS, the change in the size distribution of this population was analyzed on immunoblots. The LPS isolates from the two strains grown at different temperatures were separated by SDS-PAGE and then electrotransferred to nitrocellulose. The common antigen LPS bands were visualized with monoclonal antibody E87, which is specific for the *P. aeruginosa* common antigen (31,34). The transblots indicated that for the PAO1716 strain, the level and size distribution of the common antigen molecules was unaffected by growth temperature (Figure 1C). In addition, the AK1401 strain contained common antigen LPS, and the level and size distribution also did not change significantly with growth temperature. However, the size distribution of the common antigen LPS from strain AK1401 was somewhat different from that of the smooth strain.

Effect of salt concentrations in media on LPS composition.

Cells of H103 and H185 were grown at 37°C in TYE broth containing 0, 3, 10, 30 or 100 mM $MgCl_2$. Growth was completely inhibited for both strains in medium containing 300 mM $MgCl_2$. Cultures of H103 were also grown at 37°C in TYE containing 0, 50, 150 and 500 mM NaCl. Growth was inhibited by 1.5 M NaCl in the medium.

The size distribution of the isolates from H103 and H185 grown in various concentrations of $MgCl_2$ were analyzed by SDS-PAGE. The gels of the H103 isolates (Figure 2) and H185 (not shown) were either silver stained or were transblotted and reacted with monoclonal antibody E87 or anti-503. When these samples were applied to gels at high concentrations, the long-chain LPS could be detected. Silver-stained gels of the H103 samples indicated that there was little change in the O-antigen length with Mg^{2+} concentration except at 100 mM (Figure 2B). At this highest concentration, there was a dramatic decrease in the level of the highest-molecular-weight population of LPS. This population (population 1) represents the longest O-specific LPS molecules as detected on Western blots with the O-specific monoclonal antibody (Figure 2A). The size distribution of the common antigen LPS, measured in blots with monoclonal antibody E87 (Figure 2C), also did not change appreciable with the concentration of $MgCl_2$ in the growth medium, except at the highest concentrations. The samples from cells grown in 100 mM $MgCl_2$ had a slight increase in the longer common antigen molecules. When the samples were applied to the gel at low

Figure 2. Effect of $MgCl_2$ concentration in the growth medium on LPS size. Silver-stained gels (B and D) and Western blots (A and C) of LPS from strain H103 grown in TYE nutrient medium containing 1 (lane 1), 3 (lane 2), 10 (lane 3), 30 (lane 4) or 100 (lane 5) mM $MgCl_2$. Samples of 10 (B), 5 (A and C), or 0.05 (D) μg of LPS were applied to the wells. After electrophoresis, the gels were either silver-stained (B and D) or transblotted onto nitrocellulose and reacted with either anti-503 (A) or E87 (C) monoclonal antibody.

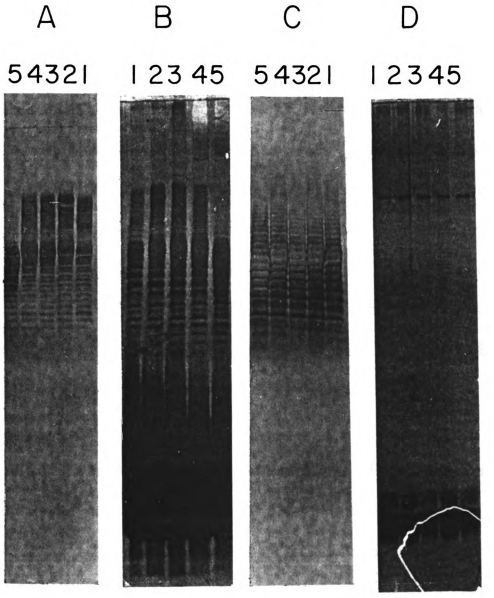

Figure 2

concentrations, the heterogeneity of the predominant, short-chained LPS could be measured. The results showed that the level of LPS containing one O repeat was not significantly altered when the Mg^{2+} concentration in the medium was increased (Figure 2D). Essentially identical results were obtained with the LPS samples from strain H185.

When the H103 cells were grown in TYE containing high concentration of NaCl, the LPS composition changed in a manner similar to that for cells grown in high $MgCl_2$. The levels of the very-long-chain O-antigen molecules decreased on cells grown in 500 mM NaCl but were unaffected by lower NaCl concentrations (Figure 3A). Furthermore, the amount and length of the common antigen also increased at the highest NaCl concentration but was unaffected by lower levels of salt (Figure 3B). The heterogeneity of the short-chain LPS was also unaffected by the NaCl concentrations (data not shown).

Effects of growth in high glycerol or high sucrose on LPS composition.

Since near-growth-limiting concentrations of salt appeared to inhibit the synthesis of the long-O-antigen molecules and to induce an increase in common antigen LPS amount and length, we asked whether this phenomenon might depend on the osmotic strength of the media. Thus, cultures of H103 were grown in TYE containing either 1.0 M glycerol or 1.0 M sucrose, which are both near growth limiting. After two transfers in these media, the cells were harvested and the LPS was isolated. When the aqueous-phase isolates were analyzed by SDS-PAGE, both

Figure 3. Effect of NaCl concentration in the growth medium on LPS size. Western blots of LPS from strain H103 grown in TYE nutrient medium containing 0 (lane 1), 50 (lane 2), 150 (lane 3), or 500 (lane 4) mM NaCl. A 10-μg sample of the LPS was applied to the wells. After electrophoresis, the gels were transblotted into nitrocellulose and reacted with either anti-503 (A) or E87 (B) monoclonal antibody.

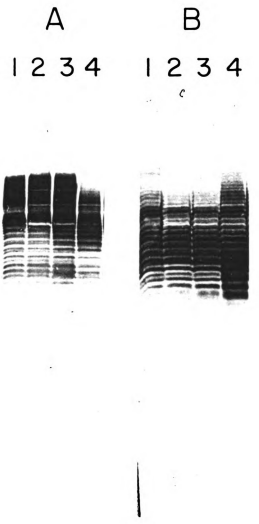

Figure 3

samples appeared to have a very long chain population that was shorter (population 1) than the LPS from cells grown in the absence of glycerol (Figure 4) or sucrose (not shown). However, the population 1 set of molecules was not completely missing in either of these isolates.

Thus, the LPS changes induced by growth in high salt are likely not triggered by high osmolarity since growth in high glycerol and high sucrose had much less of an effect on O-polymer synthesis. That high salt may induce an alteration in the LPS composition and antigen reactivity may be critical for cystic fibrosis (CF) patients infected with *P. aeruginosa*. Lam *et al.* (22) have shown that a high percentage of clinical isolates produced common antigen LPS. Of the strains which produced the A-band (common antigen) LPS, 68% could be agglutinated by anti-common antigen monoclonal antibodies. This study also indicated that during infection of a CF patient with *P. aeruginosa*, the initial isolates were serotypeable and produced long-chain LPS. However, during infection the strain became nontypeable and the cells were agglutinated with common antigen monoclonal antibody. Also, the patient produced antibody to the A-band LPS during the infection. Presumably, the environment of the lung of these patients selects for cells which produce lower amounts of long-chain, serotype-specific LPS. Others have reported that during infection of CF patients, *P. aeruginosa* strains lose their O-antigenic determinants (28,29), become nontypeable (16), and serum sensitive (16,29), and produce a new, polyagglutinable antigen (16,23,28,29). It has been suggested that the

Figure 4. Effect of 1 M glycerol in the growth medium on LPS size. Silver-stained gel (A) and Western blot (B) of LPS from strain H103 grown in TYE nutrient medium containing 0 (lane 1) or 1 (lane 2) M glycerol. A 20-μg sample of LPS was applied to each well. The Western blot was reacted with anti-503 monoclonal antibody.

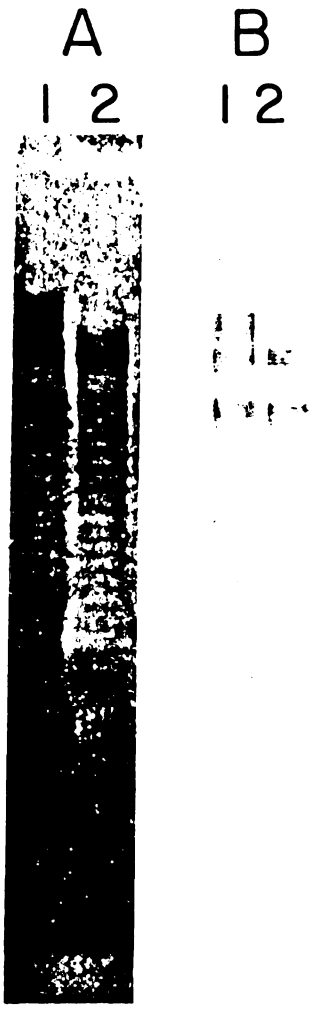

Figure 4

polyagglutinable antigen is in the core-lipid A which becomes exposed when the O polysaccharide is diminished (11). However, recent evidence indicates that the common antigen polysaccharide is present and exposed on many nontypeable strains (22). The presence of altered or elevated levels of specific ions in the sputum of CF patients (10,18) may induce a decrease in long-O-polymer LPS synthesized by the infecting *P. aeruginosa* just as we have observed for cells in culture. In addition, in the CF patient, extensive antibiotic therapy may select for β-lactam (13) - and aminoglycoside (3) - resistant strains, some of which appear rough or have decreased amounts of O polymer. Thus, there may be several selective pressures that induce *P. aeruginosa* strains infecting CF patients to lose their long O antigen.

Effects of growth in low phosphate and low sulfate on LPS composition.

The serotype-specific LPS has been shown to be high in phosphate and low in sulfate, while the common antigen LPS appears to lack phosphate but contain sulfate (31). To analyze the influence of these anions on the production of the two types of LPS, we grew cells of strain H103 and AK1401 in MBM, in MBM low in sulfate, and in MBM low in phosphate. The LPS isolates were separated by SDS-PAGE, and the gels stained with silver. When the H103 samples were loaded onto the gel at high concentration, the ladder pattern of the high-molecular-weight LPS could be detected (Figure 5C). The samples from cells grown in minimal medium and minimal medium low in sulfate appeared similar. In

Figure 5. Effect of phosphate and sulfate concentrations in the growth medium on LPS size. Silver-stained gels (C and D) and Western blots (A and B) of LPS isolated from strain H103 grown in MBM (lane 1), MBM low in sulfate (lane 2), or MBM low in phosphate (lanes 3 and 4). Samples in lane 3 were recovered from the aqueous phase, while samples applied to lane 4 were from the phenol phase. Samples of either 10 μg (A, B and C) or 0.1 μg (panel D) were applied to the gels. Western blots were reacted with either anti-503 monoclonal antibody (A) or E87 monoclonal (B).

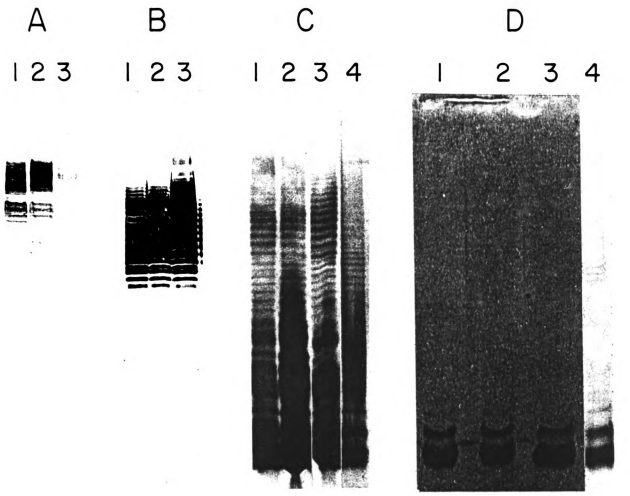

Figure 5

contrast, the LPS isolated in the aqueous phase from cells grown in low phosphate appeared to contain fewer of the molecules with long O polymers. This difference in the amounts of O-polymer-containing LPS was also detected on Western blots of these same samples reacted with monoclonal antibody anti-503 (Figure 5A). The cells grown in MBM and in MBM low in sulfate appeared to have similar amounts of the very long O-specific LPS, while the LPS from the aqueous-phase isolates of cells grown in low phosphate had a reduced amount of these long O polymers. Western blots of these samples stained with the common-antigen-specific monoclonal antibody E87 indicated little difference in the levels and size of common antigen LPS from cells grown in MBM and MBM low in sulfate (Figure 5B). In contrast, the aqueous-phase isolates of cells grown in low phosphate had a significantly greater level of common antigen LPS. Similar to the analyses described above, the LPS isolated from the phenol phase from the cells grown in low phosphate contained very little long-O-polymer LPS and was composed mainly of core-lipid A molecules (population 3) and common antigen LPS (Figure 5C). Analysis of the heterogeneity of the predominant short-chained molecules (Figure 5D) indicated that the level of capping of the core-lipid A by one or two O repeats was similar for the isolates from cells grown in MBM and in low-sulfate MBM and from the phenol-phase isolates of cells grown in low phosphate. However, the aqueous-phase isolates of cells grown in low phosphate had a lower proportion of molecules capped with one O repeat. Surprisingly, the

LPS isolated from AK1401 grown in MBM and MBM low in phosphate was essentially identical on SDS gels (data not shown).

Effect of growth at low pH on LPS composition.

To analyze the effects of medium pH on the LPS produced, we grew cultures of strains H103 and AK1401 in MBM containing citrate instead of glucose as the carbon source. The citrate buffered the medium in the acid range to within 0.2 pH units of the value set before inoculation. The growth rate of the two strains at neutral pH in minimal media did not change with the change in carbon source.

The heterogeneity of the LPS isolated from cells grown at different pHs was detected on SDS gels (Figure 6). When the samples were loaded at high concentrations, the size heterogeneity of the H103 isolates did not vary between pH 6.9 and 7.8 and the size distribution of the samples isolated at neutral pH was the same for cells grown on both carbon sources. However, the production of long-chain LPS from cells grown at pH 5.5 was severely restricted. This pH is near the lower limit for growth of this organism and appears to induce a stress-related response in the smooth strain similar to that seen with high temperature and high salt. Surprisingly, this low pH had little effect on the production of common antigen LPS by AK1401 (Figure 6A). Analysis of the heterogeneity of the dominant short-chain components (Figure 6B) showed that, for both strains, low medium pH decreased the level of capping of the core with one O repeat and

Figure 6. Effect of pH of growth medium on LPS size. Silver-stained gel of LPS from strains AK1401 and H103 grown in MBM containing 40 mM glucose or citrate. The pH of the medium was adjusted as indicated. Samples of either 12.5 μg (A) or 0.125 μg (B) were applied.

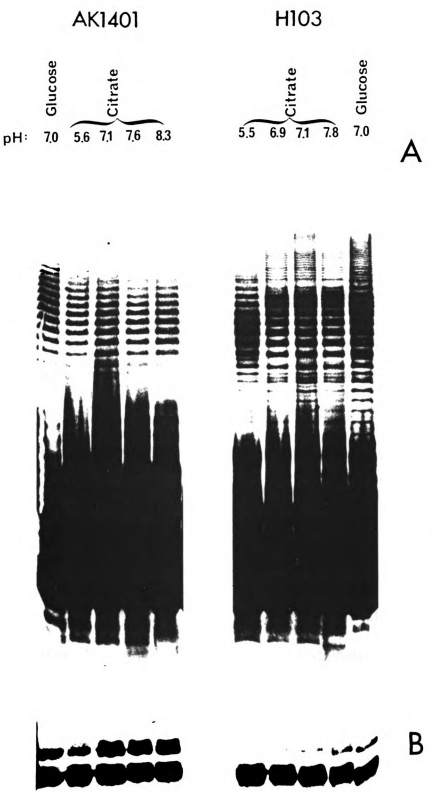
AK1401
H103
Glucose
Citrate
Citrate
Glucose
pH: 7.0 5.6 7.1 7.6 8.3
5.5 6.9 7.1 7.8 7.0
A
B

Figure 6

decreased the level of the slower-moving band in the doublet pattern; these changes are similar to what was seen in the short-chain LPS population with growth at lower temperatures (Figure 1).

Whole-cell agglutination assays.

In the studies described above, we found several growth conditions including high temperature, high salt concentrations, high glycerol and sucrose concentrations, low phosphate, and low pH which induced a change in the size heterogeneity of the serotype-specific LPS, resulting in a decrease in the number of molecules with a long O polymer. These same growth conditions either had no effect on the common antigen LPS produced or they induced an increase in the amount and size of the common antigen molecules. To determine if the reactivities of these LPS antigens on the cell surface were altered by such changes in LPS composition, we used monoclonal antibodies MF15-4 and E87 to agglutinate whole cells. Cultures of H103 were grown in the various test media at specific temperatures for two transfers. A culture of AK1401 was also grown in TYE broth as a control since it lacks serotype-specific LPS (Figure 1). The cultures were treated as described in Materials and Methods and then mixed with one of the monoclonal antibodies. Results of the agglutination assays are shown in Table 3. The H103 strain grown at 37°C in TYE broth showed no agglutination with the monoclonal antibody to common antigen even when the cells were Formalin treated, while the serotype-specific antibody, MF15-4, readily

TABLE 3. Slide Agglutination Reactions for Cells of *P. aeruginosa* Strains H103 and AK1401 Grown Under Different Conditions[a]

Strain	Medium	Growth Temp. (°C)	E87 (% Formalin)			MF15-4 (% Formalin)		
			0	0.3	1.5	0	0.3	1.5
H103	TYE	37	-	-	-	++	+++	++++
	TYE	42	++	+	+++	+++	++++	++++
	TYE + 1M glycerol	37	+/-	+/-	+	+++	++++	++++
	TYE + 0.5M NaCl	37	++	++	++	+++	+++	+++
	MBM	37	+	+	+/++	++/+++	+++	++/+++
	MBM (low PO_4^{2-})	37	+/++	++	+++	+++	+++	+++
	MBM (low PO_4^{2-}), 1x[b]	37	+	+++	+++	+++	+++	+++
AK1401	TYE	37	+++	+++	++++	-	-	-

[a]Cells were centrifuged and suspended in saline containing 0, 0.3 or 1.5% Formalin. The cells were incubated for 30 min at room temperature, washed twice with saline, and mixed with either E87 or MF15-4 monoclonal antibody. The strength of agglutination is noted by the number of +, with values greater than +++ indicating greater than 80% of the cells agglutinated.

[b]Cells were transferred only once in the growth medium before the agglutination assay.

agglutinated these cells. The agglutination with MF15-4 was strongest when the cells were pretreated with Formalin, suggesting that formaldehyde alters the O polymer to make it more accessible to antibody interaction. The lack of agglutination by common antigen-specific E87 may be due to the longer serotype molecules masking the shorter common antigen polymers. Thus, when these same cells were grown in TYE broth at 42°C or in 0.5 M NaCl or when they were grown in low-phosphate-containing minimal medium, conditions which permitted synthesis of only shorter O polymers, the cells were agglutinated with E87 antibody. The agglutination of these cells with E87 was strongest if the cells had been pretreated with Formalin, again indicating that formaldehyde alters the polysaccharide to allow for better reactivity. These same cells were also strongly agglutinated by the serotype-specific MF15-4 antibody, indicating that both antigens were present on the surface of the cells. Since greater than 90% of the H103 cells were agglutinated with serotype-specific antibodies, it appears that most of the H103 cells in the cultures grown under any of these conditions contained both types of LPS in their outer membrane.

The cells grown in TYE containing 1 M glycerol showed only weak agglutination with E87. Thus, the loss of only the longest of the very-long-chain O polymers (Figure 4) did not cause a significant increase in the exposure of the common antigen molecules on the cells. Cells grown in minimal medium also

showed only weak agglutination with E87; these cultures also produced the long O polymers which masked the common antigen. As expected, agglutination of the AK1401 strain was detected only with E87. This agglutination was very strong, presumably because these cells lack a masking O polymer.

In conclusion, analysis of LPS isolates from cells grown under different conditions showed that several stress conditions including high, near-growth-limiting salt concentrations, high, near-growth-limiting temperatures, and low, near-growth-limiting pH induce a dramatic decrease in the length of the O-specific LPS. Furthermore, for cells grown at high temperature, there was an increase in capping of the predominate short-chained population, as has been reported previously (20). Growth in high salt, either $MgCl_2$ or NaCl, also induced an increase in the amount or length of the common antigen. In contrast, growth at high temperatures did not alter the common antigen. Loss of the long O polymer allowed for reactivity of the common antigen on the cell surface, which normally is not accessible to antibody. Since $>$ 90% of all smooth cells tested were agglutinated by serotype-specific antibody and since $>$ 80% of cells from cultures grown at high temperatures or in high salt were agglutinated by common antigen-specific antibody, it appears that most cells in these cultures contain both antigens on their surface. Thus, these two LPS antigens do not appear to represent classical antigenic variation. Presumably, when high levels of the long O polymer are synthesized, the shorter common antigen is present on most cells but is covered and inaccessible to antibody.

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CHAPTER 4

Common Antigen Lipopolysaccharide of *Pseudomonas aeruginosa* Contains Sulfur.

ABSTRACT

Lipopolysaccharide (LPS) was isolated from *Pseudomonas aeruginosa* strain AK1401 which lacks O-antigen. The cells were grown in modified basal medium containing 350 μCi of ^{35}S-labelled sulfate, and the isolated LPS was separated by gel filtration chromatography into two major size populations of LPS. These fractions were characterized as a common antigen LPS (A-band) and a serotype-specific short-chain LPS (SC or B-band). The elution profile showed that the A-band and B-band LPS contained labelled-sulfur. This is in agreement with the results of an autoradiogram of the polyacrylamide gel separation of unfractionated (Uf) LPS sample. Chemical analysis of the A-band LPS showed low levels of detectable 2-keto-3-deoxyoctulosonic acid (KDO) and no phosphate. In addition, A-band LPS showed a 1:1 molar amount of sulfur to heptose suggesting that two to three sulfur atoms are present in the A-band molecule probably as sulfate. The molar amounts of sulfur detected in Uf- and SC-LPS isolates were one half the heptose content. We propose that this A-band LPS possesses an 'unusual' lipid A with the distinctive feature that the phosphate groups in the 1- and 4'-position of the diglucosamine head group are missing and may be replaced by sulfate.

INTRODUCTION

Lipopolysaccharide (LPS) is a major constituent on the surface of gram-negative bacteria and is essential for the assembly, organization (18,25), and functioning of the outer membrane (18,24). The LPS from *Pseudomonas aeruginosa*, as well as that from *Enterobacteriaceae*, can be divided into three structural regions: the lipid A, the core oligosaccharide, and the O-antigen polysaccharide (16,29). Structural similarities between LPS from *P. aeruginosa* and enterobacterial LPS include a bisphosphorylated D-glucosamine disaccharide lipid A backbone and the inner core sugars L-*glycero*-D-*manno*-heptose and 2-keto-3-deoxyoctulosonic acid (KDO) (16,18). However, LPS from *P. aeruginosa* is unique in its large number of phosphate residues, the presence of L-alanine in the core, and the presence of 2-OH- and 3-OH-dodecanoic acids and 3-OH-decanoic acid instead of 3-OH-tetradecanoic acid characteristic of enterics (9,16,29,35,36).

In smooth strains, a long polysaccharide is attached to the core, but in *P. aeruginosa* a low proportion of the LPS molecules carry the polysaccharide (30,35). Chemical differences in the O-polysaccharide (O-antigen) of different strains distinguish *P. aeruginosa* into 20 different O-serotypes (22,23).

Characterization of LPS structure has been complicated not only by chemical heterogeneity in the lipid A (2,4,35) and incomplete substitution in the core (16,24,29,35) but also by chemical heterogeneity of the O-antigen side chain (16,24,35). Beside the variability in the O-polysaccharide length (16,30), the LPS isolates of *P. aeruginosa* have been resolved into amino-sugar-rich and neutral-sugar-rich fractions (30,32,36,39).

Recently, it has been shown that strains of *P. aeruginosa* synthesize two immunologically and chemically distinct forms of LPS known as A-band and B-band LPS (27,30,31). B-band LPS is the O-antigen-containing LPS and determines the O-specificity of the bacterium, while A-band LPS or common antigen contains shorter chains of predominantly neutral polysaccharide. Others have observed a common lipopolysaccharide antigen in standard and clinical strains of *P. aeruginosa* (20,32). Arsenault *et al.* (T.L. Arsenault, D.W. Hughes, D.B. MacLean, W.A. Szarek, A.M.B. Kropinski, and J.S. Lam, Can. J. Chem., in press) demonstrated that the high neutral sugar content of the A-band LPS is attributed to an unusually high level of D-rhamnose. This is similar to the data reported by Yokota *et al.* (38,39) and Kocharova *et al.* (17) who also isolated a neutral polysaccharide from *P. aeruginosa* containing predominantly D-rhamnose. The main polymer chain of the rhamnose-rich polysaccharide is reported to consist of the repeating unit ->3)Rha-(α1->3)Rha-(α1->2)Rha-(α1- (17,38,39; T.L. Arsenault *et al.*, Can. J. Chem., in press).

In a recent study, Rivera and McGroarty (31) showed that the fatty acid

composition of the A-band LPS from *P. aeruginosa* PAO1 strains was similar to the serotype-specific B-band LPS, but that the A-band fraction did not contain KDO or phosphate. Instead, sulfate was detected in this LPS isolate. As previously suggested (31), sulfate may replace the phosphate on lipid A of A-band LPS. It is interesting to note that sulfolipids have been identified in various bacteria (10-12,15,21); they are highly hygroscopic, and occur mainly in membranes which have predominantly acidic lipids (11,12). In aqueous solutions between pH 7.5 and 9.0 there is negligible hydrolysis of the sulfate from sulfolipids (11). In this study we have demonstrated that sulfur is incorporated into A-band LPS in stoichiometric amounts.

RESULTS AND DISCUSSION

The bacterial strain used in this study was *P. aeruginosa* AK1401, an LPS defective rough mutant of the restrictionless mutant isolate OT684 (*leu*-1 *lys*-1 *res*-4, 3). This strain produces A-band LPS but is defective in the synthesis of O-antigen. Cultures were grown at 37°C in modified basal medium (MBM) (27) with the following modification: the final $(NH_4)_2SO_4$ concentration was reduced to 0.9 mM, and 0.1 mg/ml of L-leucine, L-lysine, and L-threonine, 2 μg/ml of thiamine, and 350 μCi of ^{35}S-labelled calcium sulfate were added per liter of culture. Cells were adapted to MBM for at least two transfers before growing in labelled sulfate. The cells were harvested late in the logarithmic phase. Labelled cells from 3 liters of medium were washed with distilled water and extracted with hot aqueous phenol (33). The aqueous phase was extensively dialyzed and lyophilized as described previously (27) except that 50 mM K_2SO_4 was added to the 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.5, to insure that the ^{35}S-labelled sulfate remained covalently bound.

The LPS isolate was fractionated on Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, NJ) column (69 cm by 25 mm) at room temperature by using the buffer system (pH 8.0) of Peterson and McGroarty (28). Approximately

$5.9x10^4$ cpm of ^{35}S-labelled LPS was applied to the column, and 4-ml fractions were collected at a flow rate of 6.4 ml/h. Column fractions were analyzed for amino sugar content (8) and total phosphate (1). ^{35}S counts (cpm/ml) were measured using a Beckman LS7000 Microprocessor Controlled Scintillation Counter. The column fractions were also characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the system of Laemmli (19), and the gels were silver-stained by the method of Dubray and Bezard (7). The elution profile showed three ^{35}S peaks (Figure 1, peaks 1,2, and 3) and two amino sugar peaks (Figure 1, peaks 1 and 2), while phosphate could be detected only in peak 2. Note that peak 3 contains only ^{35}S and elutes as a broad peak (fraction number 75 to 90). Presumably this peak contains either free sulfate that has been released from the LPS during the column fractionation or sulfur bound to small oligosaccharides. The dashed line indicates the position of elution of free ^{35}S-labelled sulfate suggesting the second possibility is more likely (Figure 1, arrow). The SDS-PAGE of the column fractions, when applied in order of elution, revealed a diagonal ladder pattern representing molecules of different sizes (Figure 1, A- and B-bands). The A-band LPS corresponds to the slower migrating set of bands (Figure 1, peak 1) while the second population of molecules, designated the B-band or SC-LPS (Figure 1, peak 2), corresponds to the faster migrating set of bands. As indicated in earlier studies (30,31), the SC-LPS fraction is composed of molecules containing a core-lipid A with none or only one O-repeat unit similar to that of the main serotype-determining antigen. The

Figure 1. Gel filtration profile of Uf-LPS from *P. aeruginosa* AK1401 grown in $^{35}SO_4$-MBM media. Fractions were analyzed for amino sugar (■), total phosphate (▲), and ^{35}S counts (●). Silver-stained SDS-polyacrylamide gels of column fractions are aligned under their appropriate fraction number. A represents the slow-moving set of bands, and B represents the faster moving set. The dashed line is free $^{35}SO_4^{2-}$ eluting from the column at the inclusion volume (arrow).

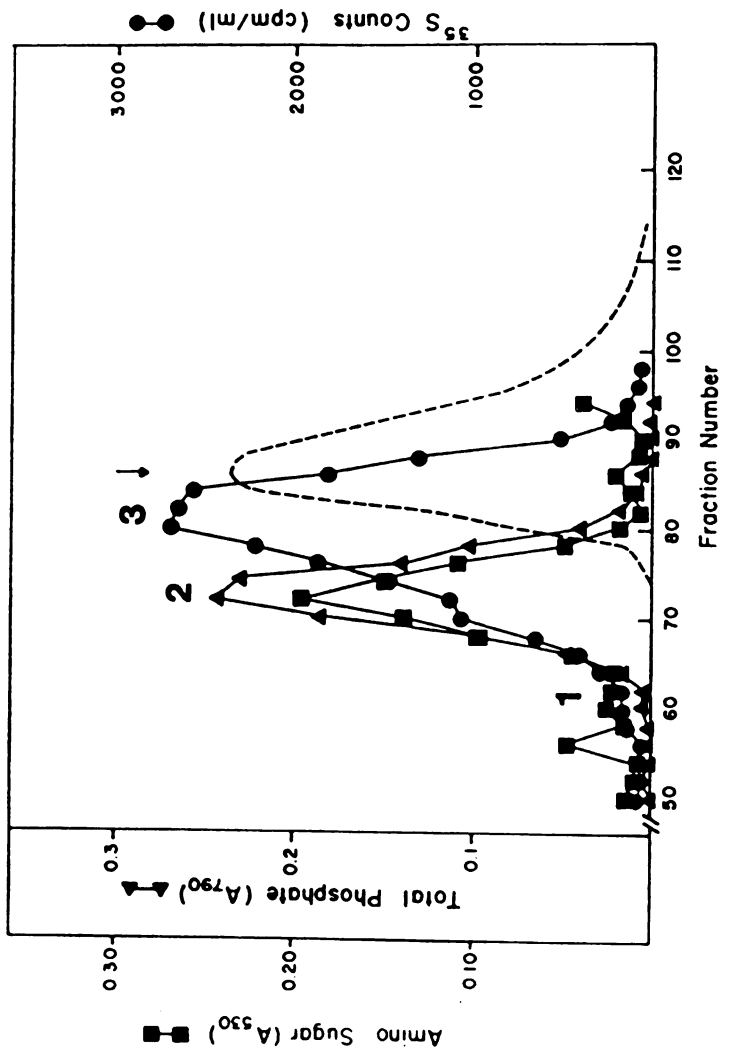
35S Counts (cpm/ml)
3000
2000
1000
Amino Sugar (A530)
Total Phosphate (A790)
0.30
0.20
0.10
0.3
0.2
0.1
1
2
3
50
60
70
80
90
100
110
120
Fraction Number

Figure 1

fractions corresponding to A- and B-bands LPS (Figure 1, peaks 1 and 2, respectively) were pooled and extensively dialyzed (12,000- to 14,000-molecular-weight-cutoff membranes), at room temperature, against a buffer composed of 50 mM Tris, 10 mM EDTA, 50mM triethylamine, and 0.02% sodium azide, pH 8.5. The pooled fractions were dialyzed further against 10 mM $MgCl_2$ and then against distilled water. The dialyzed fractions were lyophilized and stored at 4°C for further analysis.

Since the A-band LPS analyzed in previous studies was isolated from smooth strains of *P. aeruginosa*, the isolates were always contaminated with small amounts of an intermediate chain-length serotype-specific LPS (30,31). By using the AK1401 strain we obtained purified A-band LPS devoid of any O-serotype LPS contaminants. To verify that the fatty acid composition of this A-band LPS isolate corresponds to what has been reported previously (31), the fatty acid analysis of this A-band LPS, as well as SC-LPS and Uf-LPS, was performed as described elsewhere (13). For total membrane fatty acid composition, 0.2 ml of resuspended total membrane pellet was used. The identity of the fatty acids was confirmed by gas chromatography/mass spectrometry (GC/MS). GC/mass spectrometry was performed on a JEOL AX505 double focusing mass spectrometer equipped with a Hewlett-Packard 5890 GC and a capillary column (Supelco SPBI; using helium as carrier at a flow rate of 1-ml/min) interfaced directly into the ion source. The GC was programmed at an initial temperature of 150°C, and heated at a rate of 3.0 deg/min to a final temperature of 300°C, with a final hold of 10.0

min; the total run length was 60.0 min. Mass spectra were acquired at a rate of approximately 1 scan per second. Compounds were ionized by electron ionization (EI) at 70 eV. A bacterial acid methyl esters mixture (Supelco, Bellefonte, PA) was used to determine retention times as well as fragmentation patterns for the fatty acid methyl esters. The fatty acid compositions for the various LPS fractions are presented in Table 1. The fatty acids of *P. aeruginosa* strain AK1401 LPS consisted of C12:0 and the hydroxy acids characteristic of the *Pseudomonas*, 3-OH-C10:0, 2-OH-C12:0, and 3-OH-C12:0 (9). All three samples contained high amounts of 2-OH- and 3-OH-dodecanoic acid and lacked the 3-OH-tetradecanoic acid. As previously reported (31), these results show that the A-band isolate of strain AK1401 is similar to the A-band LPS isolated from smooth strains and confirm that it has the fatty acid composition typical of this species. In addition, the total cell fatty acid composition of *P. aeruginosa* AK1401 is in agreement with that of an earlier report (14).

Also, our previous studies indicated that the A-band LPS (peak X) isolated from smooth PAO1 strains of *P. aeruginosa* lacked phosphate but contained sulfate (30,31). To establish that A-band LPS from AK1401 follows the same trend, composition of the LPS isolates was analyzed for heptose (37) and phosphate (1). KDO (6) levels were determined after hydrolyzing the LPS samples in 0.5 N H_2SO_4 at 100°C for 30 min. Sulfur levels were quantitated by inductively coupled plasma emission spectrometry carried out by the Soil and Plant Analysis Lab, Madison, WI. Protein concentrations were estimated by the Pierce BCA protein

Table 1. **Fatty acid composition of LPS fractions from *Pseudomonas aeruginosa* AK1401.**

Fatty Acid	Recovery from Sample[a]			
	Uf-LPS	SC-LPS	A-band LPS	Total[b]
3-OH-$C_{10:0}$	21.3	7.9	11.9	2.1
$C_{12:0}$	8.8	8.6	3.2	1.3
2-OH-$C_{12:0}$	36.1	37.0	47.6	4.6
3-OH-$C_{12:0}$	33.9	46.6	37.4	1.2
$C_{16:1^9}$	—[c]	—	—	6.5
$C_{16:0}$	—	—	—	43.9
$C_{18:1^9}$	—	—	—	36.1
$C_{18:0}$	—	—	—	1.7
$C_{19:0\Delta}$	—	—	—	2.0

[a]Results are the averages of two or more analyses and are expressed as a weight percentage of the total.

[b]Total membrane fatty acid composition. There was less than 1.0% of $C_{14:0}$, $C_{15:0}$, i-$C_{17:0}$, $C_{17:0\Delta}$, $C_{17:0}$, $C_{19:0}$, and $C_{20:0}$ in this sample.

[c]Not detected.

assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as the standard. Analysis for protein in the pooled fractions after gel filtration indicated no detectable levels of protein, while the Uf-LPS isolate contained less than 14% (wt/wt) protein. The chemical composition of Uf-LPS and SC-LPS isolates were similar (Table 2). In previous studies we showed that all the size fractions of LPS from *P. aeruginosa* PAO strains contained heptose, a component of the inner core of most LPS isolates (30,31). The level of this sugar per weight in the isolates reflected the molecular weight of the molecules, suggesting that the heptose content is similar in the different size fractions. In contrast, the sugar KDO, another component common to the inner core of most LPS isolates, appeared to be very low in the A-band LPS isolate (Table 2). Even though the KDO levels are low in this fraction we cannot rule out the possibility that this sugar is substituted and thus not reactive in the assay (5). Furthermore, A-band LPS isolated from AK1401 had no detectable phosphate levels (Table 2), substantiating our earlier findings (31). Interestingly the three LPS isolates showed similar molar amounts of sulfur. The molar amounts detected in Uf-LPS and SC-LPS samples were approximately one-half the heptose content. Assuming that there are two to three heptose in the core region of LPS (35), then there is at least one sulfur, perhaps as sulfate, present in the SC-LPS. On the other hand, A-band LPS showed approximately a 1:1 molar ratio relative to heptose suggesting that two to three sulfurs may be bound to these molecules. This is consistent with the gel filtration profile. A possible explanation for why we are observing sulfur in the SC-LPS

Table 2. Chemical analysis of column fractions of LPS from *Pseudomonas aeruginosa* AK1401.

LPS Sample	Amount (nmol/mg)[a] of:			
	Heptose	KDO	Phosphate	Sulfur
Uf-LPS	304	200	2,538	102
SC-LPS	306	250	1,934	155
A-band LPS	163	8	0	129

[a]Average result from two or more isolates.

population may be that beside phosphate being incorporated in the core-lipid A, stoichiometric amounts of sulfur may also be attached. The probability of having an A-band type of LPS that is devoid of polysaccharide polymer that comigrates on the SDS-gel and coelutes in gel filtration with the serotype-specific SC-LPS also cannot be ruled out. Nonphosphorylated lipid As (13) and LPS lacking both phosphate and KDO (26) have been reported for a number of bacteria. As previously suggested, the A-band LPS isolate may have sulfate replacing phosphate on lipid A (31).

Finally, the common antigen LPS from smooth strains of *P. aeruginosa* has been shown to possess a regular banding pattern of intermediate polysaccharide length according to the SDS-PAGE (27,31). The AK1401 pooled A- and B-band LPS together with Uf-LPS were separated in SDS-PAGE to define their size heterogeneity and to show the presence of labelled sulfate. The lyophilized fractions were suspended in distilled water to a known weight concentration and applied to SDS-gels. Silver staining of Uf-LPS revealed two sets of bands (Figure 2A, lane 1) representing two populations of molecules differing in polysaccharide length. The slower migrating A-bands showed a ladder pattern with regular spacing. When the separated fractions were reapplied to SDS-gels, only one set of bands was observed for SC-LPS (B-band) (Figure 2A, lane 3). The A-bands showed the ladder pattern with the regular spacing of the common antigen LPS (Figure 2A, lane 2; 27) with a slight contamination of low-molecular-weight LPS. To further demonstrate that sulfur is covalently bound to this LPS, an

Figure 2. Silver-stained SDS-polyacrylamide gel (A) and autoradiograph (B) of *P. aeruginosa* AK1401. (A) Samples of 5 μg of Uf-LPS (lane 1), A-band LPS (lane 2), and SC-LPS (lane 3) were applied to a 15% acrylamide gel which had been polymerized overnight with a butanol overlay. Band sets A and B denote LPS major size populations. (B) Autoradiograph of SDS-polyacrylamide gel of Uf-LPS isolate. Approximately 5.9 x 10^4 counts ($\sim$5 mg) was applied to a 15% acrylamide gel (1.5 mm thick) and was exposed in a X-ray film for 6 weeks.

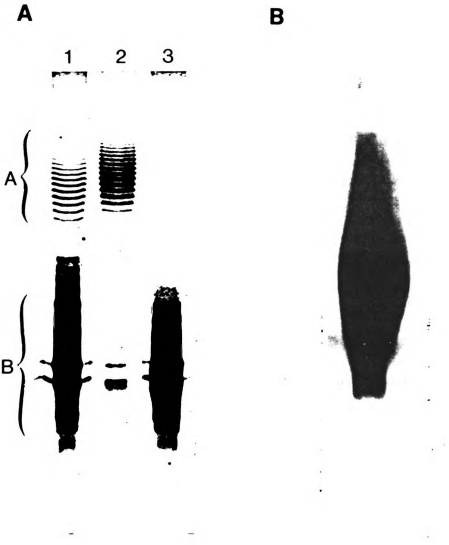

Figure 2

autoradiogram of the ^{35}S-labelled Uf-LPS separated by SDS-PAGE was analyzed. Approximately $5.9x10^4$ cpm of the sample (approx. 5 mg) was applied to a 15% SDS-gel (1.5 mm thick), with a 7.5% acrylamide stacking gel. Electrophoresis was performed as previously described. Following electrophoresis the gel was soaked in intensify-enhancer solutions (Intensify-Universal Autoradiography Enhancer, Dupont-Biotechnology Systems, Boston, MA) according to the manufacturer's instructions. A high speed X-ray film was exposed to the dried gel at -76°C for 6 weeks. The autoradiogram indicated that the Uf-LPS sample had a high amount of labelled sulfur (Figure 2B). The regions developed in the autoradiogram correspond to the components detected by silver staining in the SDS-gel (Figure 2A, lane 1). Free ^{35}S-labelled sulfate was run along with the *LPS* sample and no band corresponding to the free isotope was detected on the autoradiogram (results not shown). Presumably the labelled-sulfate diffused from the gel into the enhancer solution. These data are consistent with ^{35}S-labelled sulfur being covalently bound to LPS. Preliminary studies have also shown that *Rhizobium meliloti* incorporates sulfate into LPS (Hollingsworth, personal communication). Other microorganisms have also been reported to incorporate sulfate in their lipid-linked oligo- and polysaccharides (10-12,15,21); thus the presence of sulfur in LPS may not be confined to *P. aeruginosa*.

We have presented several pieces of evidence which indicate that A-band LPS isolated from *P. aeruginosa* AK1401 has sulfur covalently bound to LPS, perhaps as sulfate. The gel filtration column separated the two major size

populations (A- and B-band) and the A-band LPS as well as the short chain (SC) B-band LPS molecules appeared to have covalently bound sulfur. This is in agreement with the autoradiogram of the unfractionated LPS sample. Chemical analysis of the A-band LPS substantiated our earlier results which indicated low levels of detectable KDO and no phosphate (31). Furthermore, this isolate showed a 1:1 molar ratio of sulfur to heptose suggesting that two to three sulfur atoms are present in the A-band LPS molecule probably as sulfate. Given the findings reported here, we reconfirm that A-band LPS of *P. aeruginosa* contains sulfur.

The data presented in this study as well as previous reports (17,20,27,30-32,39) indicate that *P. aeruginosa* synthesizes more than one type of LPS. Furthermore, these results confirm that sulfur is incorporated into the LPS. Our results indicate that the chemical structure of the common antigen LPS differs in several respects from that of the O-serotype-specific LPS. It is reasonable to propose that the biosynthetic pathways of these two chemically distinct LPS molecules diverge at some point.

The biosynthesis of LPS has been studied in detail for *Salmonella typhimurium* and *Escherichia coli*. Several studies have indicated significant differences in lipid A synthesis among the different gram-negative bacteria (9,34; J. Lightfoot, T. Dasgupta, and J.S. Lam, Abstr. Annu. Meet. Am. Soc. Microbiol. 1991, D-69, p. 90). For example, Goldman *et al.* (9) has shown that the major lipid A precursor species from *P. aeruginosa* are completely acylated prior to addition of KDO, while enteric lipid A precursors just prior to KDO

attachment contains only 3-OH-C14:0 and lacks the other nonhydroxy fatty acids characteristic of mature LPS. It has been shown that the synthesis or expression of *P. aeruginosa* A- and B-band LPS appear to be partially independent (J. Lightfoot *et al.*, Abstr. Annu. Meet. Am. Soc. Microbiol. 1991, D-69, p. 90). Genes for A-band synthesis have been shown to map at a location different from the B-band genes. *P. aeruginosa* PAO is not the only bacterium reported to simultaneously express two LPS polysaccharide antigens. *Klebsiella pneumoniae* O1 is reported to synthesize two structurally distinct D-galactan polymers (34) and the genes involved in the expression of the two galactans are not closely linked.

Studies are now under way to chemically analyze the core-lipid A region of the A-band LPS. The characterization of the structure will give a better understanding of the function of this molecule in the mechanism of virulence and resistance to antibiotics.

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CHAPTER 5

Common Antigen Lipopolysaccharide from *Pseudomonas aeruginosa* AK1401 as a Receptor for Bacteriophage A7.

ABSTRACT

Lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* AK1401 was fractionated by gel filtration chromatography into two major size populations: the A-band or common antigen LPS and B-band or short chain (SC)-LPS. Purified A-band LPS was mixed with bacteriophage A7 and analyzed on sodium dodecyl sulfate-polyacrylamide gels. The results indicated that phage A7 recognizes the A-band polyrhamnose in the A-band isolate and within 2 h hydrolyzed the molecule to core-lipid A containing only two to three rhamnose repeat units. The phage A7 also hydrolyzed the A-band component in unfractionated (Uf) LPS. This phage A7 showed a high specificity to A-band LPS but did not alter the O-serotype-specific LPS. Chemical composition of the purified phage A7-digested A-band LPS showed low levels of heptose, 2-keto-3-deoxyoctulosonic acid, and amino sugars, and no phosphate. Reaction of lipid A from Uf-, SC-, and phage-digested A-band LPS with monoclonal antibodies (MAbs) specific for lipid A indicated that all the samples had common epitopes. Lack of reactivity of the acid hydrolyzed A-band isolate with anti-lipid A MAbs suggest that this sample is either non-reactive or is resistant to acid hydrolysis. In addition, all AK1401 LPS isolates were reactive with MAb specific to the inner core region of *P. aeruginosa*,

indicating that a common epitope in the inner core is shared by these LPS isolates. In contrast, an outer core-specific MAb raised against *P. aeruginosa*, bound only the Uf- and SC-LPS and not the A-band isolates indicating that the O-serotype LPS outer core structure is different from that of the common antigen LPS. We propose that the outer core, inner core and lipid A regions of A-band LPS are different from those of the serotype-specific LPS.

INTRODUCTION

Lipopolysaccharide (LPS) is a major component of the outer leaflet of the Gram negative outer membrane, comprising 20 to 40% of this structure by weight (25). These molecules are heteropolysaccharides covalently linked to lipid A (16,29). The heteropolysaccharide consists of three regions: a diglucosamine backbone, the oligosaccharide core, and the O-antigenic polysaccharide chain. Specific structures within the LPS molecule serve as receptors for a variety of bacteriophage (23,38). Phage absorption to its receptor is highly specific (23). Phage resistance that results from changes in LPS structure usually indicate that the LPS is the surface receptor (38), and the structural change identifies the region of the LPS comprising the receptor.

Since the structure of the O-serotype specific antigen varies from strain to strain, the host range of a phage whose receptor is the O-polysaccharide is rather narrow (16,23). One characteristic of O-specific phage is that during infection they often hydrolyze the O-antigen destroying the initial receptor (38). This enzymatic activity generally is localized in the tail-like phage attachment complex (23,38). Thus, adsorption of these phage involves the formation of an enzyme-substrate complex, the enzyme being an integral part of the phage tail and the

substrate being the O-antigen receptor.

In many strains of *Pseudomonas aeruginosa* a second LPS species is present whose polysaccharide chain differs serologically and structurally from O-antigen chain (17,21,24,30-32,41,42). This LPS has been termed A-band or common antigen LPS. The common antigen polysaccharide of *P. aeruginosa* is a regular homopolymer of rhamnose. On the basis of NMR and chemical analyses the structure has been shown to consist of the repeating unit [->3)-α-*D*-Rha*p*-(1->3)-α-*D*-Rha*p*-(1->2)-α-D-*Rha*p-(1->]$_n$ (17,41,42; T.L. Arsenault, D.W. Hughes, D.B. MacLean, W.A. Szarek, A.M.B. Kropinski, and J.S. Lam, Can. J. Chem., in press). Interestingly, the structure proposed for the repeating unit of the rhamnan chain in the common antigen LPS of *P. aeruginosa* is identical with that reported for the O-polysaccharide chain in the LPS of *P. syringae* pv. *morsprunorum* C28 (34).

The O-polysaccharide of *P. syringae* pv. *morsprunorum* C28 LPS, which is composed entirely of rhamnose (34), is specifically cleaved and released as oligosaccharides by the action of a rhamnanase borne on the typing phage A7 (33). This phage uses the LPS as its initial binding site (28,33). Thus, it is expected that the common antigen (A-band) LPS from *P. aeruginosa* will also serve as a substrate for phage A7. In this paper we present evidence to show that phage A7 binds to and hydrolyzes the polysaccharide chain of the A-band LPS from *P. aeruginosa* AK1401.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

P. aeruginosa AK1401 is an LPS defective rough mutant of the restrictionless mutant isolate OT684 (*leu*-1 *lys*-1 *res*-4; 2). This strain produces A-band LPS but is defective in the synthesis of O-antigen. *P. syringae* pv. *morsprunorum* strain No. 2168 was obtained from the National Collection of Plant Pathogenic Bacteria (Plant Pathology Laboratory, Hatching Green, Harpenden, Hertfordshire, England).

P. aeruginosa AK1401 was cultured at 37°C in tryptone (1%, w/v)-yeast extract (0.2%, w/v) broth (TYE). The cultures were harvested late in logarithmic growth phase. The phytopathogenic strain of *P. syringae* pv. *morsprunorum* was cultured in nutrient broth (NB) (1% tryptone, 0.2% yeast extract, 0.4% NaCl) at 25°C. An aliquot of an overnight culture was transferred to fresh NB media and the cells were grown to mid-logarithmic phase. This culture was used as a host for phage A7 propagation (see below).

Propagation of phage A7.

P. syringae pv. *morsprunorum* phage No. 2377 was obtained from the National Collection of Plant Pathogenic Bacteria (Plant Pathology Laboratory, Hatching Green, Harpenden, Hertfordshire, England). Phage were propagated on host cells of *P. syringae* pv. *morsprunorum* in nutrient broth-agar (NBA) (NB/1% agar) plates. Plates with 2 ml of the host (approx. 10^9 cells ml^{-1}) were overlayed with 0.2 ml of the A7 phage so as to give confluent lysis over the whole plate. After 18 to 24 h, 5 ml of sterile NB was poured onto the surface of the plate and left for 4 h to allow the phage to diffuse into the broth. The resulting phage suspension was centrifuged at 13,300 x *g* for 10 min and the supernatant solution was stored at -80°C. Yields were determined by a phage plaque counting technique (11). Titres of the order of 10^{10}-10^{11} p.f.u. ml^{-1} were obtained by this method. No significant loss of titre was observed during storage.

LPS isolation.

P. aeruginosa AK1401 or *P. syringae* pv. *morsprunorum* cells from 2 to 5 liters of medium were washed with distilled water and extracted with hot aqueous phenol (36). The aqueous phase was extensively dialyzed and lyophilized as described previously (24). In addition, the O-serotype-specific LPS from *P. aeruginosa* PAO1715 (peak 2 isolate) was isolated and purified by gel filtration on a Sephadex G-200 column as described elsewhere (30).

Column chromatography.

LPS isolates were fractionated on a Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) column (69 cm by 25 mm) at room temperature by using the column buffer system of Peterson and McGroarty (pH 8.5) (27), unless otherwise noted. Between 10 and 30 mg of LPS were applied to the column, and 4-ml fractions were collected at a flow rate of 6.2 ml/h. To remove detergent and buffer, pooled fractions were extensively dialyzed (12,000- to 14,000-molecular-weight cutoff membranes), at room temperature, against a buffer composed of 50 mM Tris, 10 mM EDTA, 50 mM triethylamine, and 0.02% sodium azide, pH 8.0. The pooled fractions were dialyzed further against 100 mM KCl and then against distilled water. The dialyzed fractions were lyophilized and stored at 4°C for further analysis.

Monoclonal Antibodies.

In order to produce core-specific or lipid A-specific monoclonal antibodies (MAbs), immunogen was prepared according to the method of Bogard *et al* (3). Briefly, cells of a rough *P. aeruginosa* strain AK1401 were suspended at a concentration of 5 x 10^9 cell per ml in 1% acetic acid, heated for 1 h at 100^0C, washed in distilled water and were lyophilized. This treatment should kill the cells and strip off cell surface polysaccharides. Core fractions of LPS from strain AK1401 were prepared by standard methods including hot aqueous phenol extraction and gel filtration fractionation using Sephadex G-50 (Pharmacia,

Uppsala, Sweden). Five mg of this core-LPS was then suspended in 5 ml of 0.5% (w/v) triethylamide followed by addition of 5 mg of the acid-treated bacteria. The mixture was stirred slowly for 30 min at room temperature and dried in vacuo with a Speed Vac centrifuge (Savant Instrument Inc., Hicksville, N.Y.). These core-LPS-coated cells were then used to immunize Balb/c mice intraperitoneally at a dose of 50 μl of core-LPS-coated cell suspension per injection in a 1:1 mixture with Freund's Incomplete adjuvant. The animals were immunized initially on days 0, 4, 9, 14, and 28. The injections were kept up once every two weeks until day 56. A test bleed was done to test for positive response against core bands of LPS with Western immunoblotting techniques. Upon detection of positive reaction to core region bands, the animals were immunized once more and sacrificed three days later to extract splenocytes for fusion with myeloma cell line NS1. The fusion protocol and isolation of hybridoma clones were precisely as described previously by Lam *et al* (20). Screening of the hybridoma cell lines was facilitated by ELISA and Western immunoblots and the use of LPS purified from the following rough LPS strains, including AK1401 (core-plus-one O side chain), AK1012 (core deficient mutant; 14), and AK44 (O-antigen deficient but with complete core; 18). A more detailed characterization of the MAbs 101 (outer-core-specific), 7-4 (inner-core-specific), and 177 (lipid A-specific) is described elsewhere (T.R. Chivers, L.A. MacDonald, and J.S. Lam, Ann. Meeting of the Can. Soc. Microbiol. 1991, Abst. No. MS4p; Manuscript in preparation). The

other MAbs 4A10 and 8A1 are specific to lipid A of enterobacteria (3) and were a kind gift of R.T. Coughlin.

Gel electrophoresis, dot blots, and Western blots (immunoblots).

LPS samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using the buffer system of Laemmli (19). The gels were silver-stained by the method of Dubray and Bezard (10). Western immunoblots of SDS-polyacrylamide gels were prepared as described previously (5,30,31,35). The gels were electrotransferred with a model TE Transphor Electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, CA) at a constant current of 290 mA for 28 h. For reaction with anti-lipid A MAbs, the nitrocellulose blots were hydrolyzed in 10% acetic acid for 2.5 h at 100°C immediately after electrotransfer. After hydrolysis the nitrocellulose blots were washed 5 times (5 min) with Tris-saline (0.9% NaCl in 10 mM Tris-HCl, pH 7.4). Lipid A was visualized on the blots, as described by Otten *et al.* (26), with either MAb 4A10 and 8A1, specific for lipid A of enterobacteria (3) or anti-lipid A MAb 177 raised against *P. aeruginosa*. In addition, Western immunoblots of the LPS samples were incubated with outer core-specific MAb, 101, or inner core-specific MAb, 7-4, both of which were raised against *P. aeruginosa*. Likewise, dot blots were analyzed by applying known quantities of LPS isolates directly on nitrocellulose with or without acid hydrolyzing the samples as above. The dot

blots were reacted with anti-lipid A MAbs 4A10 or 8A1 (3), outer core-specific MAb 101, or inner core-specific MAb 7-4.

Hydrolysis of LPS by phage A7.

Phage A7 (5.0×10^{11} p.f.u.) was pelleted from a stock suspension in NB by centrifugation at 148k x *g* for 1.5 h at 4°C and then mixed with 2 mg LPS in 2 ml of distilled water. The mixture was incubated with mild agitation at 20°C for 2, 3, 4, 5, and 24 h. An aliquot of 50 μl of the digested LPS was mixed with an equal volume of electrophoresis sample buffer (containing 4% SDS) and applied to an SDS-polyacrylamide gel. Electrophoresis was performed as previously described, and the gels were silver-stained.

In addition, a sample of approximately 10 mg of A-band LPS purified from isolated LPS of *P. aeruginosa* AK1401 by gel filtration, was resuspended in 2 ml of distilled water. Phage A7 (5.0×10^{11} p.f.u.) was added, and the mixture was incubated for 24 h as outline above. The digested LPS was treated for 24 h at 4°C with RNAse and DNAse (both at 22 μg ml^{-1}) followed by treatment with proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) (20 μg ml^{-1}) for 5 h at room temperature. The digested LPS sample was separated on Sephadex G-200 as described previously. The pooled phage A7-digested A-band fractions were extensively dialyzed, lyophilized, and suspended in distilled water

to a known concentration for further chemical analysis. A parallel experiment was performed using unfractionated LPS from *P. syringae* pv. *morsprunorum*.

Chemical assays.

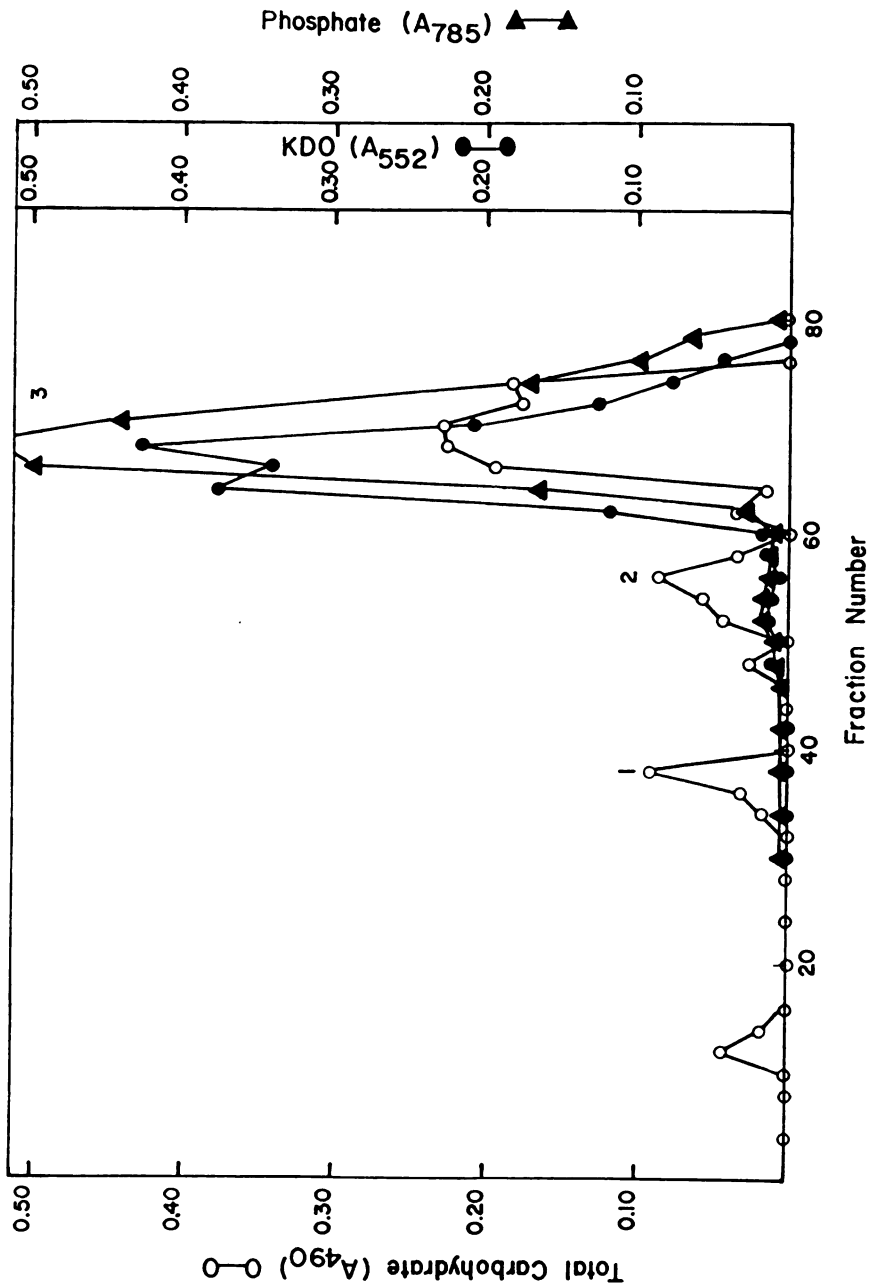
Assays for total carbohydrate (9), amino sugar (13), heptose (39), and phosphate (1) were performed as described previously. The 2-keto-3-deoxyoctulosonic acid (KDO) (8) levels were determined after hydrolyzing the LPS sample in 2.0 N H_2SO_4 at 100°C for 1 h in aqueous 1% SDS solution (6). Protein concentrations were estimated by the Pierce BCA protein assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as the standard.

RESULTS

The LPS from *P. aeruginosa* AK1401 was separated by gel filtration chromatography, and column fractions were monitored for total carbohydrate, KDO, and phosphate. The elution profile showed three carbohydrate peaks (Figure 1, peaks 1-3) while KDO and phosphate was detected only in the major peak. The SDS-polyacrylamide gel of the column fractions, when applied in order of elution, revealed a diagonal ladder pattern representing molecules of different sizes (Figure 1, A and B bands). The A-band LPS corresponds to the slower migrating set of bands (peak 2), while the second population of molecules, designated the B-band or SC-LPS (peak 3), corresponds to the faster migrating set of bands. As indicated in earlier studies (30,31), the SC-LPS fraction contains the majority of the molecules, comprising core and lipid A with none or only one O-repeat unit similar to that of the main serotype-determining antigen. The first peak in the elution profile (Figure 1, peak 1) did not appear to be LPS and was not analyzed further. Analysis of the pooled fractions for protein after gel filtration indicated a contamination of less than 1% (w/w).

To investigate the role of LPS from *P. aeruginosa* AK1401 as a receptor for phage A7, phage-binding and infectivity studies were carried out. A mid-log

Figure 1. Fractionation profile of LPS from *P. aeruginosa* AK1401 separated on Sephadex G-200. Fractions were analyzed for total carbohydrate (○), KDO (●), and phosphate (▲). Silver-stained SDS-polyacrylamide gels of column fractions are aligned under their appropriate fraction number. A represents the slow-moving set of bands, and B represents the faster-moving set.


Phosphate (A_{785})
KDO (A_{552})
Total Carbohydrate (A_{490})
Fraction Number
0.50
0.40
0.30
0.20
0.10
20
40
60
80
1
2
3


A
B

Figure 1

phase culture of AK1401 was tested as a host for the phage. A 0.1-ml aliquot of saline containing the test phage at 10^3 or 10^4 p.f.u. ml^{-1} was incubated with 0.1 ml of saline for 10 min at room temperature. At that time, 0.1 ml of bacterial culture was added with 3 ml of melted soft agar at 44°C and mixed; the mixture was then poured over the surface of an NBA plate and incubated at room temperature for 18 to 24 h. A parallel experiment was performed using *P. syringae* pv. *morsprunorum* as the host. The results showed no phage plaques with *P. aeruginosa* AK1401 whereas, with *P. syringae* pv. *morsprunorum* as host, phage plaques were observed (results not shown). We also tested the ability of purified A-band LPS from *P. aeruginosa* to inactivate phage A7 and prevent infection of *P. syringae* pv. *morsprunorum*. A 0.1-ml aliquot of saline containing the phage at 10^3 p.f.u. ml^{-1} was incubated with 0.1 ml of purified A-band LPS (100 μg) for 10 min at room temperature and was then diluted and mixed with the host cells as described above. Phage infectivity in the absence of added LPS was used as a control. The results indicated that A-band LPS produced a slight decrease in phage A7 plaque formation (less than 35% inhibition; data not shown). In addition, the SC-LPS isolate from *P. aeruginosa* was tested for its ability to inactivate phage A7 of *P. syringae* pv. *morsprunorum*, and the results indicated that there was no inhibition of phage infection with this sample (data not shown).

To show that phage A7 hydrolyses the polysaccharide chain of the A-band LPS from *P. aeruginosa* AK1401, phage A7 (5.0×10^{11} p.f.u.) was mixed with purified A-band LPS (~2 mg) at room temperature for 2, 3, 4, 5, and 24 h. The

SDS-polyacrylamide gels of the digested samples showed that the polysaccharide chain of the A-band LPS was completely hydrolyzed by phage A7 within 2 h (Figure 2A, lanes 2-7). Note that the higher molecular weight bands detected in Figure 2A (lanes 2-7) results from phage A7 protein bands (*); these samples were applied to the gel at high concentrations to detect minor LPS components. When lower amounts of LPS were applied to the gel, only the fastest-migrating bands were detected; the results show that, following hydrolysis with phage A7, the LPS consists predominantly of molecules composed of core-lipid A with presumably one to three rhamnose repeat units (Figure 2B, lanes 2-5). To demonstrate that phage A7 was specific to common antigen LPS, phage hydrolysis was also done using isolated O-serotype-specific LPS from the smooth strain of *P. aeruginosa*, PAO1715 (peak 2 isolate) (30). The results show that phage A7 does not degrade the *P. aeruginosa* O-serotype-molecule (Figure 2A, lanes 8 and 9). To define the minimum concentration of phage A7 required to completely hydrolyze 2 mg of A-band LPS from *P. aeruginosa* AK1401, samples of 1 ml of phage A7 containing 3.3×10^9 p.f.u. ml^{-1} (1:10), 1.4×10^9 p.f.u ml^{-1} (1:25), or 7.1×10^8 p.f.u. ml^{-1} (1:50) were used and hydrolysis carried out as described above. Results indicated that a concentration of at least 10^9 p.f.u. ml^{-1} is required for complete degradation of 2 mg of the polyrhamnose after 24 h (results not shown).

The hydrolysis of A-band LPS by phage A7 was further characterized using unfractionated LPS (Uf-LPS) from strain AK1401. A 0.5 mg sample of Uf-LPS was mixed with 1 ml of either 3.3×10^9 or 1.4×10^9 p.f.u. ml^{-1} at room

Figure 2. Silver-stained SDS-polyacrylamide gels of phage A7-digested A-band LPS (lanes 2-7) from *P. aeruginosa*. (A) Purified AK1401 A-band LPS was mixed with phage A7 for 2h (lanes 2 and 3), 3 h (lane 4), 4 h (lane 5), 5 h (lane 6), and 24 h (lane 7). Also, a sample of PAO1715 serotype-specific LPS was mixed with phage A7 for 2 h (lane 9). Samples of AK1401 A-band and Uf-LPS (lanes 1 and 10, respectively) as well as PAO1715 serotype specific LPS (lane 8) were applied to the gel. Samples of either 10 μg (lane 2), 4 μg (lanes 1, 3-9), or 0.5 μg (lane 10), were applied to the 15% acrylamide gel which had been polymerized overnight with a butanol overlay. A represents the slow-moving set of bands, and B represents the faster-moving set, and * indicates the phage A7 protein bands. (B) SDS-gel of AK1401 LPS after incubation with phage A7 for 2 h (lane 2), 3 h (lane 3), 4 h (lane 4), and 5 h (lane 5). Samples of either purified AK1401 A-band LPS (lane 1) or phage-digested A-band LPS (lanes 2-5) were applied to the gel at low concentrations (0.33 μg and 0.07 μg, respectively).

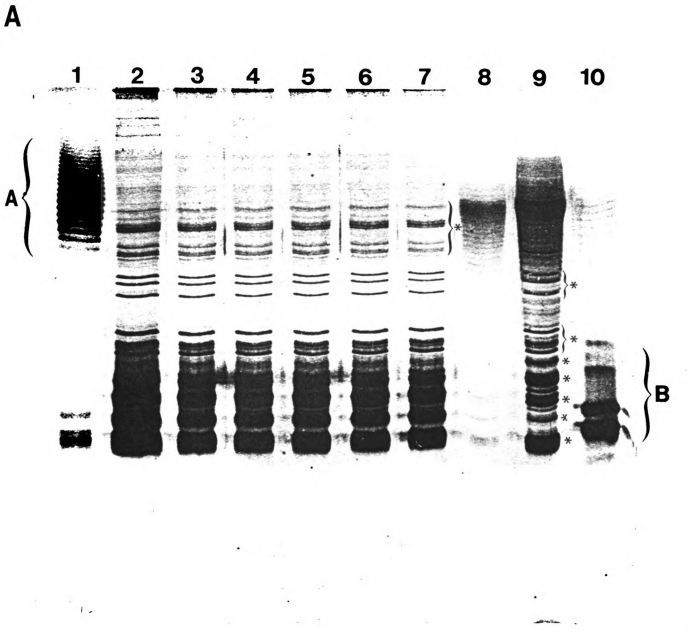


Figure 2

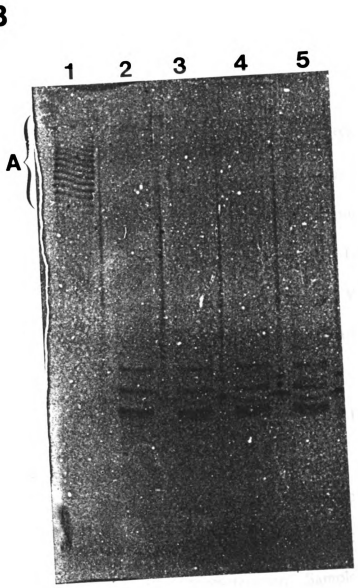


Figure 2

temperature for 24 h. Figure 3A shows the silver-stained gel of these samples. The results indicate that phage A7 hydrolyzes the A-band component in the Uf-LPS to the same extent as it does the purified A-band LPS (Figure 3A, lanes 2, 3, and 4, respectively). As indicated above, phage A7 does not appear to modify the SC-LPS (Figure 3A, lane 5). A parallel experiment was performed using *P. syringae* pv. *morsprunorum* LPS. A 1 mg sample of LPS was mixed with 1 ml of phage A7 (2.4×10^{10} p.f.u. ml^{-1}) at room temperature for 24 h. The SDS-polyacrylamide gel of the digested sample showed that the *P. syringae* pv. *morsprunorum* LPS was not completely digested after 24 h (Figure 3B, lane 2) suggesting that the phage was inactivated by this LPS isolate. Notice that the banding pattern of *P. syringae* pv. *morsprunorum* phage-digested LPS is well defined and can be used to count up to a maximum of approximately 30 rhamnose repeat units in the AK1401 A-band LPS as well as the Uf-LPS (Figure 3B, lanes 3 and 4, respectively).

It has been reported that the core-lipid A structure of A-band LPS from *P. aeruginosa* contains low levels of reactive KDO, lacks phosphate, but contains sulfur (31; M. Rivera and E.J. McGroarty, J. Bacteriol., manuscript submitted). Therefore, the lipid A component of this LPS may be antigenically different from that of the phosphorylated lipid A of the SC-LPS fraction. Samples of Uf-LPS, SC-LPS, A-band LPS, and phage A7-digested A-band LPS from *P. aeruginosa* AK1401 was separated by SDS-PAGE and then electrotransferred to nitrocellulose. A sample of an O-serotype-specific LPS from *P. aeruginosa* PAO1715 was used

Figure 3. Silver-stained SDS-polyacrylamide gel of LPS fractions from *P. aeruginosa* AK1401 and *P. syringae* pv. *morsprunorum* after incubation with phage A7. (A) A 2 mg sample of AK1401 Uf-LPS from *P. aeruginosa* was mixed with either 3.3 x 10^9 (lane 2) or 1.4 x 10^9 p.f.u. (lane 3) for 24 h. Also, 2 mg of purified A-band LPS (lane 4) and SC-LPS (lane 5) was incubated with phage A7 (1.4 x 10^9 p.f.u.) for 24 h. Samples of 5 μg of AK1401 Uf-LPS (lane 1) and phage-treated LPS (lanes 2-5) were applied to the gel. A represents the slow-moving set, and B represetns the faster-moving set. (B) A 1 mg sample of LPS from *P. syringae* pv. *morsprunorum* was mixed with 2.4 x 10^{10} p.f.u. for 24 h (lane 2). Samples of 5 μg of *P. syringae* pv. *morsprunorum* LPS (lane 1) and 2.5 μg of phage A7-digested LPS (lane 2) were applied to the gel. Samples of 5 μg from *P. aeruginosa* AK1401 Uf-LPS (lane 3), A-band LPS (lane 4), and phage A7-digested A-band LPS (lane 5) were also applied to the gel.

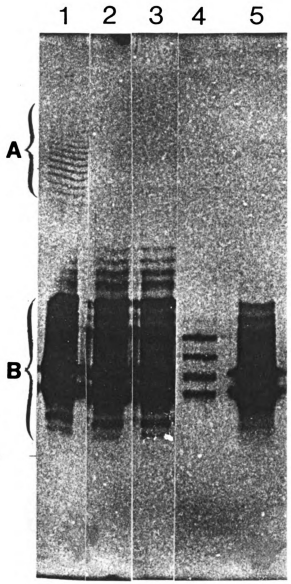


Figure 3

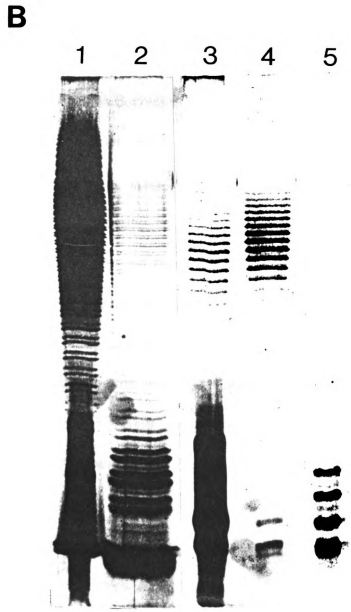


Figure 3

as a control. After acid hydrolysis of the samples on the blot, the nitrocellulose was incubated with MAb 8A1, specific for lipid A of *Escherichia coli* (3). A dot blot of the same samples was also developed under the same conditions. Figure 4 shows the Western blot (Figure 4A) and the dot blot (Figure 4B) of these LPS fractions. The Western blot indicated that MAb 8A1 reacted strongly with AK1401 Uf- and SC-lipid A derivatives, whereas phage digested AK1401 A-band lipid A derivative showed only moderate reactivity (Figure 4A, lanes 4, 3, and 1, respectively). Interestingly, AK1401 A-band isolate did not show reactivity (Figure 4A, lane 2), suggesting that this isolate may be resistant to the acid hydrolysis. The lipid A derived from the O-polymer-containing isolate showed weak reactivity (Figure 4A, lane 5). The results from the dot blot confirmed these findings (Figure 4B). Note that the AK1401 A-band isolate gave a weak reaction when 4 μg were applied; this reactivity may have resulted from the sample containing a low molecular weight contaminant, as seen in the silver-stained gel, which is immunoreactive with MAb 8A1 (Figure 2A, lane 1). In addition, this antibody reactivity might be due to non-specific binding of the A-band isolate. We also tested the binding of the MAb 4A10, specific to lipid A of *Salmonella minnesota*, to the same samples using the procedures described above. The results from the Western blot using MAb 4A10 showed that only AK1401 Uf- and SC-lipid A derivatives, and lipid A derived from O polymer gave a positive reaction (results not shown). We also tested these same samples using anti-lipid A MAb 177 specific to *P. aeruginosa* by using these same procedures. The results from

Figure 4. Western blot (A) and dot blot (B) of lipid A's derived from LPS fractions of *P. aeruginosa* reacted with MAb 8A1. (A) Samples of AK1401 phage A7-digested A-band LPS (lane 1, 40 μg), AK1401 A-band LPS (lane 2, 80 μg), AK1401 SC-LPS (lane 3, 20 μg), AK1401 Uf-LPS (lane 4, 25 μg), and PAO1715 O-serotype-specific LPS (lane 5, 40 μg) were separated by SDS-PAGE, electrotransferred to nitrocellulose, and hydrolyzed as described in Materials and Methods. The lipid A derivatives were incubated with anti-lipid A MAb 8A1. (B) Samples of PAO1715 O-serotype-specific LPS (lane 1, 4.0 μg), AK1401 Uf-LPS (lane 2, 2.5 μg), AK1401 SC-LPS (lane 3, 2.0 μg), AK1401 A-band LPS (lane 4, 4.0 μg), and phage A7-digested A-band LPS (lane 5, 2.5 μg) were applied onto a nitrocellulose strip, and was hydrolyzed as described in Materials and Methods. The lipid A derivatives were incubated with MAb A1; * indicates very weak reactivity.

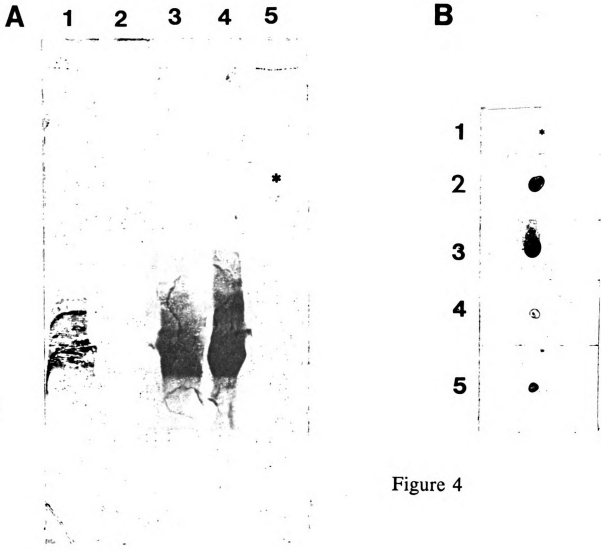


Figure 4

the Western blot showed that AK1401 Uf-, SC-, and phage-digested A-band lipid A derivatives had moderate affinity toward the MAb (Figure 5, lanes 2, 3, and 5, respectively). PAO1715 long chain LPS-derived lipid A showed very weak reactivity (Figure 5, lane 1). Again, the AK1401 A-band isolate did not show reactivity (Figure 5, lane 4) similar to the results with MAb 8A1. In addition, we performed a Western blot and dot blot immunoassay with Uf-LPS, SC-LPS, A-band LPS and phage A7-digested A-band LPS from *P. aeruginosa* as well as the O-serotype-specific LPS from *P. aeruginosa* PAO1715 using core-specific MAbs. The blots were incubated with either outer core-specific MAb 101 or inner core-specific MAb 7-4 raised against *P. aeruginosa*. When the LPS samples were incubated with the inner core-specific MAb 7-4, all the LPS samples reacted suggesting that a common epitope in the inner core is shared by these LPS isolates (Figure 6). Interestingly, when these same samples were incubated with the outer core-specific MAb 101, only the AK1401 Uf- and SC-LPS showed reactivity which indicates that the O-serotype-like LPS has an outer core structure different from that of the common antigen or A-band LPS (Figure 7).

To purify phage-digested A-band LPS for chemical analysis, the sample was treated with DNase and RNase, and then with proteinase K and separated on Sephadex G-200 column at room temperature eluting with column buffer at pH 8.0. Fractions were characterized by SDS-PAGE. The silver-stained gel revealed only one population of molecules (Dig-A-band), containing core-lipid A alone or core-lipid A with one to three rhamnose repeat units (results not shown). The

Figure 5. Western blot of lipid A's derived from LPS fractions of *P. aeruginosa* reacted with MAb 177. Samples of PAO1715 O-serotype-specific LPS (lane 1, 7.5 μg), AK1401 Uf-LPS (lane 2, 10 μg), AK1401 SC-LPS (lane 3, 5 μg), AK 1401 A-band LPS (lane 4, 15 μg), and AK1401 phage A7-digested A-band LPS (lane 5, 10 μg) were separated by SDS-PAGE, electrotransferred to nitrocellulose, and hydrolyzed as described in Materials and Methods. The lipid A derivatives were incubated with anti-lipid A MAb 177 specific to *P. aeruginosa*. Arrow indicates very weak reactivity.

Figure 5

Figure 6. Western blot (A) and dot blot (B) of LPS fractions of *P. aeruginosa* reacted with inner core-specific MAb 7-4. (A) Samples of PAO 1715 O-serotype-specific LPS (lane 1, 7.5 μg), AK1401 Uf-LPS (lane 2, 10 μg), AK1401 SC-LPS (lane 3, 5 μg), AK1401 A-band LPS (lane 4, 15 μg), and AK1401 phage A7-digested A-band LPS (lane 5, 10 μg) were separated by SDS-PAGE, electrotransferred to nitrocellulose and incubated with the MAb 7-4. Arrow indicates very weak reactivity. (B) Samples of PAO1715 LPS (lane 1, 4 μg), AK1401 Uf-LPS (lane 2, 2.5 μg), AK1401 SC-LPS (lane 3, 2 μg), AK1401 A-band LPS (lane 4, 4 μg), and phage A7-digested A-band LPS (lane 5, 4.5 μg) were applied onto a nitrocellulose strip and reacted with MAb 7-4.

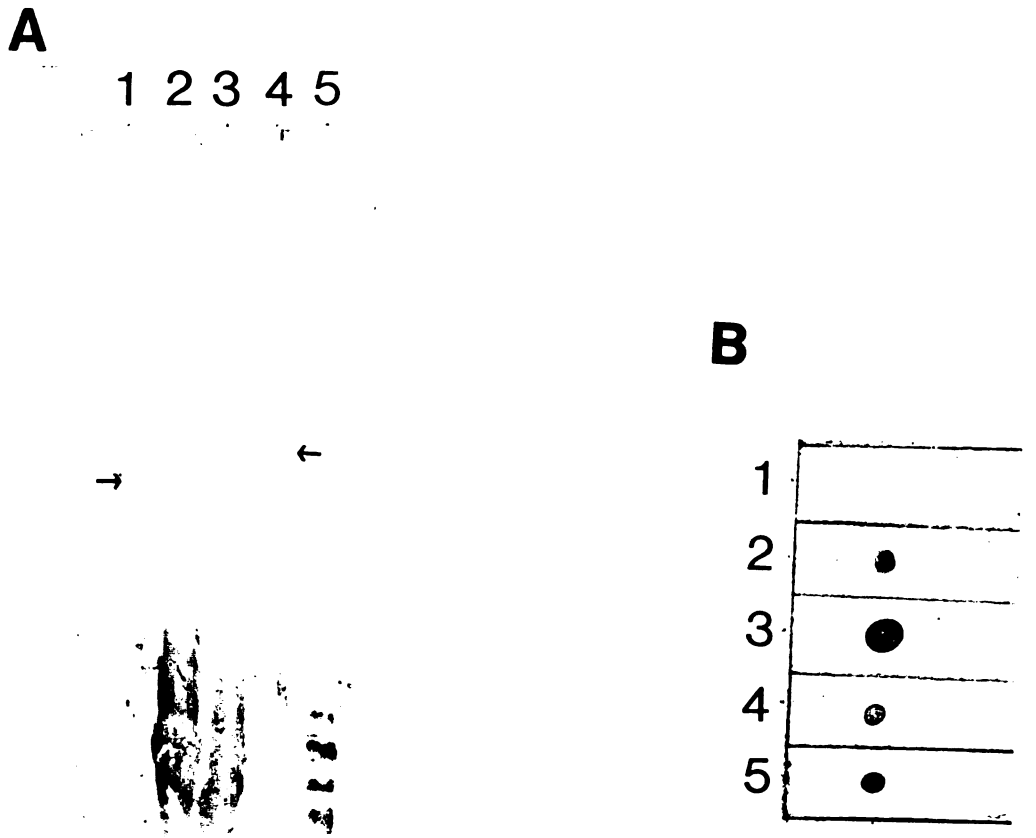


Figure 6

Figure 7. Western blot (A) and dot blot (B) of LPS fractions of *P. aeruginosa* reacted with outer core-specific MAb 101. The LPS isolates analyzed and the amounts applied are identical to those described in Figure 6.

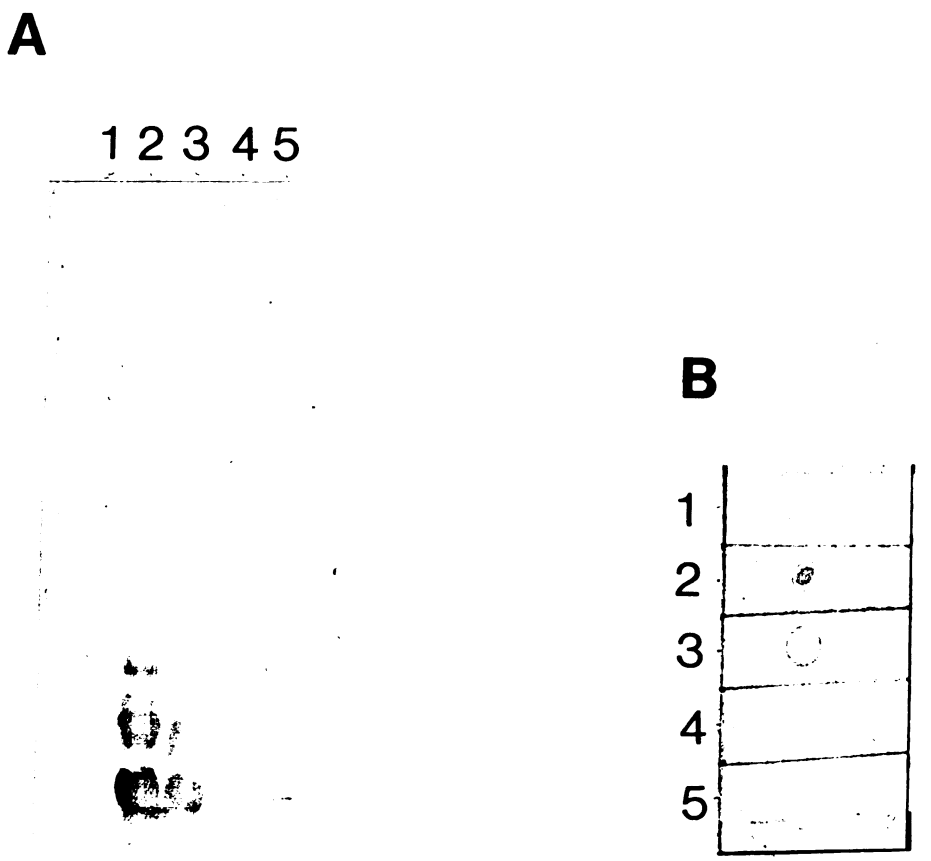


Figure 7

fractions corresponding to the major peak were pooled, dialyzed, lyophilized, and suspended in water to a known weight concentration for further analysis.

Chemical composition of the AK1401 LPS isolates were analyzed and the results are shown in Table 1. In previous studies (30,31) we showed that all size fractions of LPS from *P. aeruginosa* PAO strains contained heptose, a component of the inner core of most LPS isolates. The heptose content of the Uf- and SC-LPS were similar whereas that of A-band LPS was 10 times lower. The Dig-A-band LPS showed a dramatic decrease in the heptose level compared to that of A-band LPS isolate suggesting that rhamnose was interfering with the assay. Reaction with pure rhamnose was shown to be significant in the heptose assay (data not shown) raising the question as to whether heptose is present in the A-band isolate. Yokota *et al.* (40) have reported that the LPS of *Thiobacillus versutus* contains neutral sugars, glucosamine, KDO, and phosphorus, but is devoid of heptose. The quantity of KDO appeared to be very low in the A-band and Dig-A-band isolates confirming previous results (Table 1) (30,31). In *Acinetobacter calcoaceticus*, an octulosonic acid isomer resembling KDO is attached to lipid A and is also resistant to acid hydrolysis and does not give a positive thiobarbituric test (15). We cannot discard the possibility that an octulosonic acid isomer might be replacing the KDO residue in *P. aeruginosa* A-band LPS. Interestingly, the level of amino sugar also appeared to be very low in the A-band isolates. The molar amounts of amino sugars in Uf- and SC-LPS were approximately twice the KDO content. Furthermore, as shown previously

Table 1. Chemical composition of LPS fractions from *Pseudomonas aeruginosa* AK1401[a].

LPS sample	Heptose[b]	KDO	Phosphate	Amino sugar
Uf-LPS	440	280	1940	550
SC-LPS	300	280	1490	480
A-band LPS	44	20	ND[c]	23
Dig-A-band LPS	18	30	ND	26

[a]Levels given in nM/mg dry weight and are reported as the average of analyses from two or more isolates.

[b]Levels of heptose were corrected for D-*glycero*-D-*manno*-heptose (39).

[c]ND, not detected.

no detectable levels of phosphate was observed in the A-band and Dig-A-band LPS (30,31; Rivera and McGroarty, unpublished results).

DISCUSSION

The LPS of *P. aeruginosa* AK1401 was separated by gel filtration chromatography into two populations, the common antigen or A-band LPS and an O-serotype-like LPS which is composed of core-lipid A with none or one O-repeat unit (SC or B-band LPS). The column elution profile confirmed earlier studies which indicated that this strain is defective in the synthesis of O-antigen (2). The advantage of using this bacterial strain to isolate pure A-band LPS for chemical analysis is that the sample is not contaminated with small amounts of serotype-specific LPS as seen in previous studies (30,31).

It has been reported that the polysaccharide chain of the common antigen LPS from *P. aeruginosa* is a D-rhamnan trisaccharide unit, whose structure is similar to that of the O-antigenic polysaccharide chain of *P. syringae* pv. *morsprunorum* (17,33,34,41-43). The bacteriophage A7 is a typing phage for *P. syringae* pv. *morsprunorum*, and its primary receptor is LPS (28). Furthermore, phage A7 possesses a rhamnanase that specifically hydrolyses the O-antigenic polysaccharide chain of *P. syringae* pv. *morsprunorum* releasing it as oligosaccharide (33). Thus, we tested phage A7 for its ability to bind to cells of *P. aeruginosa* AK1401 and to digest its LPS. The results indicated that phage A7

cannot infect strain AK1401 and, when high concentrations of purified A-band LPS was added to the phage prior to mixing with host *P. syringae* pv. *morsprunorum* cells, only a moderate inhibition of phage infection was observed. Since phage A7 does not infect AK1401 cells, the moderate phage-inactivation using A-band LPS suggest that the phage may recognize the A-band LPS, but does not have the receptor needed for ejection of the nucleic acid (23,38). Smith *et al* (33) reported that the final binding site for phage A7 is within the residual core-lipid A region of *P. syringae* pv. *morsprunorum*.

Analyses of phage-digested A-band LPS from *P. aeruginosa* AK1401 (Figure 2) indicated that phage A7 recognizes the A-band polysaccharide chain as an initial receptor, hydrolysing the molecule to core-lipid A containing only two or three rhamnose repeat units. Furthermore, phage A7 reacted with Uf-LPS from AK1401 (Figure 3), indicating that the LPS of AK1401 acts as a primary surface receptor but does not have the final receptor for infection. Our results indicate that phage A7 does not completely hydrolyze all of the rhamnose polysaccharide side chain to core plus lipid A, as was suggested by Smith *et al.* (33). In fact, their results showed that the LPS from *P. syringae* pv. *morsprunorum* after phage treatment contained 2 to 4 times as much rhamnose as did the core oligosaccharide (peak II after acid hydrolysis). Our results also indicated that phage digestion of *P. syringae* pv. *morsprunorum* LPS was not complete (Figure 3B, lane 2). In the initial studies of this phage-catalyzed hydrolysis of the host LPS, the side chain digestion ended within the side chain

polysaccharide (33). Perhaps this region contains a secondary phage receptor site which induces ejection of the nucleic acid, inactivating the phage. From the digestion of the isolated host LPS we could determine that *P. aeruginosa* AK1401 A-band LPS has approximately 25 to 30 rhamnose repeat units (Figure 3B). McGroarty and Rivera (24) reported that when *P. aeruginosa* PAO strains were grown in high concentration of either $MgCl_2$ or NaCl, an increase in the amount or length of the common antigen was induced. Furthermore, Lightfoot and Lam (22) observed that the LPS from a derivative of *P. aeruginosa* AK1401 containing the pFV3 plasmid, produced A-band molecules of higher molecular weight (30 or more repeats) than that of the parent strain (max. of 20 repeats). They proposed that the pFV3 plasmid contains an A-band polymerase gene which when present in high copy number changes the A-band distribution and chain length.

Using the rhamnanase on phage A7 we can obtain an A-band LPS isolate devoided of most of the rhamnose polysaccharide to analyze the core oligosaccharide structure. In previous studies, we showed that, in contrast to the serotype LPS, the core-lipid A region of A-band LPS from *P. aeruginosa* contains low levels of reactive KDO and lacks phosphate, but contains sulfur (30,31; M. Rivera and E.J. McGroarty, unpublished results). The chemical composition of AK1401 A-band and Dig-A-band LPS showed low levels of heptose, KDO, and amino sugars, and no phosphate when compared to the serotype-specific or SC-LPS (Table 2). Nevertheless, all AK1401 LPS isolates reacted with the MAb 7-4, specific to the inner core region of *P. aeruginosa* (Figure 6). The differences

observed in the amounts of heptose and KDO between A-band, Dig-A-band, and SC-LPS apparently does not affect the binding of the MAb 7-4 to the common epitope shared by these LPS isolates. We propose that the inner core and lipid A regions of A-band LPS show some structural similarities as well as some differences compared to the serotype-specific LPS. Results from the Western blots (Figures 4 and 5) using the MAbs 8A1 and 177 indicated that all the samples have common epitopes in the lipid A. Lack of reactivity of the AK1401 A-band isolate presumably is the result of either non-reactivity or lack of acid hydrolysis. Interestingly, the phage-digested A-band lipid A derivative showed reactivity to MAb 8A1. This is unexpected since phosphate is reported to be part of the binding site for this MAb (3); the observed reactivity suggests that sulfate may be substituted as part of the epitope. Reactivity of MAb 4A10 with AK1401 Uf- and SC-lipid A derivatives, and with O-serotype-LPS derived lipid A but not lipid A from A-band isolates suggests that this MAb recognizes determinants exclusive to lipid A of the B-band type (3).

In addition to differences in the lipid A region, AK1401 A-band LPS and O-serotype-specific LPS showed structural differences in the outer core region. This was clearly shown with outer core- specific MAb 101 (Figure 7). Yokota *et al* (42) reported that the core oligosaccharide to which the common polysaccharide antigen is attached, while similar in composition to O-antigen-containing-core, also contains xylose and an unidentified 3-O-methyl-6-deoxyhexose. They proposed that there could be a separate oligosaccharide chain containing seven residues of

the O-methylated sugar and two residues of xylose. Multiple core oligosaccharide structures have been reported for other gram-negative bacteria. For example, adherent enteropathogenic *E. coli* 0119 strains are reported to have a larger LPS core than non-adherent strains, although the O-polysaccharide chains are identical, and the inner core of adherent strains reportedly has an atypical structure containing equimolar amounts of L-*glycero*-D-*manno*-heptose and D-*glycero*-D-*manno*-heptose (4).

LPS has been shown to be an important virulence factor and to have a role in pathogenesis (7). While it is not clear at present whether the A-band type of LPS found in *P. aeruginosa* serves as a virulence factor, it appeared to be a prominent surface antigen on organisms isolated from clinical sources. Lam and coworkers (21) reported that 11 of the 17 serotype strains from *P. aeruginosa* possessed A-band LPS. They also observed that 68% of the clinical isolates from patients with cystic fibrosis had A-band LPS. In cystic fibrosis, during the course of infection with *P. aeruginosa*, isolates have been shown to become nontypeable, and the O-antigen is replaced with A-band as the major LPS antigen (21). In a separate study, Sawada *et al* (32) described a MAb, E87, that bound to 80% of *P. aeruginosa* strains of various serotypes. They showed that the antigen recognized by this monoclonal antibody consisted mainly of rhamnose. As mentioned previously, the structure of this common antigen polysaccharide is a D-rhamnose trisaccharide repeating unit (17,41,42; Arsenault et al, unpublished results). This same structure is found in *P. syringae* pv. *morsprunorum* LPS O-polymer (34).

In *P. syringae* pv. *morsprunorum* there is a correlation between phage sensitivity and host specificity (12). Zamze *et al* (43) reported that loss of virulence in *P. syringae* pv. *morsprunorum* was associated with changes in the LPS structure; either modification of the side-chain polysaccharide or complete loss of the sidechains to yield a rough LPS.

In conclusion, our results show that A-band LPS from *P. aeruginosa* AK1401 serves as the initial receptor for phage A7 and is hydrolyzed by the phage to core-lipid A with only two or three rhamnose repeat units. The chemical composition of the phage A7-digested A-band LPS showed low levels of heptose, KDO, and amino sugars, and no phosphate confirming earlier work (30,31). Furthermore, the results from the immunoblots with MAbs indicate that even though the inner core region of all AK1401 LPS fractions appeared to share a common epitope, the outer core region of the A-band LPS is different from that of the B-band or SC-LPS. Further structural studies will be performed on this phage A7-digested A-band LPS fraction.

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CHAPTER 6

Summary and Perspectives

SUMMARY

In the past six years studies from several laboratories have demonstrated that many strains of *Pseudomonas aeruginosa* produce a second lipopolysaccharide (LPS) whose polysaccharide chain differs serologically and structurally from O-antigen polysaccharide (17,19,23,29,30,33,40,41). The common antigen polysaccharide is a regular homopolymer of rhamnose, and the structure has been shown to consist of the repeating unit [->3)-α-*D*-Rha*p*-(1->3)-α-*D*-Rha*p*-(1->2)-α-*D*-Rha*p*-(1->]$_n$ (17,40,41; T.L. Arsenault *et al*, Can. J. Chem., in press). In my initial studies the size heterogeneity of LPS isolates from several *P. aeruginosa* strains was defined by both gel filtration and SDS-PAGE (29, Appendix A). When column fractions were applied to SDS-polyacrylamide gels in their order of elution, molecules of decreasing size were resolved, and the ladder of molecules with different-length O-antigens formed a diagonal across the gel (Appendix A). The LPS from the PAO1 derivatives contained two distinct sets of bands, distinguished on the gels as two set of diagonals. The set of bands with the faster mobility, the B bands, represents 85 to 90% of the total LPS sample by weight; this is the O-antigen-containing LPS which determines the O-specificity of the bacterium. The slower-moving set of bands, the A-bands, represents 10 to 15%

of the total LPS sample and contains shorter chains of predominantly neutral polysaccharide. Reaction of isolated fractions with monoclonal antibody specific for the PAO1 O-antigenic side chain indicated that only the B bands from the PAO1 strains were bound. These observations led to the proposal that PAO1 strains synthesize two types of molecules that are antigenically different.

Further chemical characterization was performed on the A band and B band isolates (Chapter 2). The fatty acid profile showed that indeed the A band isolate is an LPS-like molecule. In addition, chemical analysis of the A-band and B-band LPS revealed that not only are these two LPS molecules antigenically distinct but they also differ in composition (Appendix A and Chapter 2). This is reflected in the amount of amino sugars, rhamnose, KDO, and phosphate. An important finding from this study was that A-band LPS lacked phosphate but contained sulfate groups (Chapter 2 and 4). Furthermore, the A-band LPS contains high levels of rhamnose and reacted with the polyrhamnose-specific monoclonal antibody E87. Reactivity with the E87 monoclonal antibody is found in a large number of different serotype strains (33,40) suggesting that this antigen is common among *P. aeruginosa*, perhaps as a common antigen LPS. Lam and co-workers (19) have produced seven monoclonal antibodies to the A-band LPS. Using Western immunoblot analysis, they have shown that the A band molecule is present as a common antigen on strains of many serotypes.

The size heterogeneity of LPS from *P. aeruginosa* as well as other gram-negative bacteria is reported to be altered by changes in the growth conditions,

such as temperature (2,22,25) and medium composition (10,38). Alterations in outer membrane structure induced by specific growth conditions may change cation-binding sites (10). Since a major site of cation binding in the outer membrane is with LPS, a study was initiated to analyze the influence of various growth conditions on the size heterogeneity of LPS in *P. aeruginosa* (Chapter 3). The results indicated that cells grown at near-growth-limiting conditions including high temperature, high concentrations of salt, sucrose, or glycerol, low phosphate concentration, and low pH produced decreased amounts of the very long chain population of O-antigen LPS molecules. The size and amount of common antigen LPS was either unaffected or increased slightly when the cells were grown under the above stress conditions. Also, cells grown under stress conditions, in which the long O-polymer LPS was absent, were agglutinated by both serotype-specific and common antigen-specific monoclonal antibodies. The results indicate that the long O-polymers cover and mask the shorter common antigen. However, specific growth conditions limit the production of the long O-polymer, allowing the exposure of the common antigen on the cell surface. Under certain conditions, such as prolonged antibiotic therapy, clinical isolates from *P. aeruginosa* are found to be nontypeable and appear to loose the O-polymer-containing B bands (3,11,14). Lam and coworkers (19), using immunoblot analysis, have shown that A bands were present in a high percentage of clinical isolates and appeared to be a main antigen on nontypeable strains deficient in high molecular-weight serotype-specific LPS. For such clinical isolates the A bands may become exposed and

serve as an important antigenic determinant.

Antigenic as well as chemical differences have been observed between the A and B bands, but the novelty of A-band LPS is the presence of sulfate. To corroborate that sulfur is incorporated into A-band LPS in stoichiometric amounts, *P. aeruginosa* were grown in modified basal medium containing ^{35}S-labelled sulfate (Chapter 4). We observed that the A-band LPS isolated from smooth strains of *P. aeruginosa* were always contaminated with small amounts of an intermediate chain-length serotype-specific LPS (Appendix A and Chapter 2). Therefore, the bacterial strain used for detailed structural analysis was *P. aeruginosa* AK1401 which is a rough mutant that produces A-band LPS but is defective in the synthesis of O-antigen (2). The ^{35}S-labelled LPS was separated by gel filtration chromatography into two major populations of LPS; A-band or common antigen LPS and a serotype-specific short chain LPS (SC or B-band). The elution profile as well as the autoradiogram showed that the A-band and B-band LPS contained labelled-sulfur. Chemical analysis of the AK1401 A-band LPS indicated low levels of KDO and no phosphate. In addition, A-band LPS showed a 1:1 molar ratio of sulfur to heptose suggesting that two to three sulfur atoms are present in the A-band molecule. The molar amounts of sulfur detected in unfractionated and SC-LPS isolates were one half the heptose content. Thus, A-band LPS contains covalently bound sulfur perhaps as sulfate. This functional group can be identified by FT-IR, ^{1}H NMR, and ^{13}C NMR spectroscopy. It is reasonable to propose that the biosynthetic pathways of the A-band and B-band

LPS molecules diverge at some point. It has been shown that the synthesis or expression of *P. aeruginosa* A- and B-band LPS appear to be partially independent (J. Lightfoot *et al*, Abstr. Annu. Meet. Am. Soc. Microbiol. 1991, D-69, p.90). Genes for A-band synthesis have been shown to map at a location different from the B-band genes.

Arsenault *et al* (Can. J. Chem., in press) characterized the structure for the rhamnose polysaccharide portion of *P. aeruginosa* AK1401 A-band LPS, and it was shown to be identical with that reported for the common antigen LPS (17,40,41). Interestingly, the O-polysaccharide chain in the LPS of *Pseudomonas syringae* pv. *morsprunorum* is reported to have the same structure as the polysaccharide of A-band LPS (36). The bacteriophage A7, a typing phage for *P. syringae* pv. *morsprunorum*, has LPS as its primary receptor (35). This phage A7 possesses a rhamnanase that specifically hydrolyzes the rhamnose polysaccharide chain to oligosaccharides (35). In our studies (Chapter 5) we demonstrated that A-band LPS from *P. aeruginosa* AK1401 serves as the initial receptor and is hydrolyzed by phage A7 to core-lipid A with only two or three rhamnose repeat units. The chemical composition of the core components of this phage A7-digested A-band LPS was similar to that reported previously for undigested A-band LPS (29,30; Chapter 4). This phage-digested A-band isolate was also incubated with monoclonal antibodies against either inner core or outer core epitopes of *P . aeruginosa* LPS, and the results were compared to that of the serotype SC-LPS. The results from the immunoblots indicated that, even though the inner core region

of all AK1401 LPS fractions share a common structure, the outer core region of the A-band LPS is different from that of the B-band or SC-LPS.

The identification of two chemically distinct forms of LPS in *P. aeruginosa* raises the question regarding the roles of these two fractions in virulence, antigenicity, and antibiotic sensitivity. It has been proposed that LPS is an important virulence factor and has a role in pathogenesis (6). Perhaps the A-band type of LPS found in *P. aeruginosa* common antigen serves as a virulence factor during infection in cystic fibrosis patients. Thus, it is important to characterize the structure of this A-band LPS to understand the mechanism of virulence and antibiotic resistance.

PERSPECTIVES

To analyze the core-lipid A region of A-band LPS, *P. aeruginosa* AK1401 strain will be grown at 37 °C in either TYE or ^{35}S-labelled MBM (Chapter 4). The cells will be harvested late in logarithmic growth phase and washed with distilled water. LPS will be extracted from the cells, separated by gel exclusion chromatography, and fractions purified as indicated in Chapter 5. Purified A-band LPS (5-15 mg) will be suspended in water and phage A7 will be added (10^{10}-10^{11} p.f.u.) and incubated for 24 h at 25 °C. The phage A7-digested A-band (Dig-A-band) LPS will be further purified as described previously (Chapter 5). Even though the A-band LPS is resistant to acid hydrolysis, the Dig-A-band LPS can be acid hydrolyzed to lipid A under conditions described earlier (Chapter 5). To prevent loss of sulfate, the Dig-A-band as well as B-band LPS will be acid hydrolyzed, under more gentle conditions, treating the samples either with 1% or 5% acetic acid at 100 °C for 1 or 2.5 h (15), or with 20 mM sodium acetate (pH 4.5) at 100 °C for 1h (1). Lipid A release will be monitored by thin layer chromatography (TLC; 32). The hydrolyzed products will be separated on a Sephadex G-50 column using deionized water as the eluant. The fractions will be monitored for total carbohydrate (9) and sulfate (7, Chapter 4). The fatty acid

composition of the lipid A isolate from A-band and B-band will be monitored by gas chromatography (24,39) for losses in fatty acid during the hydrolysis procedure. In addition, TLC will be used to determine the heterogeneity of the lipid A isolates; using preparative TLC we can separate and purify the lipid A mixture (16).

The structure of the lipid A as well as the ^{35}S-labelled lipid A fractions will be analyzed by soft ionization methods, namely, fast atom bombardment-mass spectrometry (FAB-MS) (16,20), plasma desorption MS (5,26), or laser desorption MS (18,34). In addition, ^{1}H and ^{13}C NMR will be used, in conjunction with Fourier transformed infra red (FTIR) spectroscopy, to analyze the structure of the lipid A isolates (12,18,21,26,27).

The complete core structure from Dig-A band LPS will be determined by using methylation analysis and degradation methods (4,28,31). The neutral sugars will be determine as their alditol acetate derivative using GC and GC-MS (28). Quantitation of amino sugars will be performed on an automatic amino acid analyzer as described elsewhere (28,31). Methylation analysis will be performed according to Hakamori (13) and Stellner *et al* (37). Methylation of the core oligosaccharide will be preceded by a reduction of the uronic acid residues using $NaBD_4$, and then will be subjected to methylation and the methylated sugars will be quantitated and identify by GC and GC-MS as the alditol acetate derivatives (28). Smith degradation of the isolated core oligosaccharide will be carried out as described elsewhere (28). Sulphation as the mode of incorporation of the ^{35}S-

label into both core oligosaccharide and lipid A isolates can be confirmed by mild acid hydrolysis and precipitation of the released counts as barium sulfate (Hollingsworth, R.I., Analyt. Biochem., submitted). Glycosyl linkages will be determined by a previously described procedure (4).

In addition, the core oligosaccharide of the Dig-A-band as well as the B-band fraction will be examined by the physical methods described above. Mass spectrometry of ^{35}S-labelled oligosaccharide can be used to define the substituents (20). Proton and ^{13}C NMR in conjunction with FTIR will reveal the anomeric configuration, the conformation of the monosacharide units, and will give relevant structural information concerning further substitution and group functionalities (4,12,20,21,28).

It has been shown previously that the cell-free preparation from *Acanthamoeba castellanii* has the ability to digest LPS from *Salmonella* Rd mutants (8). A cell-free preparation has been shown to have esterase, amidase, and phosphatase activity but no cleavage of glycoside linkages could be detected. The crude enzyme preparation acted on LPS in a specific way *in vitro*, quantitatively releasing the O-acyl residues; approximately 50% of the N-acyl residues and 70% of the original amount of phosphate was removed. Therefore, the activity of this mixed enzyme preparation can be tested on the Dig-A-band LPS isolate from *P. aeruginosa* AK1401 which will avoid unwanted chemical degradation of the LPS molecule. After purification of the enzymatic products by

extraction procedures and gel filtration, the carbohydrate fraction will be examined by both chemical and physical methods as described above.

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APPENDIX A

Heterogeneity of Lipopolysaccharides from *Pseudomonas aeruginosa*: Analysis of Lipopolysaccharide Chain Length

ABSTRACT

Lipopolysaccharide (LPS) from smooth strains of *Pseudomonas aeruginosa* 503, PAZ1, PAO1715, PAO1716, and Z61 was fractionated by gel filtration chromatography. Lipopolysaccharide samples from the first four strains, all PAO1 derivatives, separated into three major size populations, while strain Z61, a Pae K799/WT mutant strain, separated into two size populations. When column fractions were applied to sodium dodecyl sulfate-polyacrylamide gels in their order of elution, molecules of decreasing size were resolved, and the ladder of molecules with different length O-antigens formed a diagonal across the gel. The LPS from the PAO1 derivatives contained two distinct sets of bands distinguished on the gels as two sets of diagonals. The set of bands with the faster mobility, the B bands, was found in column fractions comprising the three major amino sugar containing peaks. In the sample from 503, a fourth minor peak which contained B bands was resolved. The slower moving set of bands, the A bands, were recovered in a minor peak. LPS from strain Z61 contained only one set of bands, with the higher molecular weight molecules eluting from the column in a volume similar to that of the B bands of the PAO1 strains. Analysis of the fractions of LPS from all strains indicated that less than 8% of the LPS molecules had a long attached O-

antigen. Analysis of the peak that contained mainly A bands indicated a lack of reactive amino sugar and phosphate, although heptose and 2-keto-3-deoxy-octulosonic acid were detected. Reaction of isolated fractions with monoclonal antibody which is specific for the PAO1 O-antigen side chain, indicated that only the B bands from the PAO1 strains were antigenically reactive. The bands from strain Z61 showed no reactivity. The data suggest that the A and B bands from PAO1 strains are antigenically distinct. We propose that PAO1 strains synthesize two types of molecules that are antigenically different.

INTRODUCTION

Lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria, is important in the structure (34,38) and function (34,37) of this membrane. Structural microheterogeneity has been demonstrated in several regions of LPS molecules from the *Enterobacteriaceae* (3,15,23,38,39,48,52) and *Pseudomonas aeruginosa* (34,58). Of the several methods used to separate the subclasses of LPS from individual strains, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21,25,46,48) and gel filtration (10,27,31,33,35,48) are the best. Either of these two methods by themselves, however, may be insufficient to completely characterize the high and low molecular weight fractions of LPS. Peterson and McGroarty (48) demonstrated that the SDS-PAGE of the column fractions of samples from *Salmonella typhimurium, Salmonella minnesota*, and *Escherichia coli* was instrumental in characterizing the various sized fractions. Analysis of the isolated fractions allowed for the estimation of the average number of O-antigen repeat units per LPS from each of the size fractions.

Compositional analysis of LPS from *P. aeruginosa* has indicated that the LPS molecules are structurally similar to enterobacterial LPS molecules, but

possess several distinctive features (34,58). The most outstanding differences include the unusually high phosphate content (34,59), the presence of L-alanine in the core (34,58), and the high levels of amino sugars and uronic acids in the O-side chain (9,34). The characterization of *P. aeruginosa* O-specific polysaccharides has been complicated in some cases by chemical heterogeneity of the polysaccharide chains (9,10,31,59). In some instances, the polymeric material has been resolved into amino sugar-rich and neutral sugar-rich fractions (31,59). The biological significance of such fractions is unclear, but a possible explanation is that *P. aeruginosa* strains produce multiple types of molecules with chemically distinct polysaccharide chains.

In this study, we have analyzed the size heterogeneity of LPS isolates from several *P. aeruginosa* strains by both gel filtration and SDS-PAGE. These studies have revealed that LPS isolates from PAO1 strains contain two distinct sets of bands, suggesting that PAO1 strains are capable of synthesizing more than one type of chemically and antigenically distinct molecule. We also present evidence that the percentage of core oligosaccharides carrying the O-specific polymer is less than 8%.

MATERIALS AND METHODS

Bacterial strains.

P. aeruginosa stain Z61 was a mutant derived from strain Pae K799/wt selected for antibiotic supersusceptibility; and PAZ1 *met*-28, *trp*-6, *lys*A-12, *his*-4, *ile*-226, and *abs*A was a PAO222 derivative into which *abs*A mutant gene from Z61 encoding antibiotic sensitivity has been conjugated (2). Strains PAO1716 *ade*-136, *leu*-8, *rif*-1 (revertant) and PAO1715 *ade*-136, *leu*-8, *rif*-1, *tol*A-12 (an aminoglycoside supersensitive mutant) were described previously (41). Strain PAO503 *met*-9011 is a methionine auxotroph of *P. aeruginosa* PAO1. Strains PAZ1, 1715, 1716, and 503 were 0-5 serotype. *Escherichia coli* strains D21 and D21f2 are derived from strain K-12 and were characterized as Ra and Re chemotype, respectively (4).

Growth media.

Strain PAO1716, PAO1715, Z61 and PAZ1 were grown at 37°C to mid-logarithmic phase in a 100 ℓ fermentor containing 80 ℓ of protease peptone no. 2 medium from 1 ℓ overnight culture grown in the same medium. Strain 503 was grown as previously described (5).

E. coli strains D21 and D21f2, grown as described by Coughlin *et al.* (11), were harvested in late log phase.

Isolation of LPS.

LPS from *P. aeruginosa* strain Z61 and PAO1 derivatives 1715, 1716, PAZ1, and 503 were isolated by the method of Darveau and Hancock (13), followed by two extractions in chloroform:methanol (1:1 v/v) resulting in recovery of approximately 80% of the total LPS. The LPS from *E. coli* strains D21 and D21f2 was isolated using the hot aqueous phenol (57) and the chloroform-petroleum ether (17) extraction procedures, respectively.

Gel electrophoresis.

Sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels were prepared and run using the buffer system of Laemmli (36). Unless otherwise noted, separating gels were formed with 15% acrylamide, 0.1% SDS, with a 7.5% acrylamide stacking gel. Samples were mixed 1:1 with sample buffer (containing 4% SDS) and applied to the gel. Electrophoresis was performed with a constant current of 15 mA per gel until the tracking dye entered the separating gel and then at 30 mA per gel until the tracking dye reached the bottom of the gel. LPS bands were detected by the silver staining method of Dubray and Bezard (16).

Column chromatography.

Samples were fractionated with a Sephadex G-200 (Pharmacia Fine Chemicals) column (64 cm by 25 mm) at room temperature suing the column buffer system of Peterson and McGroarty (48). Approximately 30 mg of LPS was applied to the column, and 5 ml fractions were collected at a flow rate of 8 ml per h. To remove detergent and buffer, pooled fractions were extensively dialyzed (12,000 to 14,000 molecular weight cutoff membranes) against column buffer without deoxycholate at 37°C and then against distilled water at 4°C. The dialyzed fractions were lyophilized and resuspended to a concentration of 10 mg/ml in water. All fractionations were done at least twice.

Western blots.

Western blots of SDS-polyacrylamide gels were prepared as previously described (6,55). The gels were electrotransferred with a model TE Transphor Electrophoresis apparatus (Hoefer Scientific Instruments) at a constant current of 150 mA for 18 h using the electrode buffer described by Otten and co-workers (45) unless otherwise noted. The nitrocellulose blots were visualized as described previously (45) with monoclonal anti-503 antibody (20, titer ~ 1:100,000) diluted 1:10,000 in blocking solution. In addition, dot blots were performed by applying known quantities of LPS isolates directly on nitrocellulose. The blots were washed and visualized using horseradish peroxidase conjugated goat anti-mouse IgG

antibody (Sigma Chemical Co.) as described above or using silver stain protocol identical to that described by Dubray and Bezard (16).

Assays.

Assays for amino sugars (18), heptose (60), and 2-keto-3-deoxyoctulosonic acid (KDO) (29) were performed as described previously except where noted. Phosphate analysis were performed using either a colorimetric assay (1) or by inductively plasma emission spectroscopy (11). Protein concentrations were estimated with the Pierce BCA protein assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard.

RESULTS

Silver staining of LPS from strains of *P. aeruginosa* separated by SDS-PAGE revealed a progressive ladder-like pattern of bands up the gel (Figure 1A). For the *Enterobacteriaceae* and *P. aeruginosa*, these bands have been reported to represent LPS molecules containing increasing lengths of O-antigen (5,21,46). The intensity of staining indicated three to four regions of bands representing as many as four populations of molecules (band sets 1, 2, 2a and 3, Figure 1) differing in O-antigen length. The electrophoretic pattern of LPS from strain 503 showed a set of bands (set 2a) which were closely spaced and slower migrating bands immediately above the 2a set which had greater spacing (Figure 1A, lane 2). In the banding pattern of LPS from PAO1 derivatives 1715, 1716, and PAZ1 (Figure 1A, lanes 1, 3, and 5, respectively), we observed irregularities in the spacing and intensities of bands up the gel. In contrast, LPS from strain Z61 appeared to have a regular spacing and intensity in the banding pattern (Figure 1A, lane 4). The average length of the highest molecular weight LPS of strains Z61 and PAZ1 seemed shorter than that of the other PAO1 derivatives, a phenomenon observed previously and ascribed to the *abs*A (antibiotic supersusceptibility) mutant locus (2). When low amounts of LPS were applied to the gel, only the fastest

Figure 1. (A) Silver-stained SDS-polyacrylamide gel of LPS from *P. aeruginosa* strains 1715 (lanes 1 and 8), 503 (lanes 2 and 9), 1716 (lanes 3 and 10), Z61 (lanes 4 and 11), PAZ1 (lanes 5 and 12), and from *E. coli* strain D21 (Ra, lanes 6 and 13) and D21f2 (Re, lanes 7 and 14). Samples of either 5 μg (lanes 1 to 7) or 0.1 μg (lanes 8 to 14) were applied to a 15% acrylamide gel which had been polymerized overnight with a butanol overlay. Arrows indicate the four intensively stained regions of the *P. aeruginosa* samples: band sets 1, 2, 2a and 3. (B) Western blots of LPS from *P. aeruginosa* strains 1715 (lane 15), 1716 (lane 16), 503 (lane 17), and PAZ1 (lane 18) reacted with monoclonal anti-503 antibody. Samples of 2.5 μg were applied to at 12% acrylamide gel which had been polymerized overnight with a butanol overlay. The gel was blotted as described in Materials and Methods.

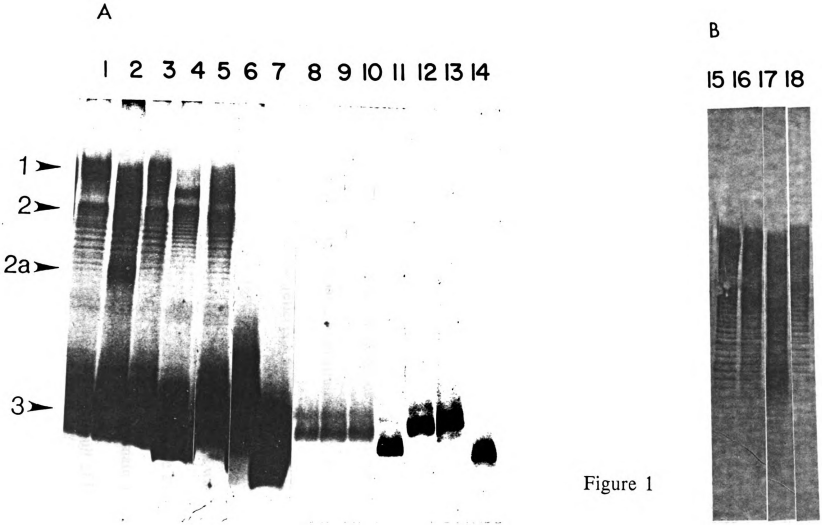

Figure 1

migrating bands were stained, and there was no difference in migration pattern of LPS of strains 1715, 503, 1716, and PAZ1 (Figure 1A, lanes 8, 9, 10, and 12 respectively). On the other hand, the low molecular weight bands from strain Z61 migrated faster than that of the other *P. aeruginosa* strains (Figure 1A, lane 11) as previously observed (35), due to an apparent truncation in the rough core of the short chain LPS molecules in this strain. LPS from *E. coli* D21 (Ra chemotype) and D21f2 (Re chemotype) was used to compare and characterize the electrophoretic mobilities of the short chain populations.

Using antibodies specific to the PAO1 O-antigen, Western blots of the LPS separated with SDS-PAGE were analyzed to help clarify the irregularities in the banding pattern. The blots of LPS isolated from *P. aeruginosa* strains 1715, 1716, 503 and PAZ1 revealed a ladder pattern of molecules that consisted of doublet bands (Figure 1B). Furthermore, the level of one of the bands in the doublet was in lower amounts in the isolates from strains 1715, 1716, and PAZ1 than from strain 503. Presumably, this reflects a difference in substoichiometric modification within the core-lipid A region of the molecules. Interestingly, spacing and intensity of the ladder pattern seen in the Western blot was much more regular than that of the silver stained gel. This suggested that the stained sample may contain bands superimposed on the ladder pattern of the main antigen.

If the irregular silver-stained banding pattern was a result of heterogeneity in the LPS samples, this heterogeneity could have been due to contamination of the culture or to true heterogeneity of the sample. The possibility that the cultures

were contaminated is very low since after growth, all cultures were streaked out onto protease peptone no. 2 agar plates to observe characteristic colonial morphologies and pigmentation and, in the cases of Z61, PAZ1 and PAO1715, tested for characteristic antibiotic supersusceptibilities. Furthermore, the irregular banding patterns were seen in samples from PAO1 derivatives (Figure 1A) isolated in two different laboratories and from several independently isolated LPS samples.

To further characterize the heterogeneity of the LPS isolates from the PAO1 strains, the samples were separated on a Sephadex G200 column. The elution profile showed three major amino sugar-containing peaks for strains 503 (Figure 2), 1715 (Figure 3), 1716 and PAZ1 (results not shown). In contrast, the elution profile of the LPS sample from the Z61 strain showed only two major peaks (Figure 4). Both gel permeation chromatography and SDS-PAGE separate molecules on the basis of size; therefore, a diagonal banding pattern should be expected across SDS-PAGE gels of column fractions when applied in the order of elution. SDS-PAGE of the column fractions of samples from each of the PAO1 derivatives studied revealed two distinct ladder patterns of apparently different sizes, the A bands (later eluting ladder) and the B bands (earlier eluting ladder) (see Figures 2 and 3 for elution profiles of strain 503 and 1715, respectively). In contrast, the LPS isolate from strain Z61 showed only one ladder set (Figure 4). Peterson and McGroarty (48) reported that the SDS-PAGE profiles of fractionated LPS from *Salmonella* species separated in this type of column as molecules of decreasing size. The presence of two distinct ladder patterns suggests that either

Figure 2. Fractionation of LPS from *P. aeruginosa* strain 503 on Sephadex G-200. Fractions were analyzed for KDO (•) and amino sugar (Δ). Silver-stained SDS-polyacrylamide gels of column fractions are aligned under their appropriate fraction number. A represents the slow-moving set of bands and B the faster-moving set.

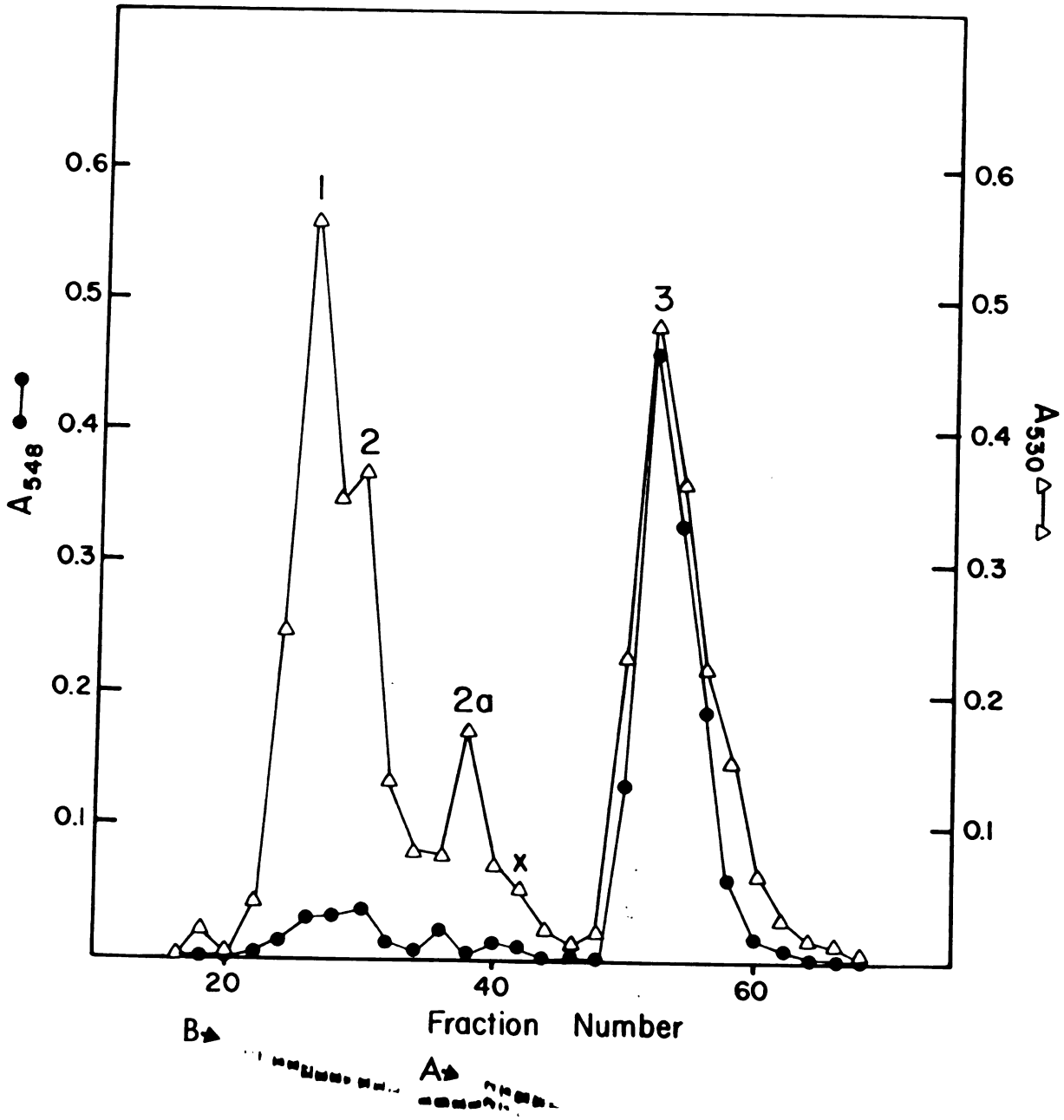

Figure 2

Figure 3. Fractionation of LPS from *P. aeruginosa* strain 1715 on Sephadex G-200. Fractions were analyzed for KDO (•) and amino sugars (Δ). Silver-stained SDS-polyacrylamide gels of column fractions are aligned under their appropriate fraction number. A represents the slow-moving set of bands and B the faster-moving set.

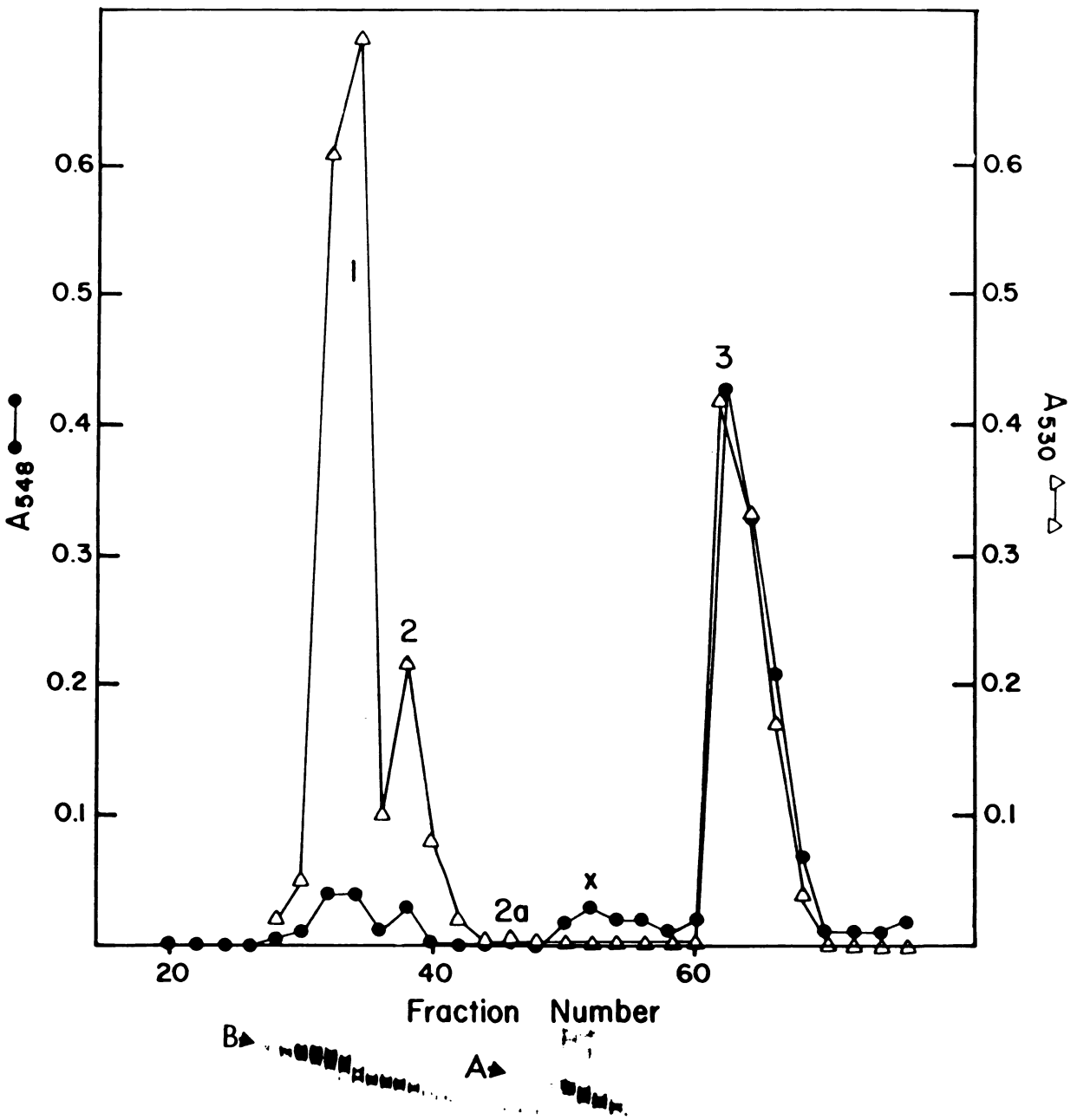

Figure 3

Figure 4. Fractionation of LPS from *P. aeruginosa* strain Z61 on Sephadex G-200. Fractions were analyzed for KDO (•) and amino sugar (Δ). Silver-stained SDS-polyacrylamide gels of column fractions are aligned under their appropriate fraction number.

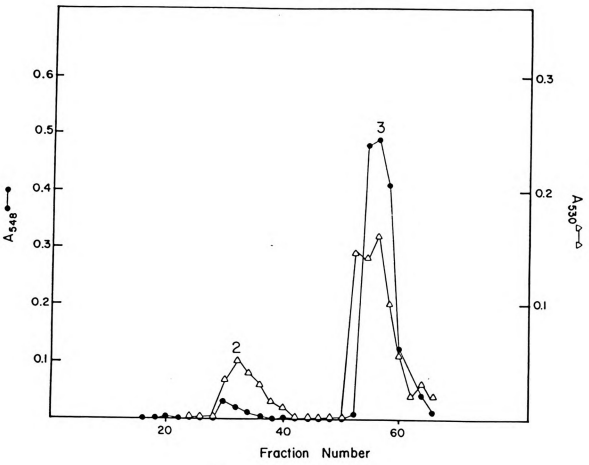

Figure 4

stable aggregates were present (48) or there were two types of molecules with different charges; electrophoresis separates molecules on the basis of both size and charge. To eliminate the possibility that A bands were aggregates of the B bands stabilized by hydrogen bonding between O-polymers, SDS-PAGE of the column fractions of samples from strain 503 was run in the presence of 4 M urea (final concentration). The same two sets of bands were observed in 4M urea-SDS gels (results now shown). In addition, two-dimensional electrophoresis of column fractions of LPS from strains 503 and 1716, which contained approximately equal amounts of A and B bands, showed that both the A and B bands ran with the same mobilities in the second dimension. Since the gels in both dimensions consisted of standard SDS gels and the gel strip from the first dimension was heated before running the second dimension, the results indicate that the bands do not interconvent (results not shown). This again indicates that one set of bands is not an aggregate or different conformational state of the other. Amino sugar analysis of our column fractions indicated that the fractions containing mainly A bands (peak X, Figure 3) lacked reactive amino sugars, while the other fractions (peaks 1, 2, 2a, and 3; Figures 2 and 3) showed reactivity.

The individual fractions from the elution profile of each of the LPS samples separated in a Sephadex G-200 column were also analyzed for KDO and phosphate content, and the relative molar ratios of amino sugar to KDO and amino sugar to phosphate were determined for the 3 or 4 major peaks. Also, the fractions corresponding to each of the major peaks in the elution profile of strains 1715 and

TABLE 1. Chemical Analysis of *P. aeruginosa* B-Band LPS Fractions

Peak No.	% P Recovered[a]	Amino Sugar/KDO[b]	Amino Sugar/P[c]	P/KDO (mole/mole)
Strain Z61[d]				
2	2.8	5.5	9.3	N.D.[e]
3	97.0	1.0	1.0	5.5
Strain PAZ1[f]				
1	2.3	24.8	30.8	4.8
2	3.2	19.5	18.8	6.1
3	94.3	1.0	1.0	5.9
Strain 1715[f]				
1	3.4	29.3	31.4	5.4
2	1.4	17.0	18.2	5.4
3	95.1	1.0	1.0	6.0

[a] Percentage of total amount in each of the peaks.

[b] Relative molar ratio; KDO and amino sugar levels were normalized to a value of 1.0 for peak 3 samples.

[c] Relative molar ratios; phosphate levels were determined for the individual fractions of the Z61 sample by using the colorimetric assay and for the pooled fractions from samples of strains 1715 and PAZ1 by inductively coupled plasma emission spectroscopy, as described in Materials and Methods. Levels were normalized to a value of 1.0 for peak 3 samples.

[d] Individual fractions from the elution profile of the LPS samples separated on a Sephadex G-200 column, as described in Material and Methods, were analyzed and the amount in all the fractions in each peak were added together.

[e] N.D., not determined.

[f] Pooled fractions corresponding to each of the peaks in the elution profile of LPS samples separated on a Sephadex G-200 column were dialyzed, lyophilized, and resuspended in distilled water for analysis.

PAZ1 were pooled, dialyzed, lyophilized and resuspended in water to a final concentration of 10 mg/ml and were analyzed as above. The data shown in Table 1 for the pooled fractions and data from the individual fractions (not shown) indicated that, using phosphate as an indicator of molar amounts of the LPS, the short chain peak 3 sample represented 92 to 97% of the total LPS molecules; peak 2a, 0.4 to 2%; peak 2, 1 to 3%; and the very long chain fraction, peak 1, 3 to 4%. The fifth population of molecules, the A bands, were resolved as a separate population on the column (peak X) in the samples from 1715 (Figure 3), 1716, and PAZ1, whereas A bands overlapped with the 2a peak of the main ladder set in the 503 fractionation (Figure 2). As stated above, strain Z61 showed only two different LPS size populations, the short chain fraction, peak 3 (97% of total), and the long chain fraction, peak 2 (2.8% of total) (Figure 4).

The O-antigen of *P. aeruginosa* is reportedly rich in amino sugars (34,35,40,58), although the reported sequence of the 05 serogroup (equivalent to Lanyi type 3a,d) indicates that most of the amino groups are acetylated (30). The phosphate and KDO assays measure residues in the core region of LPS (35), whereas the assay for amino sugars detects residues both in the core-lipid A region and O-antigen repeat region. The data reported in Table 1 show that the molar ratios of P:KDO for the three major B band-containing peaks of the pooled fractions are similar for strains PAZ1 and 1715. Also, this ratio for peak 3, calculated from the individual fractions for all strains, appeared to be relatively constant from strain-to-strain and was determined to be between 5.5 and 6.6

(mole/mole, data not shown). Since the level of phosphate or KDO can be used as a measure of the relative molar amount of LPS in each peak, comparison of the ratios of amino sugar to KDO and/or amino sugar to phosphate in the three major peaks of the pooled fractions should reflect the O-antigen length. For the individual fractions a discrepancy existed in these ratios where the values are lower than for the pooled fractions. This may be due to the elution buffer present in the fractions before dialysis. Since the pooled fractions were dialyzed to remove the detergent and salts and resuspended in H_2O to a known concentration, the pooled-fraction data were more reliable. Furthermore, the low amount of sample in the individual fractions decreased the sensitivity of the assays.

The chemical composition of the dialyzed A band samples from strains 1715 and PAZ1 was analyzed, and the results were compared with those of the three major amino sugar-containing peaks (Table 2). The results indicated that peak X (A bands) comprised 10-15% of the total LPS sample by weight. Furthermore, under the normal conditions of the assay, it appeared to have low levels of reactive KDO, amino sugars and phosphate. When the pooled samples were hydrolyzed for a longer period of time (20 min) and at a higher acid concentration (0.5 N H_2SO_4), the KDO levels detected in the B band fractions were similar to previous assays, but the KDO levels in the A band fraction increased 10-fold. Thus, the A band material appeared to contain KDO residues which are much less reactive than those in the B band isolates. Analysis of the isolates from strain 1715 indicated that the A band material had levels of heptose in between that found in

TABLE 2. Composition of the Pooled Column Fractions of LPS from *P. aeruginosa* Strains 1715 and PAZ1

Peak No.[a]	Weight as % Total Recovered		KDO[b] nmoles/mg LPS		Phosphate[c] nmoles/mg LPS		Amino Sugars[d] A_{530}/mg/ml sample		Heptose nmoles/mg LPS
	1715	PAZ1	1715	PAZ1	1715	PAZ1	1715	PAZ1	1715
1	23	14	28	31	150	150	4.2	3.4	39
2	10	15	26	31	140	190	2.3	2.6	34
3	56	55	292	272	1740	1600	1.5	1.2	272
X	11	15	2	3	7	9	0.6	0.2	83
Unfractionated LPS Sample	N.A.[f]	N.A.	162	N.D.[g]	1200	N.D.	2.7	N.D.	N.D.

a Column fractions of peak 1, peak 2, peak 3, and peak X, along with an unfractionated sample of LPS, were pooled, dialyzed against column buffer without deoxycholate followed by distilled water, and then lyophilized and weighed. The samples were resuspended in distilled water to a concentration of 10 mg/ml for analysis.

b KDO was assayed using the standard protocol.

c Phosphate levels were determined by inductively coupled plasma emission spectroscopy, as described in Materials and Methods.

d Absorption at 530 nm was corrected to that of a sample at 1 mg/ml (final concentration).

e D-glycero-D-guloheptose was used for the standard curve.

f N.A., not applicable.

g N.D., not determined.

peaks 2 and 3 (Table 2). Analysis for protein in the pooled fractions indicated less than 1.5% (w/w) in all fractions. From the SDS-PAGE of the separated fractions (e.g. Figure 3) and from the chemical characterization of the pooled samples, it was observed that the A bands of strains 1715 and PAZ1 were contaminated with only minor amounts of the B band material. The A band material is probably a glycolipid since the isolate, suspended in the absence of detergent at 10 mg/ml, was cloudy and not completely water soluble, and since it contained heptose and KDO.

To better compare the migration patterns of equivalent LPS populations from different strains, comparable column fractions of LPS containing both A and B bands from the different strains were run together on SDS-PAGE (Figure 5). The A bands from all four PAO1 strains had very similar spacings (lanes 2–5), and the spacings of the B bands of strain PAZ1, 1716, and 1715 (Figure 5, lanes 2, 4 and 5, respectively) appeared very similar. In contrast, the B bands of strain 503 (Figure 5, lane 3) appeared much more closely spaced compared to the same bands of the other strains due to an increased amount of the second band in the B band set of doublets (see also Figure 1B, lane 17). Fractions from peak 3 of LPS from strains Z61, 503, PAZ1, 1715, and 1716 were run on an 18% SDS-acrylamide gel. No differences were seen in band mobilities of the short chain isolates except for that from strain Z61 which migrated faster (results not shown), corroborating the results with unfractionated LPS (Figure 1A, lanes 8–12).

To determine the antigenic reactivity of the A and B bands, the LPS

Figure 5. Silver-stained SDS-PAGE (11% acrylamide) (lanes 1 to 5) and Western blots (lanes 6 to 10) of LPS fractions from *P. aeruginosa* reacted with monoclonal anti-503 LPS antibody. Lanes: 1 and 6, LPS from strain Z61 (fraction 36); 2 and 7, strain PAZ1 (fraction 44); 3 and 8, strain 503 (fraction 40); 4 and 9, strain 1716 (fraction 42); 5 and 10, strain 1715 (fraction 48). Samples of 100 μl were applied to the SDS-polyacrylamide gel, which had been polymerized overnight with a butanol overlay. The gel was blotted as described in Materials and Methods. Arrows indicate the A and B bands of the respective fractions.

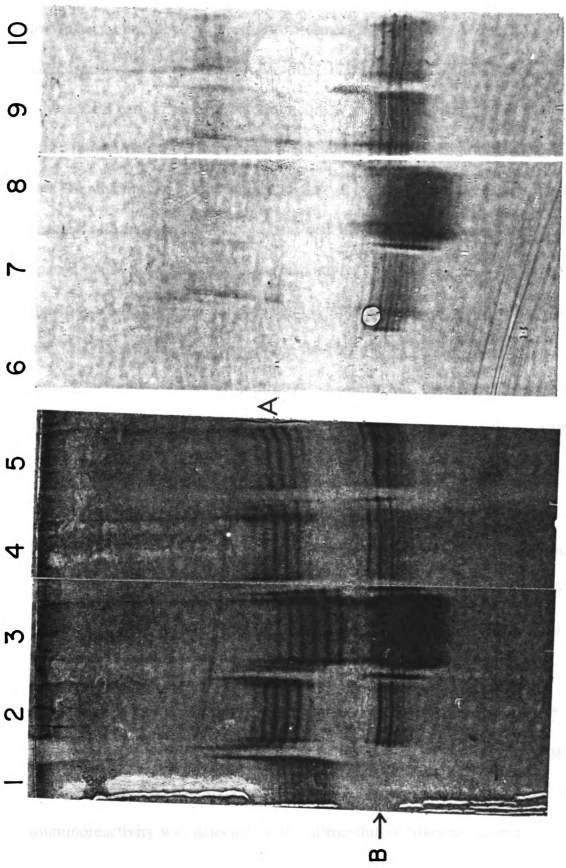

Figure 5

fractions containing approximately equal amounts of A and B bands were subjected to Western immunoblotting. Figure 5 (lanes 6–10) shows a Western blot of these LPS fractions from strains PAZ1, 503, 1716, and 1715 and a fraction of Z61 whose LPS had an electrophoretic mobility similar to that of the A bands of the other four samples. Reaction with monoclonal anti-503 LPS antibody (specific for the O-antigen side chain) indicated that only the B bands were antigenically reactive and not the A bands. There was a weak antigenic reactivity of closely spaced bands above the region corresponding to A bands which presumably was due to reaction with aggregated B band-type LPS (Figure 5, lanes 7-10). Peterson and McGroarty (48) have shown that the high-molecular weight fractions of LPS from *E. coli* can migrate as multimers. As expected, the bands from strain Z61 also showed no reactivity with the antibody since it is known that the LPS from strain Z61 is antigenically distinct from that of PAO1 derivatives (2).

To show that the lack of immunoreactivity of the A bands was not due to lack of transfer or recovery of samples on the nitrocellulose, Western blots were performed under different conditions. We used 150 and 400 mAmp and also transferred for 24 h. The gels were stained for LPS with and without transblotting to determine the level of electrotransfer of the different bands. The results indicated that, at the lower voltage, A bands did not transfer as well as B bands, consistent with their mobility on the gel. However, at the higher transblotting voltage where A band-type of molecules were removed from the gel no immunoreactivity was detected on the nitrocellulose (data not shown).

We also performed a dot blot immunoassay using the pooled column fractions to characterize differences in the antigenic reactivity of the two types of molecules. The four pooled fractions of LPS from strain 1716 (peaks 1, 2, 3 and X) were dialyzed, lyophilized and resuspended in water. A 10 μg portion of each of the fractions was spotted along the top of a nitrocellulose strip. Subsequent rows were spotted with the same volume of sample serially diluted 10-fold each row. The spots were dried and visualized with the anti-503 monoclonal antibody as described for the Western blots. The results (Figure 6A) indicated that the very long chain and long chain populations of B bands had similar reactivity, and as little as 1 ng could be detected. In contrast, the short chain population, containing no or only one O-repeat unit per molecule, showed no reactivity. Interestingly, the A band sample showed weak reactivity when 10 μg and 1 μg were applied, but no reactivity with lower amounts. The reactivity seen with 10 μg of the A bands was similar to that seen with 10 ng of long chain and very long chain fractions, suggesting that the A band fraction was contaminated with $< 1\%$ (w/w) of B band-type molecules. That the A band fraction remained bound to the nitrocellulose was shown by staining the dot blot with the silver stain used for the polyacrylamide gels. We found the intensity of staining indicated the approximate molar amount of material applied (Figure 6B). The levels detected were not changed if the blots were washed extensively with buffer or the blocking solutions used in the Western blots prior to staining (data not shown).

Figure 6. Dot blots of pooled column fractions from *P. aeruginosa* 1715 LPS. Peaks 1, 2, 3 and X were separated on Sephadex G-200, dialyzed, and lyophilized as described in Materials and Methods and suspended in distilled water to a final concentration of 10 mg/ml. In the top row, 10 μg of each samples was applied on the nitrocellulose strip, and subsequent rows were spotted with an equal volume of 10-fold serially diluted samples. The sample spots were dried, and the nitrocellulose was either reacted with monoclonal anti-503 antibody and developed as described in Materials and Methods (A) or washed with 10 mM Tris, 150 mM NaCl (pH 7.0) and reacted with the silver stain as indicated in the Materials and Methods for polyacrylamide gels (B).

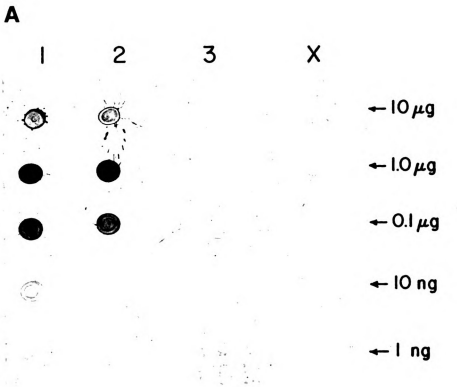

Figure 6

DISCUSSION

The LPS isolates of *P. aeruginosa* strains 503, PAZ1, 1715, 1716, and Z61 were separated by SDS-PAGE into as many as four major size populations. The ladder-like banding patterns represent molecules with increasing numbers of O-antigen repeat units (21,25,46). Other investigators have reported similar heterogeneity in the LPS from strains 503 and Z51, as well as other *P. aeruginosa* smooth strains (5,25,34,35). An O-antigen ladder pattern has recently been described for several smooth strains of *P. aeruginosa* using silver staining and immunoblotting techniques (54). Our Western blot of unfractionated LPS (Figure 1B) revealed a ladder-like banding pattern with regular spacing. The bands were resolved as doublets (Figure 1B, lanes 15–18) suggesting substoichiometric modification in the core-lipid A similar to that seen with *Salmonella* LPS (43,56). The irregular spacing that we observed in the SDS-PAGE silver-stained ladder pattern of LPS from strains 503, PAZ1, 1715, and 1716 (Figure 1A) suggested the possibility that PAO1 derivatives may be producing LPS with more than one type of O-polymers.

Peterson and McGroarty (48), using strains of *Enterobacteriaceae*, have shown that LPS molecules of different sizes can be partially separated with

Sephadex G-200 in the presence of deoxycholate and EDTA. Two or three major populations of LPS could be resolved as detected by sugar analysis. These populations represent sets of molecules with O-antigens of different lengths which are made in high amounts. Other investigators have also demonstrated, using gel permeation chromatography in combination with other methods, that the LPS from the *Enterobacteriaceae* and other gram-negative bacteria could be resolved into at least two main populations of LPS differing in the length of their O-polysaccharide chain (10,27,31,33,35). In the results presented here, we found that the LPS from strains 503 and 1715 (Figures 2 and 3, respectively), as well as strains 1716 and PAZ1, separated into three major populations (peaks 1, 2, and 3) and two minor populations (peaks 2a and X). Interestingly, the short chain population comprised more than 90% of the total sample on a molar basis (Table 1). In addition, there were greater amounts of the very long chain population from the PAO1 strains compared to the long and intermediate chain populations. These results agree with those of Wilkinson (58) and Hancock *et al.* (24), where they estimated the mole percent of S-form LPS to be between 0.2-14%. On the other hand, the *Enterobacteriaceae* show a distribution of 44-60% of the LPS molecules in the low molecular weight population of 30-50% in the high molecular weight fractions (37,48). It has been demonstrated that *S. typhimurium* synthesizes LPS molecules with over 80 O-antigen repeating units and this population constitutes about 6% of the total LPS sample (48). Since the hydrophilic O-polysaccharides extend from bacterial surface into the aqueous environment, the observed heterogeneity of O-

chain lengths suggests that the surface topography of the gram-negative bacteria is irregular and that accessibility of the lipid A head group of the LPS could vary in different regions on the surface and on different bacterial species. It has been shown that the presence of O-antigen-containing LPS influences various cell surface phenomena, including antibiotic binding to LPS (49), antibiotic susceptibility (2,5,19), LPS aggregate structure (49), bacteriophage recognition (26,28), immunochemical characterization (9,10,50), virulence (12,51), protection against the bactericidal action of serum (22,42), polyclonal B cell activation and macrophage cytotoxicity (44). The low level of LPS on *P. aeruginosa* that contains a long O-polymer, however, may be sufficient to form a uniform cover over the cell since the surface is inaccessible to rough core specific monoclonal antibodies (53).

A striking feature of PAO1 strains from *P. aeruginosa* is the presence of the A bands which constitute a significant amount of the isolated LPS (Table 2). This set of bands was observed as a slow moving diagonal banding pattern across the SDS-PAGE of the column fractions (peak X, Figures 2 and 3). LPS from strain Z61 contained only a single diagonal banding pattern corresponding to the bands in peaks 2 and 3 (Figure 4). Although SDS-PAGE separates LPS molecules according to size (27,46,48), we propose that the anomalous migration in SDS-PAGE of the A bands is due to a difference in the charge in the core-lipid A region of the molecules. The lack of phosphate substituents in the A band sample would make the molecules much less negatively charged than the B band fractions

which are high in phosphate groups. To further explore this possibility, we compared the mobility of the A and B bands of the PAO1 strains on an 11% SDS-PAGE (Figure 5). We observed a difference in the spacing between the A and B bands, and that the B bands consisted of sets of doublet bands in which one of the two differ in their staining intensity (Figures 1B and 5), while the A bands seemed to lack this doublet pattern. This doublet probably represents substoichiometric modification in the core or lipid A of the B band components. It has been reported for different gram-negative bacteria that there is microheterogeneity in the structure of lipid A (phosphate levels and types and numbers of fatty acids) (3,52,58), in the substituents in the core (15,23,37-39,58), and in modifications of the O-antigen side chain (37,38,58). The heterogeneity of LPS molecules presumably depends, in part, on the strain and on growth conditions (14,37).

We have presented several pieces of evidence which indicate that the molecules represented by the A and B bands are chemically distinct. Only the B bands from the PAO1 strains reacted with anti-O-antigen antibodies in Western blots (Figure 5). Also, the A bands lacked reactive amino sugars detected in the B band fractions. Since the pooled fractions corresponding to A bands (peak X) contained very low levels of reactive KDO unless hydrolyzed with high concentrations of acid, and no phosphate or amino sugar was detected (Table 2), there is the possibility that these A bands represent another type of molecule different from LPS, that is, an O-repeat attached to a molecule which is not lipid A. In the past 10 years the chemical structure of the lipid A's from gram-negative

bacteria other than the *Enterobacteriaceae* have been studied, and the existence of "unusual" lipid A's has been noted (40). For instance, the lipid A from *Pseudomonas paucimobilis* contains a number of sugars, in addition to glucosamine, in the "bound lipid" fraction, and phosphate as well as KDO appears to be lacking (40). Also, species of *Thermus* have been reported to make LPS that lacks detectable heptose, KDO, glucosamine, and phosphorus (40). However, negative reactivity in the thiobarbiturate assay may not reflect the lack of KDO residues. Recently, Parr and Bryan (47) demonstrated that more rigorous hydrolysis conditions were required to release KDO from *Haemophilus influenza* LPS compared to other LSP species. Subsequently, Caroff *et al.* (8) demonstrated that after treatment with aqueous hydrofluoric acid, the presence of KDO could readily be demonstrated in LPS of *Bortedellas, Bacteroides, Aeromonas,* and *Vibrios* which had been reported to be KDO-deficient. Thus, we hydrolyzed our A band sample with a higher acid concentration for longer times and observed a 10-fold increase in the KDO level. This suggests that the A bands are resistant to hydrolysis due to substitutions of the KDO units in position 4 and 5, or 5 and 7 (7). Other evidence that suggests that the A bands represent LPS molecules is the ability to silver stain the A band molecules; Kropinski *et al.* (32) and Lam (personal communication) reported than the silver staining reaction for *P. aeruginosa* LPS occurs in the lipid A rather than the core sugars.

Analysis of the Western and dot blots (Figures 5 and 6) yielded several additional interesting observations. The pooled fractions containing the higher

molecular weight LPS (peaks 1 and 2) from the PAO1 derivatives reacted with the antibody (Figure 6), indicating that the B bands comprise the serotype-specific LPS. However, *P. aeruginosa* synthesizes a significant amount of A band type molecules (Table 2) with presumably a different antigenicity. It has been shown that *S. paratyphi* B and *S. typhimurium* can synthesize a T1 polysaccharide and an O-polysaccharide attached to the same core (38,42), and that the synthesis of the two molecules is independent (38). Other *P. aeruginosa* strains may also have the ability to synthesize more than one type of LPS with different O-antigenic side chains (9,31,58,59). The high molecular weight polysaccharide released from LPS of these strains has been resolved into amino sugar-rich and neutral sugar-rich fractions when separated by gel permeation chromatography (31,58,59). And finally, Caroff *et al.* (7) have shown that *Bordetella pertussis* produces two lipopolysaccharides, one of which does not give a positive reaction for KDO under normal thiobarbituric assay conditions. This is very similar to our findings for the A and B band-type LPS of the PAO1 derivatives.

The effect of the presence of a unique, A band-type of LPS on the physical interactions within the outer membrane, as well as the immunological reaction with the cell surface, may be important. It has been proposed that bacteria synthesizing O-side chains of an unusual structure might escape the immune system of the host, which might have difficulties in producing effective antibody molecules against these O-side chains (42,51).

In summary, our data suggest that PAO1 strains from *P. aeruginosa* are

capable of synthesizing more than one type of LPS-like molecule differing in their antigenic reactivities. Although the LPS isolated from the different strains used in this study was shown to be heterogeneous on SDS-PAGE, this method by itself does not have the power to predict the presence of more than one type of LPS-like molecule with a different O-polymer. However, by combining SDS-PAGE with gel permeation chromatography, Western blots, and sugar analysis, we have been able to distinguish chemically distinct subclasses of molecules from individual strains. We have also demonstrated that the percentage of core oligosaccharides carrying the O-specific polymer is less than 8%.

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