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Douglas Raymond Dixon

Lipid A heterogeneity within *Porphyromonas gingivalis* and other oral bacteria: Effect of lipid A content on hTLR4 utilization and E-selectin expression

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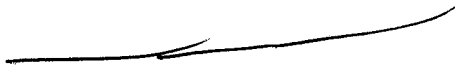
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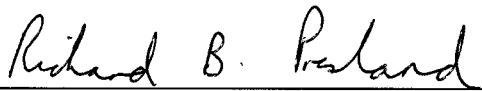
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Abstract

Lipid A heterogeneity within *Porphyromonas gingivalis* and other oral bacteria: Effect of lipid A content on hTLR4 utilization and E-selectin expression

Douglas R. Dixon

Chairperson of Supervisory Committee

Professor Richard P. Darveau

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Lipopolysaccharide (LPS) is an outer membrane molecule for virtually all Gram-negative bacteria. Specific components of the lipopolysaccharide (LPS) molecule, once thought to be highly conserved, are in some bacterial species surprisingly variable. One such portion of the LPS molecule displaying this surprising variability is the lipid A portion. Some examples of lipid A structural variability are seen within bacterial pathogens such as *Yersina pestis*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* which have been linked to significant alteration in host biologic activity. However, what has yet to be determined is the presence, extent or mechanism behind lipid A heterogeneity in oral bacteria. This project was therefore developed to test the hypothesis that any structural changes observed within lipid A of oral bacteria would alter host responses. Results confirmed our initial hypothesis that lipid A structure from *P. gingivalis* was highly

variable (heterogenic) as compared to other oral bacteria sampled. *P. gingivalis* lipid A structure was altered by growth phase, environmental pH, as well as by insertional inactivation of the PhoP transcriptional activator, which is part of a two component regulatory mechanism recently identified within the genome of *P. gingivalis*. It was also observed that application of phenol or base chemicals (especially in the presence of heat during extraction procedures) resulted in loss of structural components or degradation of the lipid A molecule in both oral and enteric bacteria. Reduction in the number of fatty acids and/or phosphate groups from the disaccharide backbone of oral lipid A, as a result of the experimental procedures, resulted in the reduction of E-selectin expression in HUVECs and hTLR4 utilization in transfected HEK 293 cells whereas unaltered lipid A structure retained full biologic activity. In addition, selective removal of fatty acids within different oral bacterial lipid A resulted in partial antagonism of both *E. coli* and *P. gingivalis* LPS. These results suggest that under specific environmental conditions, certain bacteria which naturally possess the ability to alter its own or neighboring bacterial lipid A structure, would be able to manipulate the local environment to potentially favor colonization, growth and survival within host tissues.

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DEDICATION

This dissertation is dedicated to my family: To my wife and her special gift of strength and encouragement when I am weak, tolerance when I am intolerant and unfailing love, has given me the intention as well as the fortitude to complete this project. To my daughter whose compassion, humor, and willingness to learn have inspired me to understand what was unknown or forgotten. To my son, who has shown me that courage, strength, and the ability to overcome adversity can occur at any age.

Background

What is lipopolysaccharide (LPS)?

Lipopolysaccharide (LPS) is an essential outer membrane molecule for virtually all Gram-negative bacteria. It has been historically described as a heat stable, non-proteinaceous, endotoxic microbial cell wall component consisting of highly variable as well as highly conserved segments (Rietschel, *et al.*, 1992b; Rietschel and Westphal, 1999). Conserved regions of LPS represent critical molecules that are shared between bacterial species that assist in either the development and/or maintenance of a component or structure that is essential for survival of the bacterium. The variable regions represent segments with less “essentialness” for the bacterium allowing for evolutionary variation without catastrophic consequences. Changes within either region can result in either simple modifications of LPS, such as minor alteration in the length of the segment, or can have dramatic effects like changing chemical configuration / arrangement, composition, or attached charge groups which, in turn, can affect overall LPS structure.

Conservation within structural components, like LPS, serves as the basis for host identification and response by creating “molecular signals” that are unique to prokaryotic cells thus allowing for host distinction between self and non-self (Medzhitov and Janeway, 2000). However, new insights within the relatively recently discovered Toll-Like Receptors (TLRs) and co-associated molecules have revealed that even subtle

differences in “conserved” LPS structures and their consequent molecular signals can be identified by host cells resulting in profound host effects. For example, specific synthetic alteration within conserved segments of LPS, were shown to be not only recognized by host cells but capable of blocking or down-regulating the response towards other LPS forms normally associated with robust innate cell activation. Furthermore, observation of naturally occurring LPS heterogeneity of specific pathogenic bacteria, resulting from either altered bacterial growth or environmental conditions, was shown to result in differential and altered innate host cell responses *in vitro* that could potentially represent or confer some inoculation or evasion advantage resulting in survivability and sustained pathogenicity for the bacterium *in vivo*.

Schematic

The majority of Gram-negative bacteria contain various amphiphilic molecules within the outer membrane; however, Lipopolysaccharide (LPS) represents the main surface antigen possessing both microbiologic and immunologic significance. Although there are some notable exceptions, many species contain a common form or architecture that consists of specific components: An outer or distal segment, the O-chain or O-antigen; the outer core segment; an inner core segment; and finally the medial lipid A portion (Luderitz, *et al.*, 1981; Morrison and Ryan, 1992). **Figure B.1** illustrates the major components of LPS and its relative position on the bacterial outer membrane surface.

LPS (O-antigen) region: Features/Functions/ Host responses

General features within the O-antigen segment of LPS consist mainly of the fact that the repeating residues that forms the carbohydrate polymer is a glycosyl residue and that by its nature, the O-chain is only found in smooth type Gram-negative bacteria. Diversity, therefore, within this segment is the characteristic feature. Variation among certain species can range from as much as 50 separate but repeating oligosaccharide subunits (comprised of one to eight sugar units) (Caroff and Karibian, 2003) to those species such as *Bacteroides fragilis*, *Neisseria meningitidis* and *Haemophilus influenzae* which lack the O-chain altogether.

The O-specific or O-antigen region represents a unique segment of LPS that contains multiple biologic functions and activities that, due to its diversity, both activate and down-regulate host functions. For example, one of the earliest host recognized structures, generated by bacterial LPSs, came through studies performed with different *Salmonella* species. From these studies, it was determined that one mechanism by which the host can identify individual bacteria was mediated through recognition of specific epitopes (O-factors) that were present within the repeating residues incorporated into the O-chain segment of the LPS. For example, it was determined that the O-chain segment contains unique molecular conformations due to different, chemical linkage types between sugar groups as well as additions or substitutions of sugar residues within this region. Remarkably, the host is able to detect these minor chemical alterations such that a “serological specificity” – the individual identification to the host – is conferred for

different LPS types and their corresponding parental bacterial strain (Rietschel, *et al.*, 1992b; Rietschel and Westphal, 1999). This serologic specificity led to serotype classification and agglutination schemes that are the basis for *Salmonella* identification assays still in wide use today (Rietschel and Westphal, 1999).

Additional functions for LPS O-polysaccharides stem from continued interaction between the host and the O-polysaccharide itself. This dialogue arises again from interaction(s) between: carbohydrates within the O-polysaccharide (Grossman and Leive, 1984; Raetz and Whitfield, 2002), O-factors expressed (Rietschel, *et al.*, 1992b), the length of the chain (Burns and Hull, 1998; Raetz and Whitfield, 2002), as well as the overall amount (Joiner, 1988). Benefits for the microbial organism, from these interactions with the host, result from down-regulation of specific mechanisms (C3b mediated phagocytosis) within the alternate complement pathway (Grossman and Leive, 1984; Rietschel, *et al.*, 1992b) altered or reduced attachment of the C5b-9 host membrane attack complex (Grossman and Leive, 1984) leading to a “shielding effect”(Joiner, *et al.*, 1984), as well as disrupting certain neutrophil functions. (Weiss, *et al.*, 1986) Furthermore, O-chains seem to help certain bacteria adhere to host tissues such as the lung (Paradis, *et al.*, 1994), and protect it from certain antibiotics (Banemann, *et al.*, 1998; Caroff and Karibian, 2003).

It has been suggested that activation of the alternate complement pathway is an important strategy utilized by the innate immune system during microbial “non-self” recognition (Medzhitov and Janeway, 2002). Once C3, a key component of complement, binds to a recognized microbial component, it activates the complement cascade which is then

capable of microbial cell lysis or phagocytosis (Austen and Fearon, 1979). Host cells are protected from this destructive process by possessing gene products capable of inhibiting C3 initiated protease complexes (Austen and Fearon, 1979). However, as stated previously, it is well known that there is a list of human pathogenic organisms such as: *Bacteroides fragilis*, *Bordetella species*, *Haemophilus influenza* as well as *Neisseria meningitidis* contain either a substantially reduced or lack a complete or O-specific chain altogether (Caroff, *et al.*, 2002; Griffiss, *et al.*, 1988). It is therefore interesting to speculate that although the presence of a full length O-chain can be beneficial to certain types of bacteria, significant alteration *via* reduction, absence or heterogeneity within this segment of LPS, could potentially confer a selective colonization or survival advantage for certain known pathogens through innate host non-recognition mechanisms related to the absence or *in vivo* alteration of the O-chain itself (Preston, *et al.*, 1996).

LPS (Outer core) region: Features/Functions/ Host responses

A major feature of the outer core is that this region is present in bacterial species that produce long segments or smooth forms of LPS (S-LPS) and is distinguished from the O-segment by genetic determination, internal structures, biosynthesis and reduced variation (within species) as compared to the adjacent O-segment (Raetz and Whitfield, 2002; Rietschel, *et al.*, 1992b). Common chemical constituents of the outer core in bacterial species such as *Salmonella*, *Escherichia*, and *Shigella* consist of a 1-6 oligosaccharide unit that is linked to heptose (Caroff and Karibian, 2003). Exceptions to this vary and include different forms, linkages to, or numbers of heptose units which alters attachments

site of the O-chain (Vinogradov, *et al.*, 2002; Vinogradov and Perry, 2000; Vinogradov and Perry, 2001; Vinogradov and Sidorczyk, 2002). The outer core polysaccharide segment of LPS functions as a linkage point for the O-antigen segment.

Elucidation of the overall core region was initiated through the discovery of enterobacterial mutants with a defect in biosynthesis of the O-chain resulting in a truncated LPS and the characteristic rough morphotype. These rough mutants (R-mutants), were first described in *Salmonella* (Fukushi, *et al.*, 1977; Galanos, *et al.*, 1979) and interestingly, have a reduced virulence when compared directly to the full length parental S-LPS (Holst and Brade, 1992). This observation might lend credence to the suggestion that O-chain is not essential for bacterial virulence per se (Holst and Brade, 1992), but rather aid the bacterium in some other role such as survivability and establishment within the host.

LPS (Inner core) region: Features/Functions/ Host responses

At the cytoplasmic membrane, O-chain and core-lipid A synthesis are occurring separately. Core synthesis usually occurs after the formation of a mature lipid A, catalyzed by the Rfa protein (Roncero and Casadaban, 1992). Once these pieces are assembled, they are ligated and then transported to the outer membrane for incorporation as a major outer membrane structure. Again, rough mutants which result in truncation of LPS yield varying lengths of core LPS as depicted in **Figure B.1** result in rough-phenotype colonies when grown on agar plates. Wild type bacterial strains, with the full

length O-chain polysaccharide yield the characteristic smooth colonies, and are termed S-LPS.

Generalities within the inner core of most bacterial species, therefore, reveal that the majority of the chemically analyzed inner core LPSs contain Kdo and heptose groups. In *Salmonella*, inner core polysaccharides consist mainly of ketodexoyoctonate (Kdo), and a series of heptose sugar groups: N-acetylglucosamine, galactose, and glucose. These inner core structures are more conserved than the O-chain polysaccharide and outer core segments and also serves as a linkage point between outer core structures, if present, and the lipid A portion of LPS. Interestingly, the bond between the Kdo of the inner core and the medial lipid A is sensitive to mild acid hydrolysis which allows for chemical separation of the polysaccharide portion from the lipid portion of LPS (Caroff, *et al.*, 1988; Osborn, 1963).

The presence of Kdo and heptose coupled with hydroxytetradecanoic acid were deemed essential components for bacteria and used as an identifying features during compositional analysis of bacterial LPS (Caroff and Karibian, 2003). However, recent evidence in specific bacterial species (*Neisseria meningitides*) has suggested that heptose groups were not essential *in vitro* (Makela and Stocker, 1984). Furthermore, the 3-deoxy-oct-2-ulosonic-acid (Kdo) group, in certain non-enteric species, could be reduced to D-glycero-D-talo-oct-2-ulosonic acid (KO) resulting in a non-lethal change (Caroff and Karibian, 2003). Recently, Steeghs et al. created a non-lethal mutant within *Neisseria* that was void of a core structure and grew *in vitro* (Steeghs, *et al.*, 1998).

However, this phenomena might be associated with a protective function, within a polysaccharide capsule, expressed in some but not all bacterial species.

Bio-function for the core segment of LPS was once thought to be limited to serving as a linker region, functioning as a spacer between two bioactive components (O-antigen and lipid A). However, physiologic evidence suggests that that chemical groups attached to core structures affect the presence of divalent cations associated with maintenance and stability of the bacterial outer membrane, consequently certain inner core structures are considered essential for bacterial viability. Therefore, in agreement with host recognition of structures essential for bacterial survival, it has been shown that recognition of specific core elements can and does modulate the bioactivity of lipid A (Caroff, *et al.*, 2002; Haeffner-Cavaillon, *et al.*, 1984; Holst and Brade, 1992; Muroi and Tanamoto, 2002; Rietschel, *et al.*, 1971; Rietschel, *et al.*, 1994).

Lipid A: “Classic” lipid A structure is represented by *E. coli*

Lipid A is a gluco-configured hexosamine-based phospholipid (Zahringer, *et al.*, 1999) which serves as the hydrophobic anchor of lipopolysaccharide (LPS) on the majority of Gram-negative outer membranes (Raetz, 1990; Raetz and Whitfield, 2002). The majority of Gram-negative bacterial lipid A structure is conserved and consists of a mono or biposphorylated disaccharide backbone which has been acylated with C₁₂-C₁₄ length hydroxy and non-hydroxy fatty acids at specific positions (C2, C3, C2', C3').

Furthermore, if present, the hydroxy groups of these fatty acid chains can be further

esterified by additional fatty acids (secondary substitution) (Sforza, *et al.*, 2004). Lipid A structural differences such as hydroxylation, secondary substitution as well as the presence of longer than average length fatty acid chains (C₁₆) might be potentially regulated by the presence of specific environmental factors (pH, divalent cations) and specific regulator mechanisms that respond to these factors (Caroff and Karibian, 2003; Garcia Vescovi, *et al.*, 1996; Guo, *et al.*, 1997; Preston, *et al.*, 2003). The “classic” structure of lipid A is represented by *E. coli* and is depicted in **Figure B.2**. This lipid A contains a 1, 4'- biphosphorylated β (1-6)-linked D-glucosamine disaccharide backbone (D-GlcN I, D-GlcN II) that is hexa-acylated *via* primary ester and amide linkages (Erwin and Munford, 1990) with secondary substitution on specific hydroxyl groups (Rosner, *et al.*, 1979; Seydel, *et al.*, 1984). Known gene regulation of specific components of lipid A are described within the figure legend of **Figure B.2**.

Lipid A is the “bioactive center” (Endotoxin) of LPS

During the late 1980’s experimentation with LPS and separated lipid A fractions revealed that that lipid A was, in fact, the bioactive component of LPS, responsible for the majority of IL-1 induction and immunoregulation in human mononuclear cells within the pg/ml range (Loppnow, *et al.*, 1989; Loppnow, *et al.*, 1990). These experiments were based on and correlated with findings from earlier and contemporary experimentation with synthetic lipid A components (Kotani, *et al.*, 1985) and Re-mutants strains (Kasai and Nowotny, 1967; Luderitz, *et al.*, 1966) – both of which are devoid of any additional polysaccharide (PS) additions – which resulted in full endotoxic activity when compared

to the original LPS forms (Kim and Watson, 1967; Kotani, *et al.*, 1985; Rietschel and Westphal, 1999). Results from these and other pivotal experiments (Galanos, 1975) confirmed the role of lipid A as the active LPS constituent relegating other subcomponents of LPS as non-essential for endotoxicity (Rietschel and Westphal, 1999).

Endotoxic effects of lipid A

The biologic effects of LPS-lipid A have been well documented. (Morrison and Ryan, 1979; Raetz, 1990; Raetz and Whitfield, 2002) However, during the mid 1980's, results from chemically synthesized lipid A helped to delineate the biologic effects that were distinctly inherent to the lipid A itself. This discovery came from a number of international laboratories that centered their efforts in defining structural components with bioactivity. Before this distinction, the emphasis was placed on defining the LPS-lipid A complex, which was deemed an important virulence factor for host activity. Now, it is widely believed that to have "endotoxic" capabilities, synthetic or natural lipid A must be comprised of specific structural features. For example, active lipid A must contain: (1) a $\beta(1-6)$ -linked D-glucosamine disaccharide core; (2) phosphorylation at either positions 1 or 4' of the core disaccharide; (3) and appropriate number and length of 3-acyloxyacyl groups on each monosaccharide. (Kotani and Takada, 1990; Takada and Kotani, 1989) One of the earliest breakthroughs in lipid A study was the creation of a synthetic lipid A (synthetic compound 506) with not only the same structural features as a strain of *E. coli* (labeled F515) but more importantly, possessing the same endotoxic and related bioactivities. (Kotani, *et al.*, 1985; Takada and Kotani, 1989) During the same

time frame an additional synthetic compound, labeled (Lipid IV_A) was synthesized by Galanos' group. Here, this synthetic lipid was created that was structurally similar — lacking only the additional ester-linked fatty acids on R2' and R3' — but shared only partial and sometimes dramatically different bioactivity when compared to the naturally occurring *E. coli* reference strain. (Galanos, et al., 1985) **Figure B.3** displays the salient structural features of both compound 506 and Lipid IV_A.

What is interesting to note is that the structural differences and potential bioactivities between 506 and IVA relate to the secondary substitution of additional fatty acid residues. Compound 506 displayed full endotoxic activities that were identical or greater than that of the reference lipid A from *E. coli*. (Kotani, et al., 1985) On the other hand, without these residues, IVA possessed an endotoxin antagonistic capability in human monocytes against LPS extracted from a variety of different Gram-negative bacteria. (Golenbock, et al., 1991; Raetz and Whitfield, 2002) This suggests that the number, position and/or length of the fatty acid residues that differ between the synthetic compounds and the natural species represent a lipid A heterogeneity that has biologic significance. Furthermore, *Rhodobacter sphaeroides* lipid A, which naturally presents a intermediate lipid A moiety between that of compound 506 and IVA demonstrated similar antagonistic capabilities of IV_A. In addition, it has recently been shown that *Pseudomonas aeruginosa* and *Erwinia carotovora*, can alter their lipid A structure in direct response to changing environmental factors. (Fukuoka, et al., 2001; Hajjar, et al., 2002) These Lipid A moiety modifications were also shown to be recognized differently by the host — depending on TLR expression — indicating natural intrinsic ability within

specific bacteria to modulate lipid A structure resulting in direct modulation of the host inflammatory response.(Fukuoka, et al., 2001; Hajjar, et al., 2002)

LPS and Periodontal pathogenesis

Periodontal disease affects well over 125 million adults within the United States, approximating 80% of the entire adult population. Periodontal diseases of the oral cavity are extremely difficult to resolve due to a number of factors. The first of which is that the oral environment is host to well over 400-600 bacterial species. (Socransky, *et al.*, 1998) During the course of disease, a pathogenic shift in bacterial organisms occurs resulting in a change from aerobic Gram-positive bacteria to predominately anaerobic Gram-negative species. This transition undermines the effectiveness of identification of specific bacteria responsible for the initiation of disease. Secondly, periodontitis is a disease that has periods of progression and remission. Determination of specific bacteria involved in these disease stages have proven difficult due to the clinical challenges of diagnosis and variable host responses to these invading organisms. Lastly, pathogenesis of periodontal disease is further complicated by bacterial complexes, opportunistic species and multiple virulence factors of oral bacteria. However, what is common to all Gram-negative oral bacteria is the presence of LPS/lipid A on their outer membrane surface. Therefore, this work focused on the conserved segments and observed heterogeneity within specific components of LPS, such as lipid A, and the biologic ramification(s) thereof.

The effort described in this dissertation was designed to test the hypothesis that lipid A heterogeneity exist within specific oral bacteria which can affect levels of innate immune modulators from host cells. The chapters within this dissertation were written as complementary but separate sections however, these chapters contain some overlapping material which may be repetitive. Each chapter describes work which addressed each of the specific aims of the project:

Specific Aim 1 to determine the relative amount of lipid A structural heterogeneity within pathogenic and commensal oral Gram-negative bacteria and determine if there is any correlation with host responses.

Specific Aim 2 to determine the contribution of altered environmental conditions on lipid A structure within *P. gingivalis*

Specific Aim 3 to determine the effect of selective removal (de-acylation) of specific fatty acids, linked to the lipid A molecule, from selected oral bacteria and determine the affects within innate immune host based assays and,

Specific Aim 4 to determine the effect of different LPS extraction techniques on lipid A structure using oral and enteric Gram-negative bacteria and relate these changes to altered host functions.

The work reported here demonstrates that *P. gingivalis* is a unique microbial member of the oral cavity due to heterogeneity within its lipid A structure that is specific for the “Bacteriodes” family. Specific structural alterations of the lipid A molecule were linked to environmental conditions, specifically those conditions associated with *in vitro* changes in pH values that have also been shown to occur *in vivo* during acute and chronic

periodontal infections in the mouth. Third, chemically or physically induced deacylation of ester-linked fatty acids, linked to the core disaccharide of the lipid A molecule, was shown to have a dramatic effect with host based assays. Lastly, specific extraction technique and reagents were also shown to chemically degrade the lipid A molecule thereby adding to the reported lipid A heterogeneity of certain Gram-negative bacteria. These observations of naturally occurring lipid A heterogeneity, as well as the environmental conditions or enzyme activity that initiates lipid A structural alteration in *P. gingivalis* or other bacteria contained in the oral biofilm, represents a novel finding and invites further investigation.

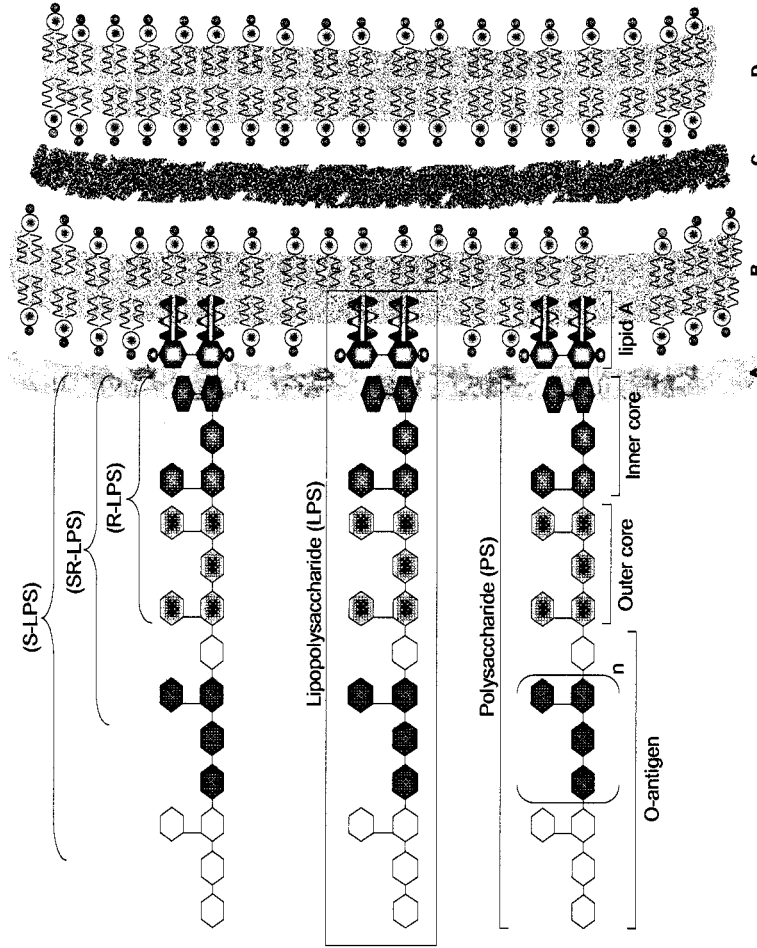


Figure B.1 General overview of lipopolysaccharide (LPS) on the outer membrane of a gram-negative bacterium.

LPS consists of 3 major components: the highly variable outer O-antigen segment; a more conserved core which is divided into an outer in inner segment; and the bioactive lipid A portion. Variation within the length of the LPS, due to mutational absence of specific structures not only changes the phenotypic appearance of the bacterium (i.e. smooth [S], Semi-rough [SR], or rough [R]), but may also change some bioactive responses by the host to the bacterium itself. (A) Some bacterial species contain an outer capsule that protects the bacterium from host defenses such as complement, lysis and phagocytosis. (B) Outer lipid bilayer with LPS which is approximately 8nm in width (C) Peptidoglycan layer (D) Inner bilipid membrane. Note: additional lipoproteins, porin complexes, and additional membrane proteins established within and surrounding the inner and outer membranes have been removed to simplify the diagram. (Caroff, *et al.*, 2002; Raetz, 1992). Figure adapted from Dixon and Darveau. LPS heterogeneity: Inmate host responses to Bacterial Modifications of Lipid A Structure. *Critical Reviews in Oral Biology and Medicine*. JDR Aug 2005 *In Press*.

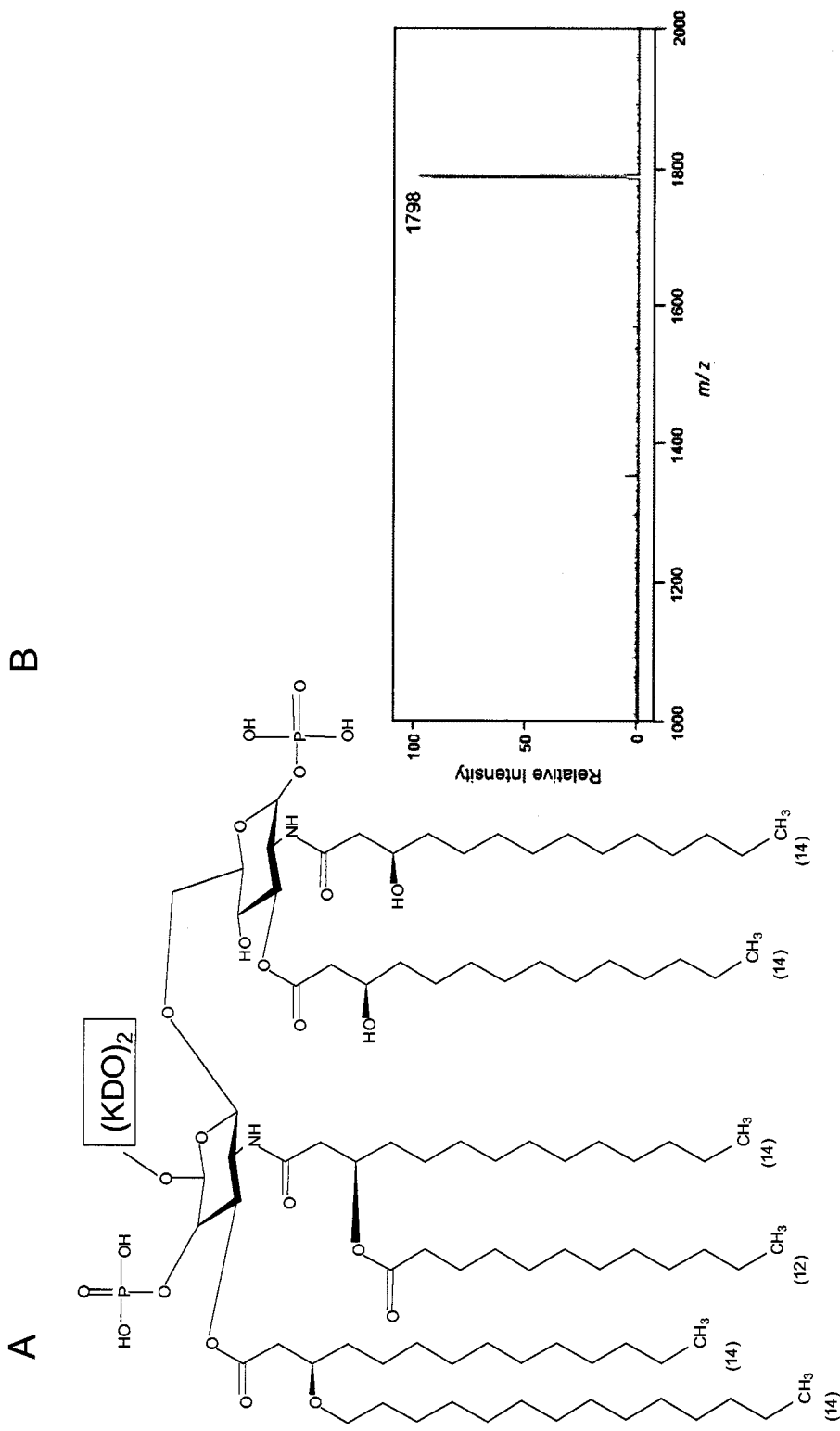


Figure B.2 (A) Representative lipid A structure for *E. coli*. Adapted from: Lipopolysaccharide Endotoxins. Annu Rev. Biochem. 2002. 71. 635-700. (B) MALDI-TOF analysis of *E. coli* JM83 lipid A after TRI-CAROFF extraction.

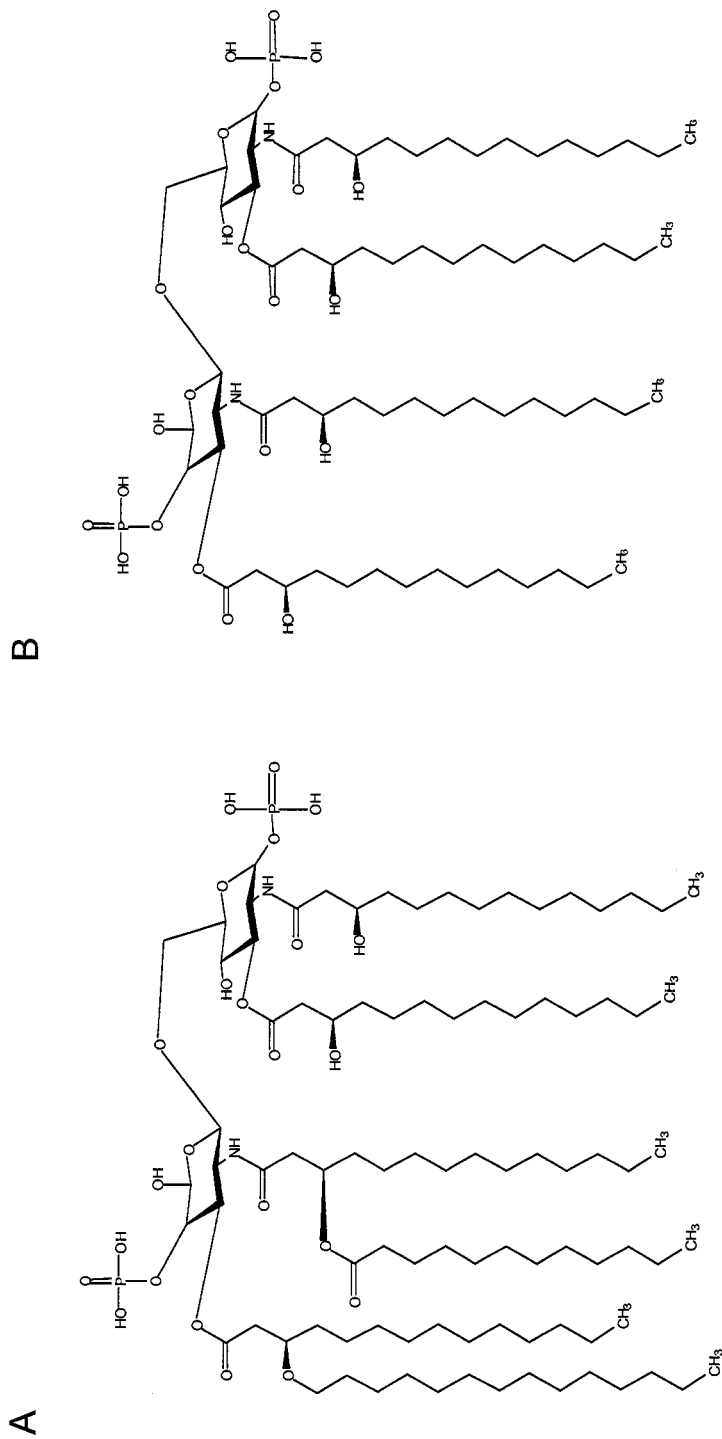


Figure B.3 (A) Compound 506. Adapted from: Kotani, Takada, Tsujimoto et al. Synthetic Lipid A with Endotoxic and Related Biologic Activities Comparable to Those of a Natural Lipid A from an *Escherichia coli* Re-mutant. Infection and Immunity Jul 1985. P225-237. (B) Lipid IV_A. Adapted from Golenbach, Hampton, Qureshi, Takayama, Raetz. Lipid A-like Molecules that Antagonize Effects of Endotoxins on Human Monocytes. Journal of Biologic Chemistry Vol 266, No. 28. pp19490-19498.

CHAPTER 1: Characterization of oral Gram-negative lipid A profiles

lipid A structure

INTRODUCTION

As stated within the introduction, lipid A has been described as a glucosamine-based phospholipid which serves as the hydrophobic anchor of lipopolysaccharide (LPS) on the majority of Gram-negative outer membranes. (Raetz, 1990; Raetz and Whitfield, 2002) The majority of Gram-negative bacteria lipid A structure is conserved and consists of a mono or diphosphorylated disaccharide backbone which has been acylated with hydroxy and non-hydroxy fatty acids at specific positions on the lipid A sugars. Furthermore, the hydroxy groups of these fatty acid chains can be further esterified by additional fatty acids and is referred to as secondary substitution. (Sforza, et al., 2004) The representative example of lipid A was demonstrated with *Escherichia coli* in **Figure B.2**.

Lipid A heterogeneity – the appearance of separate lipid A forms that are distinctly different from each other within the same bacterial preparation – have been reported for many bacterial species. These lipid A moieties have been explained as partial structures of the parent or mature form of lipid A that can occur due to incomplete biosynthesis or chemical degradation during LPS extraction and purification procedures. (Rietschel, *et al.*, 1992) Others have suggested that lipid A heterogeneity can result as a response towards fluctuating environmental conditions that can render specific advantages to bacterial

species during colonization, growth or in the presence of antimicrobial peptides.(Ernst, *et al.*, 1999; Fukuoka, *et al.*, 2001; Hajjar, *et al.*, 2002)

Although evidence for the presence of LPS structural alteration exists within bacterial species, what is not well understood is the extent of lipid A heterogeneity within bacteria that cultivate, persist and become potentially pathogenic within the oral cavity. In regard to this, an obvious starting point would be the bacterium, *P. gingivalis*, since its LPS structure has been previously reported as well as it's documented isolation from gingival sites with multiple forms or stages of periodontal disease.(Socransky, *et al.*, 1998)

The basic structure of lipid A for *P. gingivalis* 381 was initially described by Ogawa as a monophosphorylated tri-acylated disaccharide with a negative ion FAB MS-MS mass ion located at m/z 1195 which displayed low endotoxic activity. (Ogawa, 1993) Soon after this was published, Kumada et al. began to describe additional lipid A moieties such as a lipid A species containing four to five fatty acid chains with a negative ion FAB MS-MS mass ion(s) located at m/z 1435, 1449, and 1769 respectively. (Kumada, et al., 1995)

Likewise, we have also shown the ability to successfully cleave the lipid A (endotoxin) portion from LPS from *Porphyromonas gingivalis* 33277 and then characterize the biochemical structure of this lipid A via MALDI-TOF and GC analysis. Recently, it has been reported from our laboratory that within the same preparation, different structural forms of *P. gingivalis* lipid A do exist and can be characterized as separate and distinct lipid A moieties with dramatically different host responses.(Darveau, *et al.*, 2004) What

has yet to be determined is if this observed heterogeneity within the lipid A structure is consistent (i.e. same mass ion profile) between clinical isolates or laboratory strains of *P. gingivalis*. In addition, other oral Gram-negative bacteria containing lipid A structure(s) have yet to be characterized. Furthermore, we have identified biologic activity that is directly related to specific forms within the proposed biochemical structure of *P. gingivalis* endotoxin. For example, *P. gingivalis* 33277 contains a predominate lipid A species that has been identified to contain a monophosphorylated, penta-acylated disaccharide that is a potent activator of E-selectin from human umbilical endothelial cells (HUVEC). In addition, we have also identified a tetra-acylated species of *P. gingivalis* 33277 that possesses antagonistic properties against other bacterial lipid A as well as it's own. Yet, it is not known whether this lipid A heterogeneity exists in all oral Gram-negative bacteria or is it limited to specific species or isolates within a specific oral genus such as Porphyromonas.

To address this question, we employed the use of mass spectral analysis on different strains and isolates of *P. gingivalis* as well as several other oral Gram-negative bacteria. MALDI-TOF spectrometry analysis has proven to be a powerful tool to discriminate bacterial taxa, not only at the species level but also at the subspecies level as well. Therefore, with the use of this analytical tool we tested our first hypothesis that there is no difference in lipid A spectral profiles between clinically obtained isolates or laboratory strains of *P. gingivalis* and that lipid A spectrum profiles would not vary from one oral Gram-negative bacteria to another. Our initial results, however, suggests that there is a

significant difference in lipid A composition between oral Gram-negative bacteria, corresponding to different host responses, and that this difference might aid or enhance the ability of specific pathogens to evade and/or regulate the immune inflammatory response. Furthermore, it could be hypothesized that those oral bacteria that have not evolved the ability to modify/manipulate their own lipid A structure are more easily identified, and contained or eliminated by the innate host defense system.

MATERIALS AND METHODS

Selection of Oral bacteria

It is well known that a relationship exists between specific oral bacteria and inflammatory disease states of tissues within the oral environment. As a result of this, bacterial classification systems, based on association to specific inflammatory disease states of the periodontium, have grouped together certain bacterial species into pathogenic and non-pathogenic microbial complexes. (Socransky, *et al.*, 1998; Socransky, *et al.*, 1988)

Within these particular studies, a color code was established that ordered or ranked oral bacteria in relation to their association with periodontal disease. This color code, which represents specific bacteria, has been described as follows: RED > ORANGE > YELLOW > GREEN > MAGENTA where RED indicates those bacteria with the highest association with disease states and MAGENTA representing the lowest association. Therefore, in an effort to sample a wide range of oral bacteria, bacterial species from

each of the microbial complexes, as well as some additional bacterial samples, were used in this study. Specifically, *Porphyromonas gingivalis* ATCC 33277, W83, 7436, and MP4-504, as well as four additional clinical isolates and *T. forsythensis* ATCC 43037 were used as a representative from the [Red-Cluster #1] complex. *B. fragalis* ATCC 25285 was used as a type species of Bacteroides. The [Orange Cluster #2] complex was represented by *Fusobacterium nucleatum* ATCC 22586, *Provetella intermedia* ATCC 25611, and *Campylobacter rectus* ATCC 33238. The [Green Cluster #4] complex consisted of *Capnocytophaga sputigena* ATCC 33612 and *C Capnocytophaga ochracea* #25 as well as *E. corrodens* ATCC 23834 and *Actinobacillus Actinomycetemcomitans* serotype “A” ATCC 43717 , and the [Magenta Cluster #5] was represented by *Veillonella* sp.4409. The [Yellow Cluster #3] as well as *Treponema denticola*, *Peptostreptococcus micros*, *Eubacterium nodatum* and *Actinomyces odontolyticus* were excluded from this study by being Gram-positive bacteria thus void of LPS.

Growth requirements

P. gingivalis ATCCC 33277, W83, 7436, *T. forsythensis* ATCC 43037, *B. fragalis* ATCC 25285, *F. nucleatum* ATCC 22586, *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 33563, *C. rectus* ATCC 33238, *C. sputigena* ATCC 33612, *A. actinomycetemcomitans* serotype “a” ATCC 43717 were obtained from the American Type Culture Collection. *P. gingivalis* MP4-504, *P. gingivalis* isolates 1-4, *C. ochracea* #25, and *Veillonella* sp.4409 were clinical isolates obtained from stock cultures. The majority of the bacteria were grown in the same basal medium. Specifically, *P. gingivalis* 33277, MP4-504, isolates 1-

4, W83, 7436, *T. forsythensis* ATCC 43037, *B. fragalis* ATCC 25285, *C. sputigena* ATCC 33612 were grown in Trypticase soy broth (30g/L, Bectin, Dickinson BBL) and supplemented with: yeast extract (5g/L, Difco), sodium lactate 1mL/L (Sigma), sodium succinate (0.5gm/L, Sigma), sodium fumerate (1.0gm/L, Sigma). Hemin (Sigma) and menadione (Sigma) were added from a 10 mL and 0.2 mL stock solution to a final concentration of 0.005 gm/L and .001 gm/L respectively. *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 33563, and a separate *P. gingivalis* 33277 preparation were grown in the above media with a modification to include adding 0.4 gm/L of anhydrous sodium carbonate (Sigma) and cysteine HCL (Sigma), 1g/L of glucose (Sigma) and only 1g/L of Yeast extract (Difco). *C. ochracea* #25 was grown in Trypticase soy broth (30g/L, Bectin, Dickinson BBL) and *F. nucleatum* was grown in mycoplasma broth base (21g/L, BBL). Hemin (Sigma) and menadione (Sigma) was added as described above. *C. rectus* ATCC 33238 was grown, as described by Tanner (Tanner, 1987), with brain heart infusion 37g/L plus: yeast extract (5g/L, Difco), sodium fumerate (1.0gm/L, Sigma) and hemin were added as described above. *E. corrodens* ATCC 23834 was also grown as previously described for *C. rectus* with the exception that potassium nitrate 1.0gm/L was substituted for sodium fumerate.(Tanner, 1987) Veillonella sp.4409 was grown in heart infusion broth 25g/L, yeast extract 5g/L (Difco), 1g/L of Glucose (Sigma), sodium lactate 1mL/L (Sigma), sodium succinate 0.5gm/L (sigma). *A. actinomycetemcomitans* serotype “a” was grown in brain heart infusion media 37g/L. All mediums were pH balanced to 7.0-7.2 and autoclaved before use. Cultures were obtained from sterile frozen isolates

and were incubated and passed in their appropriate medium for up to 24-48 hrs at 37°C under either microaerophilic, anaerobic or aerobic conditions as appropriate.

Preparation of bacterial LPS and lipid A (Crude LPS TRI-reagent extraction)

LPS-lipid A from each strain of Gram-negative bacteria was extracted using the TRI-reagent extraction protocol. (Yi and Hackett, 2000) Briefly, 50 mg of lyophilized whole bacterial cells were suspended in 1mL of TRI-reagent. Complete mixing of this initial suspension was accomplished by vortexing and sonication as needed. Samples were then allowed to incubate for 15-30 minutes at room temperature to completely homogenate. After incubation, 200 uL of chloroform was added and the mixture was vortexed again and subjected to further incubation at room temperature for 15-30 minutes. Aqueous and organic phase separation was then accomplished by centrifugation. Only the aqueous phase, which contains the LPS complex, was removed and saved. Repeated LPS extractions from the organic phase was conducted by adding endotoxin free distilled water to the original remaining organic phase, vortexing, incubation and centrifugation as before. All additional aqueous phase separation was then removed and combined with the first aqueous extraction, frozen and lyophilized (Freezone 4.5, Labconco Corp. Kansas City Mo.).

Lipid A isolation

For all the bacterial samples, lipid A was isolated using the technique described by Caroff. (Caroff, *et al.*, 1988) Briefly, crude TRI-reagent LPS-lipid A samples were

resuspended in sodium acetate, in the presence of SDS, heat and low pH, to effectively cleave the lipid A from the residual LPS. Next, after being frozen and lyophilized, these dried samples were further purified by repeated washes in acidified and non acidified ethanol. Purified lipid A samples were then frozen again, lyophilized, weighed and stored at -20°C.

Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis

Analysis using MALDI-TOF techniques require ionization and separation of bio-molecules, based on their mass-to-charge ratios. In brief, these bio-molecules from different Gram-negative bacteria were mixed with a laser absorbing organic matrix which facilitates crystallization in a spatial array on a stainless steel plate. These samples were then exposed to a fixed, pulsed laser beam. The energy from the laser beam is absorbed by the matrix which results in ionization and liberation of a portion of the sample. Ions from this sample travel through a flight tube to a detector during which time differences in mass-to-ionic charge ratios result in their separation. Results from multiple laser pulses are collected electronically and are then converted from time of flight (TOF) measurements into mass:charge values. Calibration to known standards then allows for analysis and profiles of characteristic mass-spectral profiles of the samples. For the purpose of this work, this information served as a “library” of lipid A spectral profiles for direct comparison between and within species of Gram-negative bacteria. Additionally, modification and manipulation of bacterial growth variables were employed to create specific spectral profiles for further evaluation. For example, altering the hydrolysis

times during the cleaving of lipid A from LPS or adding/deleting specific media components in certain bacteria has shown to modify the characteristic spectral profile of the bacteria itself. Those samples that indicated changes or modification of the lipid A were then applied to host assays for analysis of effect. Negative ion MALDI-TOF mass spectral analysis was utilized to determine individual lipid A spectral profiles from each sample discussed in the preliminary data section. (Shah, *et al.*, 2002; Yi and Hackett, 2000) Here, spectral analysis was acquired in the negative-ion mode using a Bruker Autoflex-III reflectron time of flight mass spectrometer (Bruker-Franzen, Bremen, Germany) as detailed by Yi and Hackett. (Yi and Hackett, 2000) 5-Chloro-2-mercaptobenzothiazole (CMBT, Aldrich Chemical Co., Milwaukee, WI) was dissolved in a mixture of chloroform and methanol (1:1 v:v). Typically, lipid A samples were prepared for MALDI-TOF analysis by depositing 2.5 μ L of a premixed solution containing the lipid A sample and CMBT onto the sample plate and allowed to air dry at room temperature. Mass ion values are described in m/z units and have routinely been rounded to the nearest whole number in both text and figure for ease of description.

Host Assays: (HUVEC E-selectin expression assay)

Host activation assays have been used routinely to describe the effects of endotoxic properties of bacteria. Here, LPS preparations from different bacteria will be examined for their ability to directly stimulate E-Selectin (via an ELISA based assay) from human umbilical vein endothelial cells (HUVEC). The basic protocol for the HUVEC E-selectin expression assay was described by Darveau *et al.* and was followed. (Darveau, *et al.*,

1995) Briefly, HUVECs were plated on fibronectin-precoated 96 well plates the day prior to the stimulation. On the day of the assay(s) purified bacterial lipid A was suspended in M-199 stimulation media at the concentrations indicated in the figures. All lipid A suspensions and combination suspensions were premixed, at their appropriate concentrations, prior to adding to the HUVEC monolayer. After a 4 hr incubation interval, HUVEC cells were rinsed, fixed in glutaraldehyde, and blocked overnight. The following day, anti-E-selectin antibody (R&D systems) was applied to each well followed by the application of a goat anti-mouse immunoglobulin G-specific horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Labs, West Grove, PA) after non specific blocking and incubation of the primary antibody. A chromogen reagent system (3,3', 5,5'-trimethylbenzidine dihydrochloride in substrate buffer, Genetic Systems, Redmond, WA) was employed and the reaction was stopped with 0.1 mL of 1 N H₂SO₄ per well. The 96 well ELISA plate was read in an ELISA reader spectrophotometer (Kinetic microplate reader, Molecular Devices Corporation, Sunnyvale, California) at a dual end 450-650 nm wavelength.

RESULTS

Identification and characterization of intrinsic *P. gingivalis* heterogeneity

To determine the extent of heterogeneity within *P. gingivalis*, MALDI-TOF analysis of lipid A samples of different laboratory strains of *P. gingivalis* as well as low passage

clinical isolates was performed. Laboratory strains of *P. gingivalis* (33277, MP4-504, W83, 7476) as well as 4 clinical *P. gingivalis* isolates cultured from separate patients, were grown in TYHK media supplemented with fatty acids plus hemin and menadione. All samples were grown under anaerobic conditions. Bacterial cells were grown in liquid culture then harvested, frozen and then lyophilized. LPS-lipid A was then extracted as described in the Research and Design section. MALDI-TOF analysis revealed the presence of the m/z 1435/1450 complex as well as the m/z 1690/1770 mass ion complex in each of the laboratory as well as the low passage clinical isolates tested. **(Figure 1.1: A)** In all of the *P. gingivalis* samples, the major mass ion grouping was either the m/z 1690 complex (corresponding to a mono-phosphorylated penta-acylated lipid A or the m/z 1435/1450 complex (corresponding to a mono-phosphorylated tetra-acylated lipid A). In addition to the presence of the aforementioned major mass ion peaks, the presence of minor adjacent sub-peaks that surround the major peaks were also observed in each of the samples. Although the majority of the samples contained the same major and minor spectral groups there were some unique differences. For example, *P. gingivalis* 33277 and 7476 contain a mass ion peak at m/z 1440 that was not routinely found to be present in either MP4-504 or W83. In addition MP4-504 contains a significant peak at m/z 1480 whereas the other laboratory strains do not. Interestingly, the clinical isolates obtained from dental patients contained the majority of the major and minor mass ion peaks described but revealed specific differences that might assist in strain or isolate identification for *P. gingivalis*. **(Figure 1.1: B)** The most interesting finding, however,

was the presence of multiple forms of lipid A within all laboratory and clinical isolates of *P. gingivalis*.

Characterization of other Gram-negative lipid A of selected bacteria as compared to *P. gingivalis*

To determine the major mass ion characteristics of other oral Gram-negative bacteria, MALDI-TOF analysis was again employed. Bacterial samples were selected based on strength of association with periodontal disease as described by Socransky et al.

(Socransky, *et al.*, 1988) Here, lipid A from bacteria strongly associated with periodontal disease were directly compared to lipid A samples from bacteria that were not normally associated with diseased states. Perhaps the most interesting finding, after MALDI analysis of other oral bacteria, was that the majority of oral bacteria possessed the larger penta and even hexa-acylated mono or diphosphorylated disaccharide lipid A. **(Figure 1.2)** In addition, there was minimal heterogeneity within these samples, as evidenced by the majority of oral lipid A spectra displaying only one major mass ion grouping, as compared to the spectra seen with the *P. gingivalis* laboratory and clinical isolates.

(Figure 1.1: A, B)

To standardize, each of these bacteria were grown up in their respective media and the LPS-lipid A was extracted using the same (TRI-CAROFF) technique used for the *P. gingivalis* samples. (Note: The majority of these Gram-negative bacteria use the same basal ingredients for growth therefore the differences in growth media for each of these samples were minimal) In addition, additional experimental LPS/Lipid A extractions and

MALDI-TOF profiles of *Veillonella* grown in three different types of medias revealed no significant differences in the spectral profiles (data not shown). Consequently, the basal experimental media was standardized as much as possible to allow for assay consistency while allowing for proper growth of the different bacterial samples.

Two of the oral Gram-negative bacteria screened displayed a surprisingly similar lipid A profile when compared directly to the *P. gingivalis* samples. *P. intermedia* ATCC 25611 as well as *B. fragalis* ATCC 25285 displayed similar higher and lower major mass ion groups indicating the presence of a tetra as well as a penta-acylated, mono phosphorylated lipid A for *B. fragalis* and both a mono and diphosphorylated, tetra and penta-acylated lipid A for *P. intermedia*. **(Figure 1.3)** In addition, the same minor adjacent “sub-peaks” (with the same or very similar mass ion values) were also observed in these two bacteria as was recorded in the laboratory as well as the clinical isolates of *P. gingivalis*. Furthermore, it was also noticed that the *major* mass ion for these bacteria was either centered around or was below m/z 1690 which, being similar to *P. gingivalis* but differed considerably to the other oral Gram-negative bacteria tested, confirms that there is substantial variability (number of fatty acids or phosphate groups) within lipid A structure from species to species. **(Compare Figure 1.3 to Figure 1.2)**

Although routine TRI-CAROFF procedures resulted in successful extraction of lipid A from a variety of oral bacteria, we were unable to successfully extract lipid A (even after multiple attempts) from two samples, *T. forsythensis* ATCC 43037 as well as *E.*

corrodens ATCC 23834 using the TRI-CAROFF extraction technique. The significance of this observation is unknown at this time.

Host Assay: hTLR4-E-Selectin

TRI-extracted LPS samples from pathogenic and non-pathogenic Gram-negative were applied to the HUVEC E-selectin assay which, as a cell line, contains basal hTLR4 expression. Interestingly, those bacteria possessing lipid A (i.e. *P. gingivalis*, *P. intermedia* and *B. fragalis*) whose mass ions centered around m/z 1690 and contained a lower grouping centered at or near m/z 1450, responded with lower E-selectin stimulation than comparable amounts of LPS from those bacteria that contained only one high mass ion dominant lipid A moiety. **(Figure 1.4)** These differences were reflected in additional dose response assays (data not shown) as well but was significant at the $1\ \mu\text{g/ml}$ data set as reported here. This suggests that bacterial species that contain lower lipid A species and increased heterogeneity, might potentially harbor *intrinsic* innate host hTLR 4 modulation ability. Although it is recognized that small amounts of residual lipoproteins may exist within these full structure LPS samples, (that could have presumably have gone undetected due to limitation in the sensitivity of currently used assays to detect small amounts of protein) similar results have been seen with a subsample of highly purified lipid A preparations extracted from these LPS samples. In addition, even if present, contaminating lipoproteins are thought to activate innate cells through a hTLR2 dependent manner which would be absent in this particular cell line. (Muzio and Mantovani, 2001)

DISCUSSION

MALDI-TOF analysis of *P. gingivalis*

One of the first studies discussing the structural features of lipid A suggested that *P. gingivalis* strain 381 possessed limited lipid A structural heterogeneity with LPS preparations consisting of only a tri-acylated monophosphorylated form with a negative ion mass of m/z 1195 (Ogawa, 1993). Later, Kumada et al. reported additional *P. gingivalis* lipid A structural isoforms from a clinical isolate, however, in this particular study, certain forms predominated including a tetra- and penta-acylated monophosphorylated lipid A with molecular mass ion(s) of m/z 1435, m/z 1449 and m/z 1690 respectively, as well as a minor m/z 1770 mass ion. (Kumada, *et al.*, 1995) These structures are depicted in **Figure 1.5** and differ from the canonical *E. coli* lipid A structure as depicted in **Figure B2** in the number of phosphates, and the number or length as well as the position of the fatty acid chains linked to the lipid A disaccharide. The reasons for the apparent discrepancies in the predominant type of *P. gingivalis* lipid A found between these two studies was not clear at the time of their publication. Recently, two studies from our laboratory confirmed that the majority of *P. gingivalis* lipid A isoforms previously reported (m/z 1195, 1435, and 1450) (Bainbridge, *et al.*, 2002), as well as others (m/z 1690 and 1770) (Darveau, *et al.*, 2004), were present in the same purified LPS preparations of *P. gingivalis* strain 33277.

The results obtained from this experimental procedure, using a standardized extraction procedure (TRI-CAROFF) to evaluate the extent of heterogeneity within *P. gingivalis* confirmed the presence of multiple forms of lipid A within all laboratory strains and clinically obtained isolates tested and disproved the theory that heterogeneity was unique to a single strain or subset of *P. gingivalis* isolates. **(Figure 1.1)** This result occurred regardless of sample origin (wild type isolates vs. laboratory strains). Each of the clinical isolates was grown from frozen aliquots taken from dental patients with varying stages of periodontal inflammation (gingivitis through chronic periodontitis). The laboratory strains represent four commonly used ATCC strains that have been widely reported within the literature and ATCC 33277 has been shown to be indistinguishable from strain 381 using Multilocus Enzyme Electrophoresis (MLEE) and DNA fingerprinting. (Loos, *et al.*, 1993) It was therefore interesting that the mono-phosphorylated tri-acylated lipid A, as described by Ogawa using strain 381 (Ogawa, 1993), was apparently absent in all of the *P. gingivalis* samples tested. Furthermore, the minor mass ion peaks adjacent to the major mass ion peaks have been identified in other Gram-negative bacteria (Que-Gewirth, *et al.*, 2004) and are likely due to the addition or subtraction of carbon units to the ends of the fatty acid chains that add additional heterogeneity to the overall lipid A structure.

MALDI-TOF comparison of other oral Gram-negative lipid A

Little is known about the structure of lipid A from the majority of oral Gram-negative bacteria that inhabit the mouth. Gas Chromatography-Mass Spectrometry (GC-MS) data

suggest that most oral bacteria contain lipid A with 3-hydroxylated and nonhydroxy fatty acids with varying chain length (C12-C17) in either ester or amide linkages. (Mashimo, *et al.*, 1985) *Actinobacillus actinomycetemcomitans* serotype “A” ATCC 43717, for example has 3-hydroxytetradecanoic acid as the main amide-linked fatty acid with the presence of 3-hydroxytetradecanoic acid as well as non-hydroxy tetradecanoic acid in ester linkage with tetradecanoic and hexadecanoic acid in secondary substitution creating a mass ion value of approximately m/z 1826. **Figure 1.6** displays a proposed lipid A structure calculated from reported fatty acid composition (Mashimo, *et al.*, 1985) and our experimentally observed MALDI-TOF analysis indicating a diphosphorylated, hexa-acylated lipid A with the predominate mass ion at m/z 1827. The mass ion value of m/z 1746 can be accounted for chemically with the loss of one of the phosphate groups at the C1 or C4' positions. *F. nucleatum* ATCC 22586 contains 3-hydroxyhexadecanoic as the main amide-linked fatty acid with the presence of 3-hydroxytetradecanoic acid as well as non-hydroxy tetradecanoic acid in ester linkage with tetradecanoic and hexadecanoic acid in secondary substitution creating a mass ion value of approximately m/z 1881. **Figure 1.7** displays a proposed lipid A structure calculated from reported fatty acid composition (Hase, *et al.*, 1977; Mashimo, *et al.*, 1985) and our experimentally observed MALDI-TOF analysis also indicates a diphosphorylated, hexa-acylated lipid A. MALDI-TOF results obtained from these bacteria as well as the majority of the lipid A samples from other oral Gram-negative bacteria also revealed the apparent lack of structural heterogeneity, as exemplified by *P. gingivalis*, by the absence of lower mass ion peaks that would mathematically equate to the loss of certain phosphate groups or specific fatty

acid chains in either primary or secondary substitution. Another interesting finding was that the presence of the major mass ion grouping observed for the majority of the samples was well above that observed for *P. gingivalis* confirming that some of oral Gram-negative bacteria were hexa-acylated rather than penta or tetra-acylated like *P. gingivalis*. We are unaware of any literature in regard to the chemical analysis of lipid A structure for *Campylobacter rectus*, *Capnocytophaga ochracea* and *sputigena* or *Veillonellaceae* therefore our MALDI-TOF analysis represents novel information concerning the overall lipid A structure of these oral bacterial species and apparent lack of structural heterogeneity.

MALDI-TOF analysis from *B. fragalis* and *P. intermedia* indicated that there was some lipid A structural similarities to *P. gingivalis* which would not be unexpected since these bacteria are closely related. However, in the case of *B. fragalis* as well as *P. intermedia*, the majority of the major mass ion peaks were reduced by approximately 15-30 mass ions, indicating that the enzyme responsible for adding hydrocarbon units to the acyl chains was set to a slightly different base number. For example, in *E. coli*, the acyltransferase responsible for placing carbons on the fatty acid chains is set to an even number, (ie. C₁₄) and there are different acyltransferase enzymes that incorporate either higher (C₁₆) or lower (C₁₂) even numbers of carbon acyl chains, each specific for that number and possessing different rates of incorporation. (Olsen and Roderick, 2001; Wyckoff, *et al.*, 1998) However in *Pseudomonas aeruginosa*, the enzyme is set to measure 10 hydrocarbon units (Dotson, *et al.*, 1998) which creates a much different lipid

A structure when compared directly to *E. coli*. (**Figure B2**) The proposed chemical structure for *P. intermedia* ATCC 25611 and *B. fragalis* ATCC 25285 are represented in **Figures 1.8 and 1.9**, respectively. The absence of one ester bound phosphate group at either the C4' position has been previously reported for *B. fragalis* which has been linked to low endotoxic activity.(Weintraub, *et al.*, 1989)

Furthermore, being that the *major* mass ion was centered around or was below m/z 1690, indicates that these bacteria share similar lipid A structure characteristics (penta and tetra-acylated lipid A) that apparently make these species unique within the realm of oral Gram-negative bacteria. At present, it is not known if *oral* Gram-negative bacteria have evolved the means to purposefully modify structures within its LPS through enzymatic activity or whether this observation indicates some labile pattern of chemical bonds existing with the lipid A molecules themselves that are just simply different between bacteria. Either way, the resulting structural changes that do or could occur as a result of environmental changes or enzymatic activity would have significant effects on a host response system which has been germ-line encoded to recognize highly specific bacterial structures or patterns.

HUVEC data

It has been well documented that the lipid A structure required to produce the highest or most potent bioactivity(Kotani, *et al.*, 1985; Takada and Kotani, 1989; Takada and Kotani, 1992; Takahashi, *et al.*, 1987), include: (1) a β , (1'-6)-linked D-glucosamine

disaccharide backbone; (2) phosphorylation at positions 1 and 4' on the disaccharide unit; (3) an appropriate number (two was determined to have maximum effect) (Shimizu, *et al.*, 1987) of 3-acyloxyacyl groups, with the position of these 3-acyloxyacyl groups on either the reducing GlcN I or distal GlcN II residues of the disaccharide (Takada and Kotani, 1992); and (4) an appropriate number (usually 6) of hydrophobic fatty acid chains with a suitable carbon length (C₁₂-C₁₈) (Kumazawa, *et al.*, 1988; Nakatsuka, *et al.*, 1989) with or without hydroxylation. **(Figure B2)** Interestingly, these structures, which yield the most potent endotoxic effects, are highly conserved within enteric and non-enteric Gram-negative bacteria and are often referred to as the canonical lipid A structure. Furthermore, alteration in any of the canonical structure, such as changing the number, position, or length of the primary or secondary acyl groups, or removal of the phosphate or monosaccharide groups results in dramatic alteration of biologic effects (Loppnow, *et al.*, 1989; Schumann, *et al.*, 1996; Takada and Kotani, 1992).

Recent advancements in lipid A research have resulted from work in synthetic preparations. Arguably the most important came when a synthetic lipid A preparation — labeled either compound 506 (Kotani, *et al.*, 1985) or compound LA-15-PP (Kanegasaki, *et al.*, 1986) containing the same lipid A disaccharides, phosphorylation patterns as well as the 3-hydroxy- and 3-acyloxy-tetradecanoyl groups at the C2, C3 and C2', C3' positions as the *E. coli* strain F515 — was shown to exhibit equal to or greater activity than the reference *E. coli* strain in both the rabbit pyrogenicity test and lethal toxicity assays in galactosamine-sensitized mice, as well as the local Shwartzman reaction (Kotani,

et al., 1985). **(Figure B3: A)** More importantly, it was also noted that chemical alteration of these preparations, either by number or length of the acyl chains, phosphorylation pattern (addition or subtraction) or overall chemical symmetry of the lipid A structure resulted in intermediate or low biologic activity within the same biologic assays.

Interestingly, *significant* alteration in the acyl pattern, such as those created in compound(s): IV_A, LA-14-PP or 406, which represent intermediate precursors of lipid A biosynthesis and contain only four, symmetrically placed (R)-3-hydroxytetradecanoyl (C₁₄-OH) groups, possessed very weak activation properties within mice assays (Kotani, *et al.*, 1985) or, in the case of IV_A, also contained antagonistic properties when mixed with LPS samples on human cells (Golenbock, *et al.*, 1991; Raetz, *et al.*, 1985). **(Figure B3: B)** This dual agonistic and antagonistic effect of compounds such as IV_A, was partially explained by the presence or absence of human Toll-Like Receptor 4 (hTLR4) and MD-2 expressed by the specific target cell type being assayed (Lien, *et al.*, 2000; Poltorak, *et al.*, 2000; Raetz and Whitfield, 2002).

Further complicating the field of LPS research is the growing body of evidence that has suggested that extraction of LPS from Gram-negative bacteria using highly disruptive agents like Phenol (Tsang, *et al.*, 1974) or high concentrations of bases or acids that can disrupt the integrity of some potentially environmentally labile chemical bonds of the lipid A molecule itself. When comparing data from one research group to the next it is difficult to evaluate biologic activity data because different extraction or purification procedures are frequently used which might affect the LPS/lipid A structure. However,

in these experiments presented, each bacterial species underwent the same extraction and purification procedure which allowed direct comparison of the data. Furthermore, within the HUVEC system, basal expression of hTLR4 without hTLR2 has been reported (Dixon, *et al.*, 2004; Muzio and Mantovani, 2001) with the E-selectin molecule as a reporter of signaling activation. This eliminates the potential of additional TLR expression (i.e. TLR2) which could be activated from lipoproteins contained within the whole LPS structure but separate from the lipid A molecule.

E-selectin expression, with a subset of oral bacteria responding considerably lower than the majority of the other oral LPS(s) tested, becomes more intriguing when one considers the fact that *P. gingivalis* and *P. intermedia* have been highly associated with periodontal disease states whereas the majority of the others have moderate to low associations with disease (Socransky, *et al.*, 1998; Socransky, *et al.*, 1988). (**Figure 1.4**) One could speculate that if a bacterium, under significant environmental stressors, could manipulate its own structural features to dampen host identification and response, then presumably these bacterial species would gain a considerable advantage — compared to those without these adaptive capacities — within a competing bacterial consortium. In theory, those bacteria whose lipid A structural features mimic *E. coli* lipid A in terms of numbers or type of fatty acids, phosphate groups, or secondarily substituted 3- acyloxyacyl groups would be predisposed to increased recognition and host response, *via* pattern recognition, leading to bacterial clearance. On the other hand, lipid A from certain bacteria possessing modified features might result in altered identification by the same pattern

recognition system within innate cells, leading to an absence of clearance by the host, ultimately resulting in a chronic state of infection.

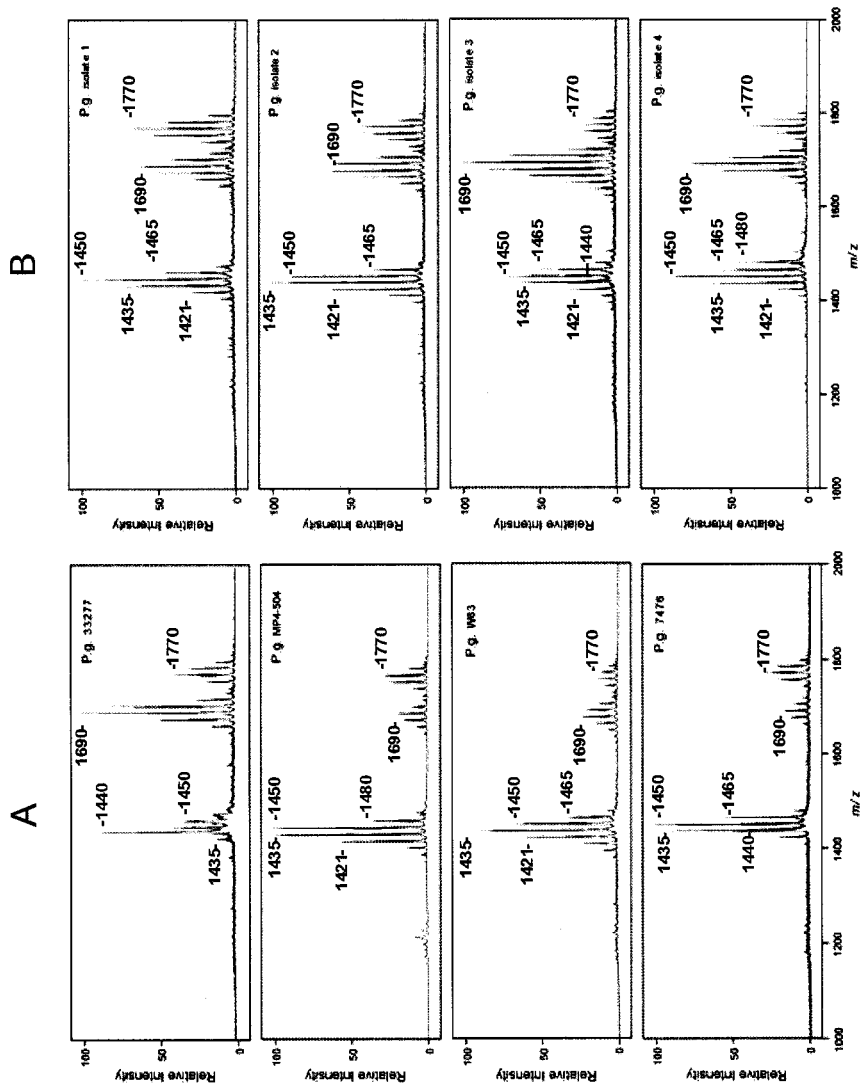


Figure 1.1: (A) MALDI-TOF characterization of different laboratory strains of *P. gingivalis*. (B) Different low passage clinical isolates of *P. gingivalis* from dental patients with gingival inflammation ranging from gingivitis to periodontitis. A general pattern of 2 separate major mass ion groupings were observed (m/z 1435-1450/1690-1770) and was consistent between all samples surveyed with minor additional differences highlighted in red.

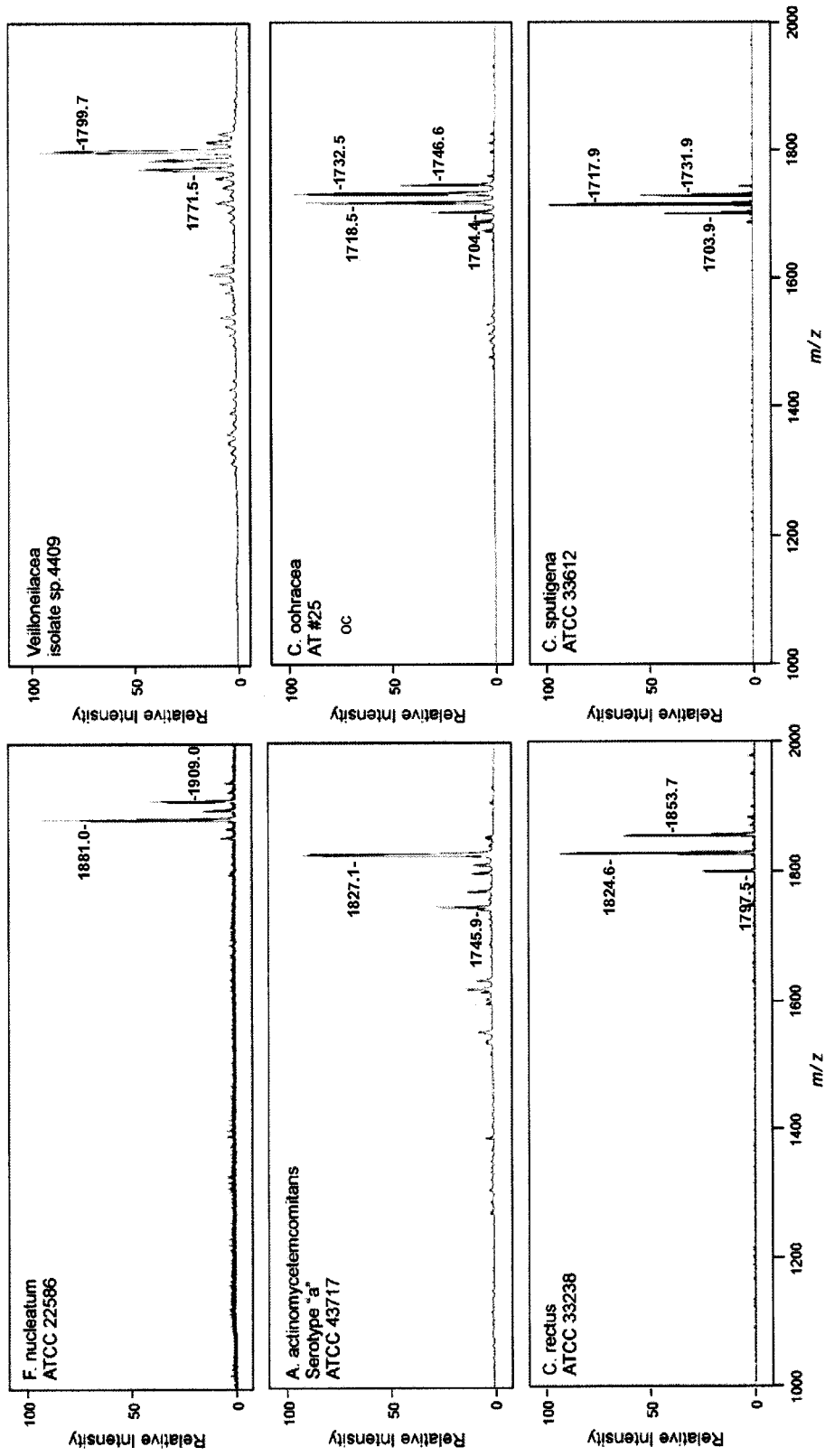


Figure 1.2: MALDI-TOF characterization of other common oral gram negative bacteria reveal the presence of a dominate mass ion grouping that clusters at the high end of the spectrum range. These results suggest the presence of a dominate penta or hexa-acylated lipid A structure with reduced heterogeneity when compared to *P. gingivalis*.

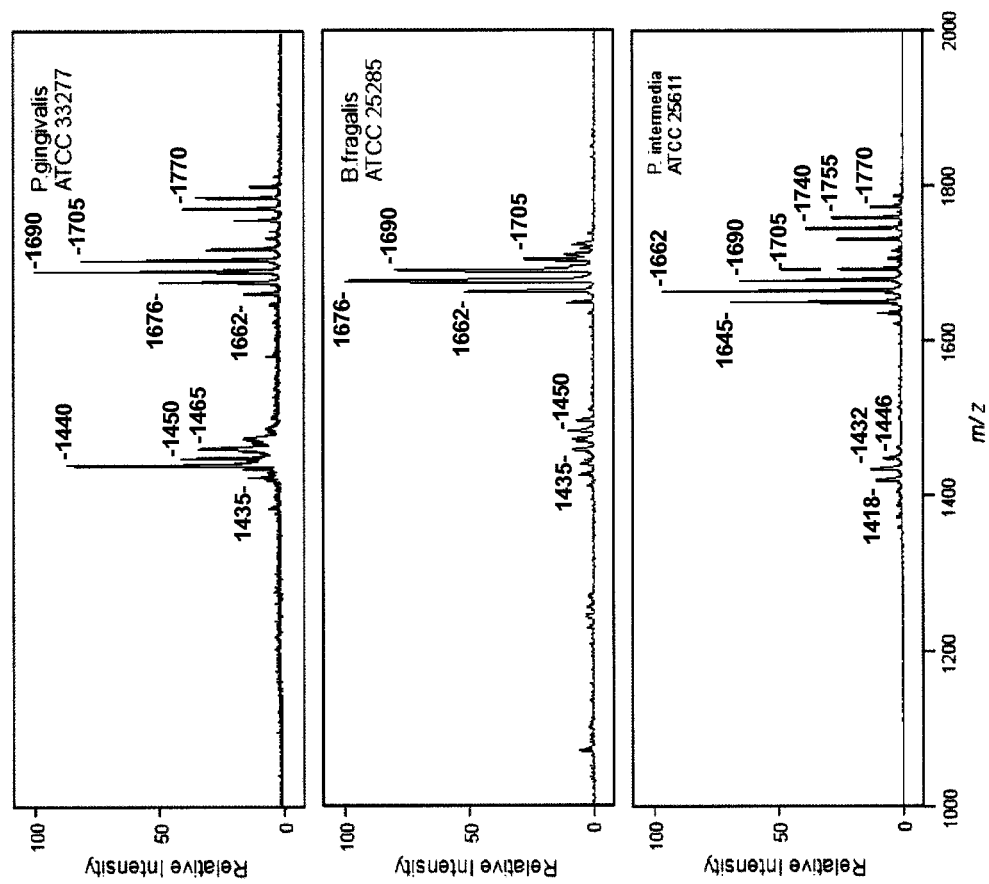


Figure 1.3: MALDI-TOF characterization of *P. gingivalis* ATCC 33277 compared to *B. fragilis* ATCC 25285 and *P. intermedia* ATCC 25611. The same general *P. gingivalis* pattern of 2 separate major mass ion groups (m/z 1435-1450/1690-1770) was also observed within *B. fragilis* and *P. intermedia* as well as a dominate mass ion peak centered around m/z 1690.

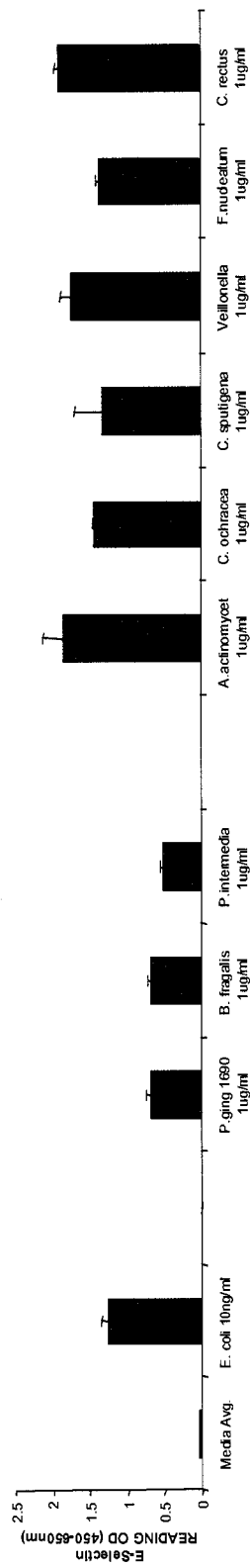


Figure 1.4: E-selectin assay using LPS from various oral Gram-negative bacteria. Results suggest that specific oral bacteria whose lipid A structure consists mainly of upper mass ions (similar to *E. coli*) with little heterogeneity are more potent in terms of E-selectin expression than those oral bacteria with lower mass ions and increase lipid A heterogeneity.

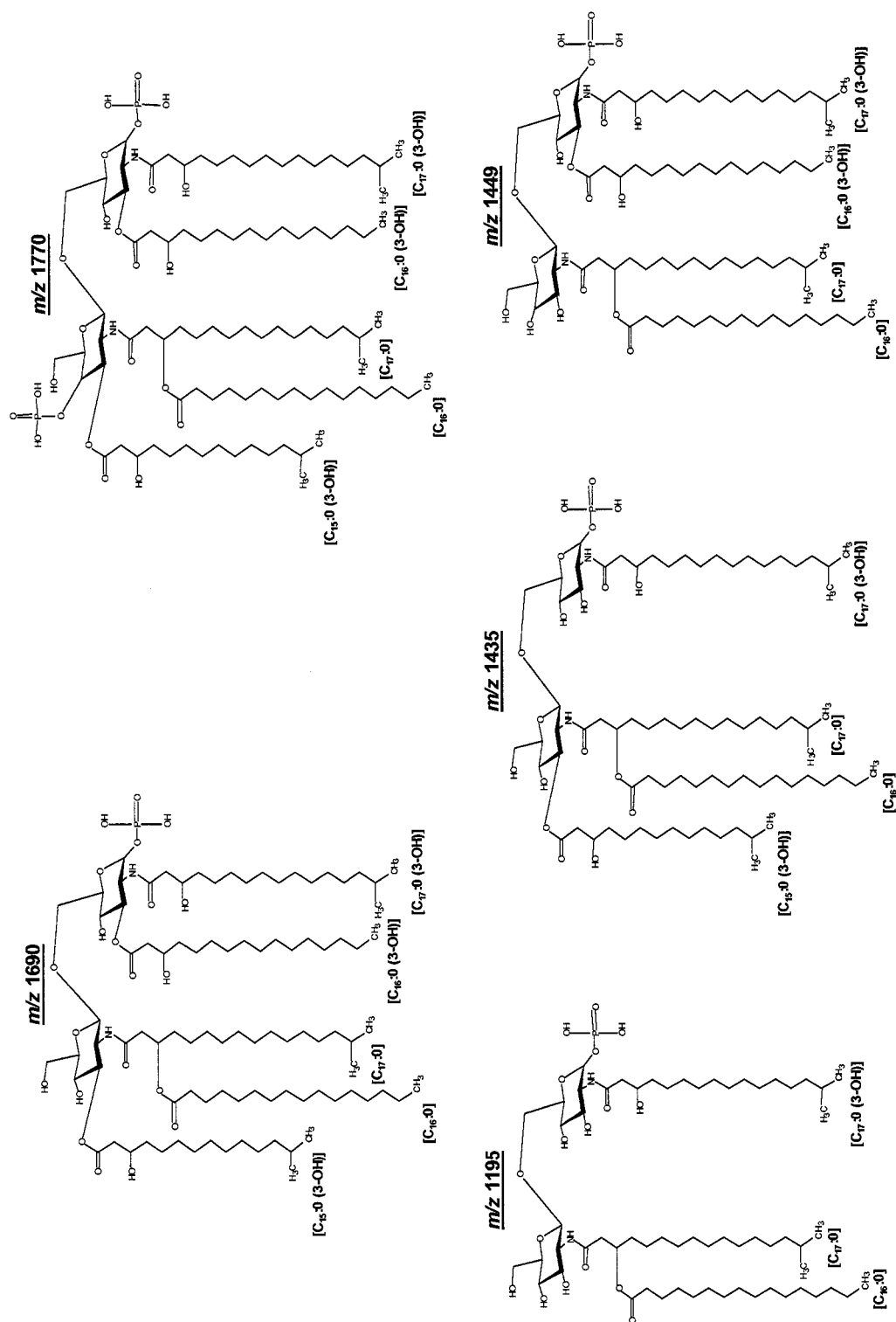


Figure 1.5: Chemical structure and associated MALDI-TOF mass ion values (m/z) of lipid A isoforms reported for *Porphyromonas gingivalis*

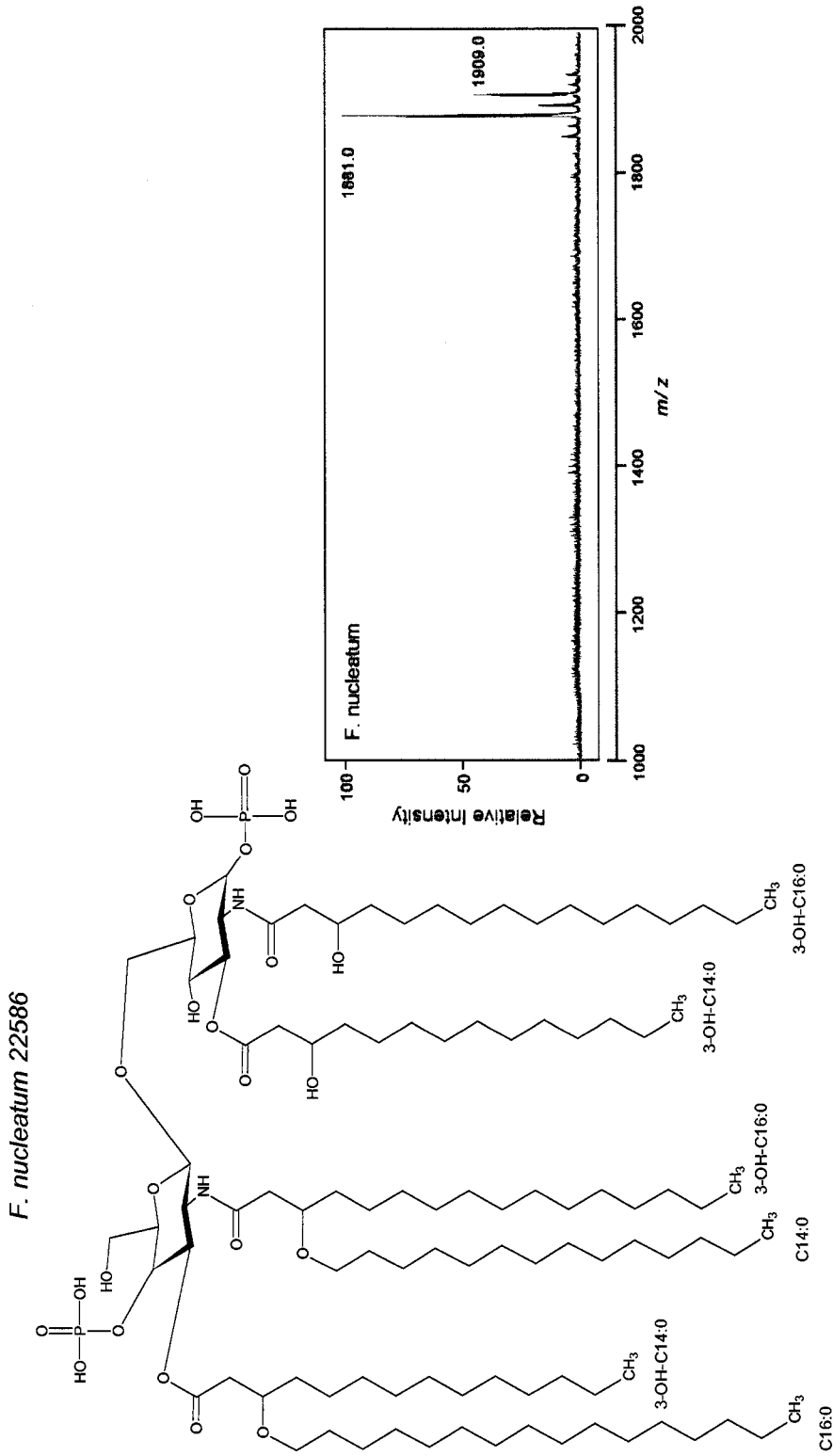


Figure 1.7: A proposed chemical structure calculated from associated MALDI-TOF mass ion values (m/z) of lipid A isoforms observed and GC-MS data reported for *Fusobacterium nucleatum*. (Reference Mashimo et al. *Microbiol Immuno* 1985; 29(5): 395-403.

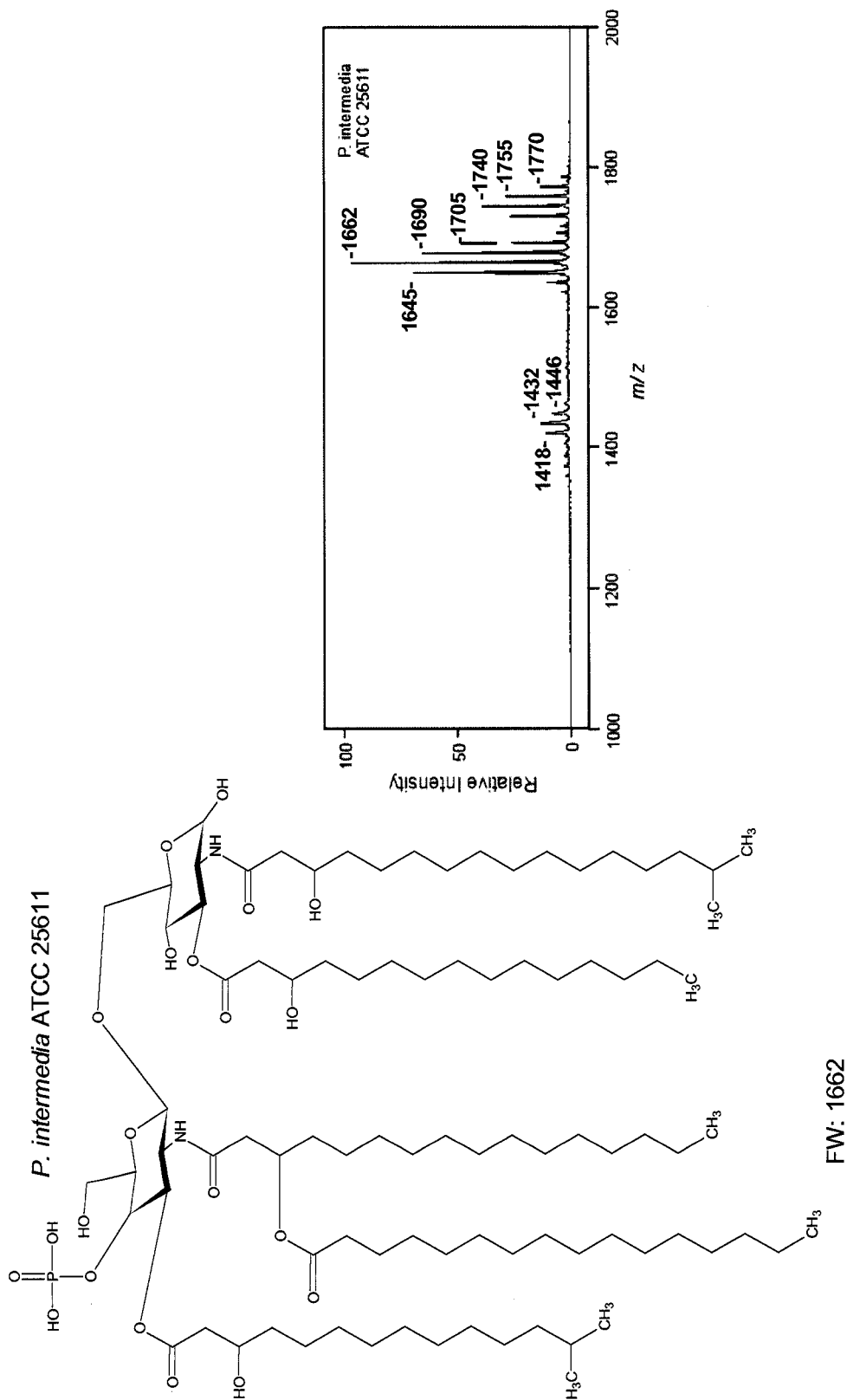


Figure 1.8: A proposed chemical structure calculated from associated MALDI-TOF mass ion values (m/z) of lipid A isoforms observed and GC-MS data reported for *Providentia intermedia*. (Reference Hashimoto et al. *FEBS letters* 543 (2003) 98-102.

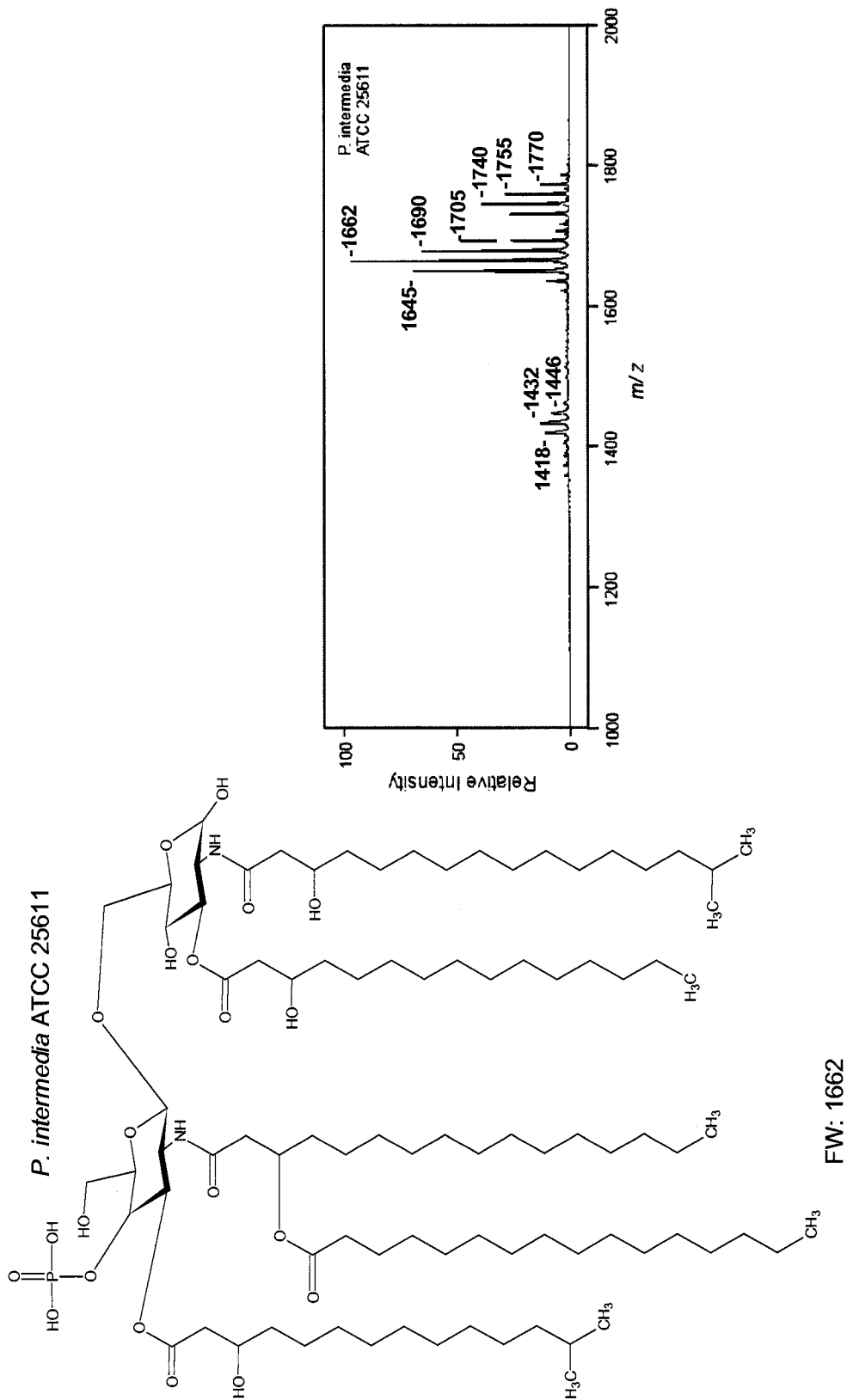


Figure 1.8: A proposed chemical structure calculated from associated MALDI-TOF mass ion values (m/z) of lipid A isoforms observed and GC-MS data reported for *Providentia intermedia*. (Reference Hashimoto et al. *FEBS Letters* 543 (2003) 98-102.

CHAPTER 2: Environmental affects on

P. gingivalis lipid A structure

INTRODUCTION

The most prevalent natural habitat for *P. gingivalis* within humans is the oral and nasopharyngeal environment with the gingival sulcus being the major site of colonization. (Tanner, *et al.*, 1992) Although early identification of *P. gingivalis* was hindered due to taxonomic groupings with other *Bacteroides* species and difficulty in isolation due to specific and restrictive anaerobic culturing identification techniques, inflamed oral tissue has proven to be the best reservoir for the presence and proliferation of the species, *P. gingivalis*. (Rietschel, *et al.*, 1992) Because of the high isolation prevalence within inflamed and diseased gingival tissues, *P. gingivalis* has also been associated with multiple forms of periodontal disease. However, *P. gingivalis* has also been isolated from clinically healthy tissue, such as buccal mucosa, tongue epithelium and tonsils indicating that this particular organism has evolved mechanisms to survive in multiple oral locations and environmental conditions.

P. gingivalis survival *in situ*, as with any microorganism, is dependent on growth which, in turn, is regulated and dependent on a range of host environmental factors and conditions that the microorganism must continuously adapt to. It has been reported that *P. gingivalis* growth both *in vitro* and *in vivo* is environmentally fastidious, being

affected by factors such as: binding to solid surfaces or other bacteria, substrate or cation concentrations, oxygen tension and redox potentials, as well as local pH and temperature. In general, rapid microbial response to changing environments is essential for the survival of the organism resulting in reports of phenotypic alteration within many bacterial species in all types of ecosystems. (Marsh, *et al.*, 1993) Taking advantage of this fact, specific environmental parameters can therefore be manipulated during the *in vitro* growth of *P. gingivalis*, allowing for evaluation of the resulting physiologic alterations or changes that occur within the microorganism.

In the present study, three separate environmental conditions were selected and specific alterations were constructed to examine the possibility that environmental changes or fluctuations alter lipid A structure of *P. gingivalis*. It has been estimated that *P. gingivalis* growth within the oral cavity is likely to progress at relatively slow rates. (Marsh, *et al.*, 1993) Therefore, the first set of experiments were designed to determine the effects of exponential growth vs. stationary growth, as well as post stationary growth on lipid A structure. Due to the fact that *P. gingivalis* most commonly grows attached to other bacteria within a biofilm superimposed on either oral tissue or surfaces of the teeth, a second group of experiments were devised to characterize the lipid A changes that might occur if the bacterium was allowed to grow in liquid culture vs. bacteria grown on a solid medium. The third group of experiments evaluated the lipid A effects of bacteria grown in different pHs conditions that might mimic *in situ* pH disturbances seen in chronic *P. gingivalis* infections or inflamed epithelial tissue. As detailed below, each of

these *in vitro* experimental systems resulted in alteration of lipid A structure. The results also suggest that within the limitations of batch culture, specific physiologic events occurred, such as significant pH alteration which has been reported to occur *in vivo* and has also proven to significantly alter multiple virulence factors possessed by *P. gingivalis*. The data within this Chapter represents a potential link between environmental conditions and lipid A structure that is novel for *P. gingivalis* and supports a new body of evidence for natural lipid A heterogeneity that is potentially occurring within the oral environment.

MATERIALS AND METHODS

Growth phase experiments

To determine the effects of experimental growth conditions on lipid A in *P. gingivalis*, preliminary growth phase experiments were performed. Here, early logarithmic phase growth or logarithmic phase growth was compared to stationary and post stationary growth followed by LPS extraction, lipid A separation and finally, MALDI-TOF analysis of the lipid A profiles. Specifically, *P. gingivalis* MP4-504 was grown in trypticase soy broth 30g/L (Bectin, Dickinson BBL) and supplemented with: yeast extract 5g/L (Difco), sodium lactate 1mL/L (Sigma), sodium succinate 0.5gm/L (sigma), sodium fumarate 1.0gm/L (Sigma). Hemin (Sigma) and menadione (Sigma) were added from a 10 mL and 0.2 mL stock solution to a final concentration of 0.005 gm/L and .001 gm/L respectively

(TYHK + FA). For early/logarithmic and stationary growth samples displayed in **Figure 2.1, A**, frozen stocks of pure isolates were thawed and TSA blood agar plates (Remel, Lenexa, KS USA) were inoculated and samples were allowed to grow in an anaerobic environment at 37° C for 3-5 days. Two 10ml tubes containing the selected media were then inoculated under the same environmental conditions and allowed to grow for 2 days to achieve an OD₆₆₀ value of 2.0. This culture was then used to inoculate an additional 180ml bottle which achieved an OD₆₆₀ reading at 18-24 hours. This volume of culture (approx 200mls) was then used to inoculate the next media sample to a final volume of 1 L (each inoculum averaged 10% of the next higher volume) and harvested at the indicated time point according to OD₆₆₀ values. For post stationary growth samples as displayed in **Figure 2.1, B**, the experimental procedure described above was followed however each liquid growth was extended to 48-72 hours and confirmed by OD₆₆₀ values to ensure bacteria were in post-stationary growth phase (minimum of 72 hours growth) before being used as an inoculum for the next batch culture. Final experimental samples were then harvested at different time points which are indicated by the red arrows in **Fig 2.3, A**. These arrows correspond to logarithmic (4-20 hours of growth), stationary (22-30 hours of growth), and post-stationary growth phases (52-100 hours of growth) predetermined (OD₆₆₀ readings and bacterial counts) and reconfirmed with additional growth curve experiments using OD₆₆₀ readings. Routinely, for these experiments, optical density (OD₆₆₀) was used to confirm phase of bacterial growth. Gram-staining and CO₂ and anaerobic check plates were performed for each phase as well as additional trypsin and fluorescence assays from selected check plates to ensure pure cultures. Crude

LPS was extracted using the phenol / guanidinium thiocyanate in aqueous solution (TRI-reagent) based extraction reagent as described in Chapter 1. Lipid A was further isolated and purified from these LPS samples using the Caroff procedure and samples were analyzed using MALDI-TOF as further described in Chapter 1.

Plate vs planktonic growth

Liquid Media consisted of the standard liquid trypticase soy broth plus fatty acid basal media with the addition of hemin and menadione (TYHK + FA). pH was adjusted to 7.2 as previously described. Agar plates A portion of the liquid media was saved and to it, 15g/L of agar (ex BBL 12304 agar) was added before autoclaving. Media + agar was then allowed to cool to 58° C and equilibrated in a water bath. Once equilibrated, this media + agar was then poured into polystyrene plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) and allowed to cool. Media plates were then sealed and stored at 4° C until ready for use.

To answer the question whether stationary plate growth of *P. gingivalis* would alter the lipid A profile, *P. gingivalis* 33277 and MP4-504 were first grown in the liquid culture to an appropriate optical density indicating pre-stationary phase (1.6-1.8 OD₆₆₀). These initial samples served as a baseline for comparison and were harvested, washed, frozen and lyophilized. Next, before harvesting, live culture samples from these harvests were used to inoculate 20 agar spread plates containing the same basal media as was used in the liquid culture. (**Figure 2.2**) These *P. gingivalis* samples, obtained from the liquid

growth, were then allowed to grow on solid surface agar plates until an adequate growth was achieved (spread lawn confluency) for harvest (approx 4-5 days growth). Growth on agar plates occurred in the same anaerobic environment as the liquid cultures. Finally, samples from these spread plates were used to inoculate new liquid media, grown to the same optical density as the first liquid growth samples and harvested. Spectral analysis of lipid A patterns were performed and profiles characterized as described in the preliminary results section

pH experiments

P. gingivalis MP4-504 was initially selected for this experiment. Frozen stock isolates were used to inoculate TSA blood agar plates and grown for 3-5 days as described above. 750 ml of TYHK + FA media supplemented with hemin and menadione as previously described was used as the growth medium and aliquoted into three 250 ml samples and then the pH was adjusted with 1N HCL or 1 N NaOH to 6.8, 7.2, 8.0, respectively. Samples were then further aliquoted into 10 ml tubes and labeled according to initial pH. These media aliquots were then autoclaved, cooled and then placed in an anaerobic chamber (5% CO₂, 5% H₂, 90% N₂, Coy anaerobic chamber, Coy laboratory Inc, Ann Arbor MI) to reduce for 12 hrs (overnight). The pH was recorded after autoclaving and reducing step for each sample. *P. gingivalis* isolate MP4-504 was then used to inoculate each aliquot creating parallel sets for each pH sample. These samples were then allowed to grow for up to 72 hours. OD₆₆₀ values as well as sample pH were recorded during the 24, 48 and 72 hour mark. Inoculated media samples that were used for pH determination

were first centrifuged at 1000 rpm for 2 min (Beckman GS-GR Centrifuge, USA) to remove gross cellular debris before pH probe was inserted.

To determine the effect of pH on lipid A structure, *P. gingivalis* isolates 33277 and MP4-504 were chosen and initially grown from frozen isolates as described. Here 2 L of TYHK + FA media supplemented with hemin and menadione as used previously was adjusted to either an initial pH of 6.5, 7.2, 8.2 or 9.0. This media was autoclaved and reduced according to the above protocol. All subsequent media was created and pH adjusted in the same manner. Final samples were harvested in pre-stationary phase (approximately 14-18hrs growth and OD₆₀₀ verification) and samples were washed and lyophilized as described. LPS and lipid A was extracted using the TRI-CAROFF technique and lipid A structure was analyzed via the MALDI-TOF analysis protocol.

RESULTS

Effect of growth phase on lipid A profile

P. gingivalis strain MP4-504 and 33277 were grown and described previously. Samples were then harvested at different time points as indicated by the red arrows in **Figure 2.3, A**. These arrows correspond to logarithmic, stationary and post-stationary growth phases predetermined and reconfirmed with growth curve experiments. Optical density (OD₆₆₀) was used to confirm phase of bacterial growth during these experiments. MALDI-TOF

analysis of MP4-504 lipid A components is depicted in **Figure 2.3, B**. Here, during logarithmic growth, when the bacterium is growing very rapidly and surrounded by an environmentally rich media, three major mass ion grouping exist (centered around m/z 1450, 1690 and 1770) which correspond to a mono-phosphorylated tetra-acylated and penta-acylated lipid A and a di-phosphorylated penta-acylated lipid A, respectively. Relative intensities of these mass ion groupings suggest that the majority of the lipid A is mono-phosphorylated tetra-acylated followed by mono-phosphorylated penta-acylated lipid A and then di-phosphorylated penta-acylated lipid A. At stationary growth, mono-phosphorylated tetra-acylated and di-phosphorylated penta-acylated lipid A are at equal intensities with a reduction of mono-phosphorylated penta-acylated lipid A. Finally, during post stationary growth, di-phosphorylated penta-acylated lipid A is the predominate lipid A moiety with almost the complete absence of mono-phosphorylated tetra-acylated and penta-acylated lipid A.

Comparison of plate vs. planktonic growth conditions on lipid A structure

To determine whether stationary plate growth of *P. gingivalis* would alter the lipid A profile, *P. gingivalis* 33277 and MP4-504 were first grown in the standard liquid TYHK + FA basal media then used to inoculate solid media plates. Once grown on solid surfaces, samples were used to inoculate fresh liquid media. Bacteria were harvested during each growth condition and resulting lipid A structure was evaluated via MALDI-TOF. The results indicated that a unique MP4-504 (m/z 1480) peak was altered during this environmental growth changes. (**Figure 2.4**) Specifically, during liquid growth

conditions a peak at m/z 1480 appears that has not been characterized in any other *P. gingivalis* species to date. Interestingly, when this liquid grown bacteria is transferred to immobile plate growth (mimicking oral *in vivo* growth) this peak all but disappears. Furthermore, upon return to liquid culture, we then see a reemergence of the m/z 1480 peak. **(Figure 2.4)** Results also suggest that the mass ions centered around m/z 1770 (corresponding to di-phosphorylated, penta-acylated lipid A) is a significant moiety in the liquid phase, in terms of relative intensity, when compared to a 50% reduction in intensity of this mass ion when the bacterium is grown on a solid surface. Furthermore, monophosphorylated, penta-acylated lipid A (m/z 1690) increased 50%, in relative intensity, during solid media growth when compare to initial liquid and subsequent liquid growth phases. **(Figure 2.4)**

Effect of environmental pH changes on lipid A profile

Experimental results revealed that pH values dropped immediately after normal autoclaving at 121°F for 21 minutes, then again after reducing in the anaerobic chamber overnight. **(Figure 2.5)** The average pH drop for all samples was 3.6% after autoclaving and 6.0% after reducing as calculated from initial pH adjustments. Here, the more acidic the baseline the less pH drop during autoclaving and reducing procedures whereas the more basic the starting pH the larger the pH drop. **(Table 2.1)** Once inoculated, pH values for each sample began to rise during the 24, 48 and 72 hour timepoints. Interestingly, the more acidic pH environment resulted in the largest alkaline shift compared to neutral and alkaline baseline mediums. **(Figure 2.5 and Table 2.1)**

Specifically, the more acidic baseline media rose 13.8% to achieve a final pH of 7.4 compared to a 10.9% and 6.9% rise in pH from the neutral and basic mediums achieving a final pH of 7.6 and 7.8 respectively. (**Table 2.1**)

To determine if more basic medium conditions altered lipid A structure, two different pH adjusted mediums (7.2 and 8.2) were initially compared. This initial range was selected to ensure maximal growth from reported pH ranges reported for *P. gingivalis*. (Marsh, *et al.*, 1993) MALDI-TOF analysis revealed the potential drop of the mass ion peaks at m/z 1690 as well as m/z 1770 within samples grown in the more basic medium when compared to the standard neutral 7.2 pH adjusted medium. (**Figure 2.6**)

Using this information in conjunction with the reported optimum pH growth range for *P. gingivalis* (Marsh, *et al.*, 1993; McDermid, *et al.*, 1988), the next experiment utilized two specific starting pH adjusted mediums, 6.5 and 9.0 to determine if more extreme pH ranges were required for LPS or lipid A alteration. Care was taken to choose two extreme pH ranges that would still permit adequate growth. Calculations made from **Table 1** suggested that after autoclaving and media reduction within the anaerobic chamber, initial pH adjusted media of 9.0 and 6.5 would reduce to approximately 8.1 and 6.2 respectively which is within the reported pH range for *P. gingivalis*. (Marsh, *et al.*, 1993) Results displayed in **Figure 2.7** reveal that when *P. gingivalis* 33277 is grown under acidic conditions (initial pH of 6.5), there is a loss of the diphosphorylated, penta-acylated lipid A species located at m/z 1770 and a significant reduction in the m/z

1435/1450 mass ions. In contrast, when *P. gingivalis* 33277 is grown in more basic conditions (initial pH of 9.0), there is a large increase in tetra-acylated lipid A as evidenced by the substantial increase in the m/z 1435/1450 mass ion complex and complete absence of any penta-acylated lipid A at m/z 1690 or 1770.

DISCUSSION

Affect of Growth phase on LPS

Researchers have reported that extracellular vesicle formation, a normal bacterial byproduct of cell walls, was increased in some bacterial species during slow or inhibited growth rates induced by stress conditions created during experimental or *in vivo* growth limitations. (Minhas and Greenman, 1989) Similarly, bacterial growth rate has been shown to affect both the specific activity of different bacterial enzymes, such as trypsin, chymotrypsin and collagenase, as well as their location and distribution in various cell fractions. (McDermid, *et al.*, 1988; Minhas and Greenman, 1989) Changes in environmental substrate availability can alter the growth rate which can, in turn, affect the enzyme profile of the organism. Additional enzymes like deacylases or acyl-transferases, produced by bacterial cells, can alter lipid A structure by adding or removing selected fatty acid chains and could also be affected by bacterial growth rates and/or substrate availability fluctuations during bacterial phases of growth. Therefore, it

was hypothesized that LPS, being a major component of the cell wall, might be altered during similar growth limiting conditions or stressors that affect bacterial enzymes. Initial MALDI-TOF results suggest that during logarithmic growth lower mass ion groups (centered around m/z 1450) are produced and represent a dominate spectral group. **(Figure 2.3)** During post-stationary growth, when the bacteria have depleted media components and cell numbers are decreasing, a spectral shift occurred in which the upper mass ion groups became predominate. Stationary growth results show an intermediate spectral pattern of lower and upper mass ion groups.

Interestingly, additional experiments in which post-stationary bacteria were continuously passed (representing a continued long phase growth pattern) showed reversal of this spectral shift to a more evenly distributed spectral profile. **(Figure 2.1, C)** This initial data might indicate a bacterial induced lipid A modification relative to environmental signals such as cell numbers or duration of growth. Such structural changes in lipid A could arise from specific enzymes responsible for adding a specific number of carbons to the fatty acid chains linked to the lipid A sugar groups, such as lipid A acyltransferase(s) produced by the bacterial cell. In other words, if one specific acyltransferase is faster or less inhibited than another, in creating specific fatty acid lengths, then one lipid A spectral profile might be dominate during different phases of growth. However, although specific lipid A structural changes may occur during environmental changes, care must be exercised in evaluation of MALDI-TOF results due to its limitations in quantitative assessment by revealing only relative intensities of particular lipid A mass ions.

Therefore, additional experiments to include gas chromatography mass spectrometry (GC-MS) analysis of the amounts of each of these fatty acid chains will be performed (in relation to an internalized standard) to determine the exact total of one lipid A species vs. another within these respective samples.

Effect of solid vs. liquid growth on lipid A structure in P. gingivalis

In vivo, *P. gingivalis* grows stationary on a biofilm surface that has been previously established by specific oral bacteria. (Socransky and Haffajee, 2002) Standard microbial growth procedures to generate adequate amounts of bacteria for processing require that *P. gingivalis* be grown in liquid media and not on a solid agar surface. However, *P. gingivalis* does not grow naturally in a liquid environment (*in vivo* data). In the oral environment, *P. gingivalis* is a late colonizer of the oral biofilm, usually requiring a preexisting, immobile bacterial colonization for binding purposes. Within this biofilm, it has been suggested that nutrient transport is altered and surface exposure is different between solid surface growth compared to liquid growth leading to phenotype changes within the bacterium itself. (Stoodley, *et al.*, 2002). Furthermore, bacteria have evolved ways to release and perceive chemical signals (autoinducers) allowing cell to cell “communication” regarding environmental conditions and this phenomenon has been termed “quorum sensing.” Quorum sensing autoinducers also reveal information regarding cell density within the local environment, therefore, quorum sensing regulated gene expression might be altered due to lower density of cell growth on plate vs. liquid growth. (Socransky and Haffajee, 2002) Results from the plate vs. planktonic growth

experiment suggest that alteration in the lipid A profile has occurred and, in the case of the m/z 1480 mass ion, increases in this peak would correspond to the *lpxA* addition of an extra carbon to one of the fatty acid chains in the tetra-acylated lipid A species that was first described by Kumada.(Kumada, *et al.*, 1995) Relative intensity changes observed in the m/z 1690 peaks could potentially indicate a labile state of one of the attached phosphate groups to the disaccharide core of the lipid A. Any alteration in fatty acid number, distribution or length of fatty acids, or number of phosphate groups attached to the lipid A sugars has shown to alter the toxicity of this molecule.(Silipo, *et al.*, 2002; Takada and Kotani, 1989) These experimental observations may potentially indicate that lipid A modification, by the bacterium, may be linked to environmental signals from growth on a solid surfaces similar to naturally occurring biofilm formation and growth *in vivo*.

Effects of *in vivo* pH on growth and lipid A structure

Bacterial growth is affected by many biophysical factors, pH being one of the most critical. In fact, extreme pH ranges have been shown to dramatically affect cell surface structures in *Salmonella* and was suggested to be a major regulator of virulence within clinical isolates.(McDermid, *et al.*, 1996) However, most bacterial species only grow within a vary narrow range of pH, with the majority growing optimally around neutral pH values (pH 7.0). (Costilow, 1981) The reported pH range for *P. gingivalis* is 6.3-8.3 with 7.5 determined as the value for optimum growth.(loos, *et al.*, 1993; Marsh, *et al.*, 1993). From the experimental results displayed in **Table2.1**, it is interesting that regardless of

the starting or intermediate pH values, all samples achieved a final pH value after 72 hours growth that averaged to 7.6, which is in agreement with the previously reported optimal growth pH for *P. gingivalis*. These initial experimental results confirmed that *P. gingivalis* possesses the ability to significantly modify environmental pH factors, in batch culture, to ensure maximum growth.

In the mouth, it has been reported that the pH of healthy sulci is approximately neutral (Bickel and Cimasoni, 1985; Forscher, *et al.*, 1954) and becomes more alkaline during gingival inflammation. (Eggert, *et al.*, 1991; Marsh, *et al.*, 1993) (Bickel and Cimasoni, 1985) (Borden, *et al.*, 1977) It has been suggested that asaccharolytic or non-fermentative microorganisms, metabolize lactic acid during growth and produce bases like ammonia in the process. (Bickel and Cimasoni, 1985; Borden, *et al.*, 1977) *P. gingivalis* is one such microorganism, requiring lactic acid as a media component for growth. Additional alkaline shifts could occur during protein catabolism within the gingival crevicular fluid (by proteases from certain bacteria, such as *P. gingivalis*) which generates carbon dioxide and amino groups as metabolic products. (Bickel and Cimasoni, 1985) pH values displayed in **Figure 2.5** revealed that *P. gingivalis*, grown in the same media with different initial starting pH values, created an alkaline shift during all phases of growth. This affect occurred immediately upon inoculation and seemed to counteract the initial pH drop seen after autoclaving and media reduction.

Deepening sulcular depth, due to loss of junctional epithelial integrity and attachment to the tooth and tooth root surface, coupled with alveolar bone loss, increased gingival

crevicular fluid and connective tissue destruction seen during inflammatory disease states significantly disturbs the local periodontium. This dramatic environmental alteration results in altered patterns of nutrients available for metabolic consumption by bacteria. This places significant pressure on bacteria currently residing within the sulcus to adapt to these changes for survival. Those bacteria that can adapt have a selective advantage over others less capable species. *P. gingivalis* has been shown to produce a large repertoire of other virulence factors like cytotoxins and enzymes capable of tissue destruction that have been shown to be influenced by environmental pH conditions.(McDermid, *et al.*, 1988; Slots and Genco, 1984)

Results from the described pH experiments suggest that within different isolates of *P. gingivalis*, environmental conditions that are more basic, in terms of pH values, might potentiate the effects of a deacylase-like enzyme that would produce a lipid A structure that is predominately tetra-acylated as compared to the penta-acylated lipid A structure normally found at neutral pH values. In agreement with this, the potential for the deacylase enzyme to exist with the genome of *P. gingivalis* was recently identified by our laboratory. (Unpublished data) In addition, when the pH in culture medium is reduced, MALDI-TOF analysis reveals the loss of a phosphate group from the core lipid A disaccharide suggesting the presence of a natural phosphatase activity or the labile nature of this bond to acidic conditions. These results suggest an exciting link between environmental conditions or host responses and lipid A structure that have been recently described in other Gram-negative bacteria (Ernst, *et al.*, 1999) or host immune

cells.(Erwin and Munford, 1990) The following Chapter (Chapter 3) deals with altered host responses towards changes in lipid A structure from biologic preparations of *P. gingivalis* as well as other oral Gram-negative bacteria.

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Table 2.1 pH alteration during growth of *P. gingivalis* MP4-504. Calculated percent change in pH within culture media for *P. gingivalis* during growth. (A) Indicates growth phase using OD₆₀₀ values for each sample with different starting pH media values. (B) Reveals the pH reduction that occurred during normal autoclaving and media reducing procedures as well as the increase in pH values for all samples during *P. gingivalis* growth.

	Initial pH		
	6.8	7.2	8.0
After autoclaving ^a	↓ 1.6%	↓ 2.8%	↓ 6.3%
After O/N reducing ^a	↓ 4.4%	↓ 4.9%	↓ 8.6%
24hr growth ^b	↑ 6.2%	↑ 5.9%	↑ 1.4%
48 hr growth ^c	↑ 7.7%	↑ 8.0%	↑ 4.1%
72hr growth ^d	↑ 13.8%	↑ 10.9%	↑ 6.9%

^a: Before the presence of bacterial inoculum.

^b: After bacterial inoculum and growth continued for 24 hours before harvest.

^c: After bacterial inoculum and growth continued for 48 hours before harvest.

^d: After bacterial inoculum and growth continued for 72 hours before harvest.

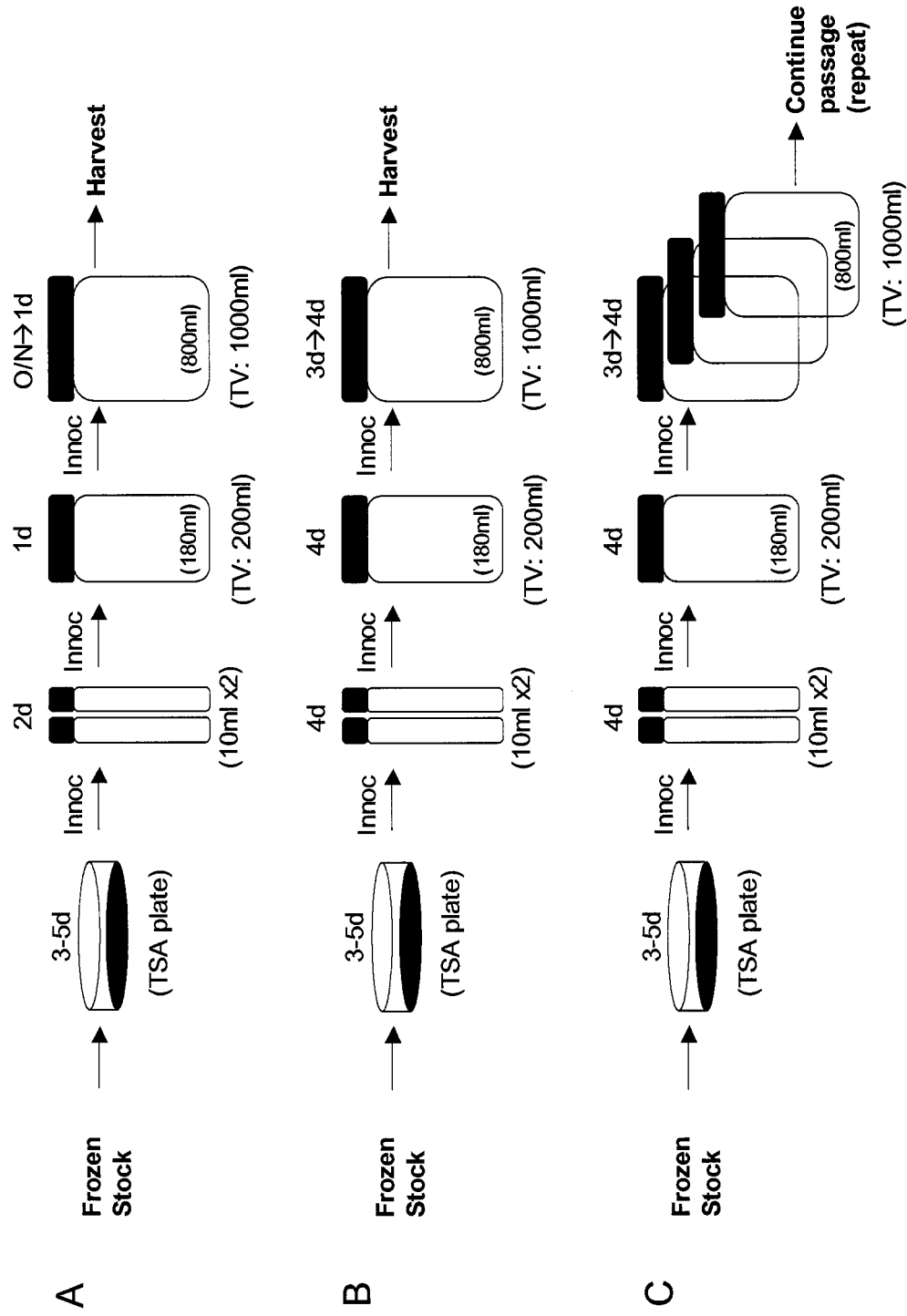


Figure 2.1: (A) Basic procedure for inoculation of liquid media from frozen aliquots for *P. gingivalis*. (B) Post stationary growth inoculation passage protocol. (C) Repeat passage of long growth phase inoculum protocol

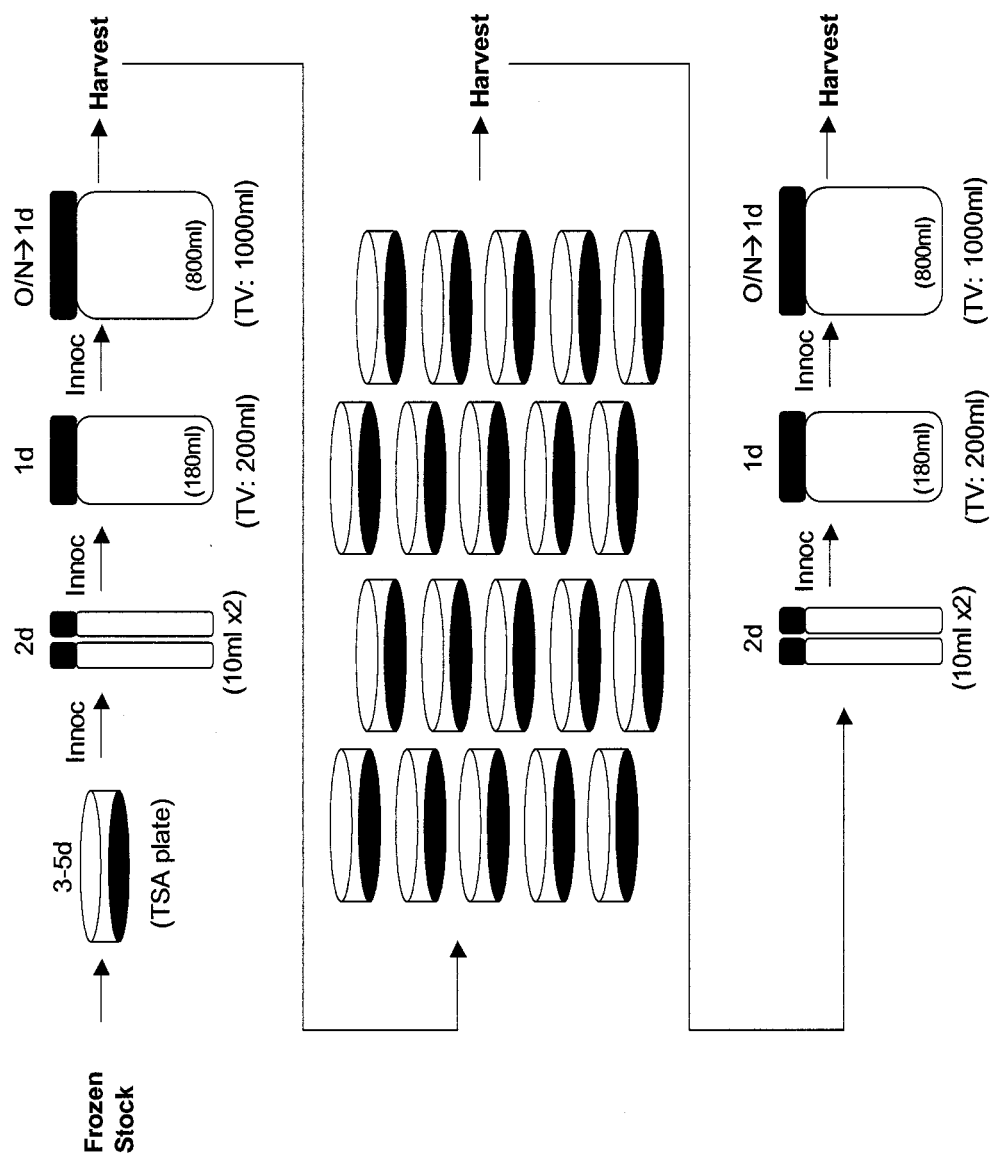
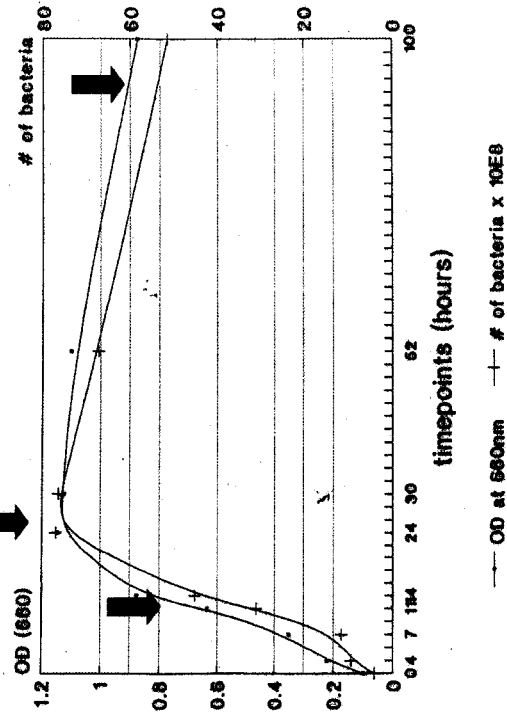


Figure 2.2: Protocol for inoculation of solid media after initial growth in liquid culture. A pure culture from the solid media plates was then used to re-inoculate additional liquid media. Samples from each growth condition were harvested and evaluated for structural alteration of lipid A.

A

P. gingivalis growth curve

Pg growth curve



B

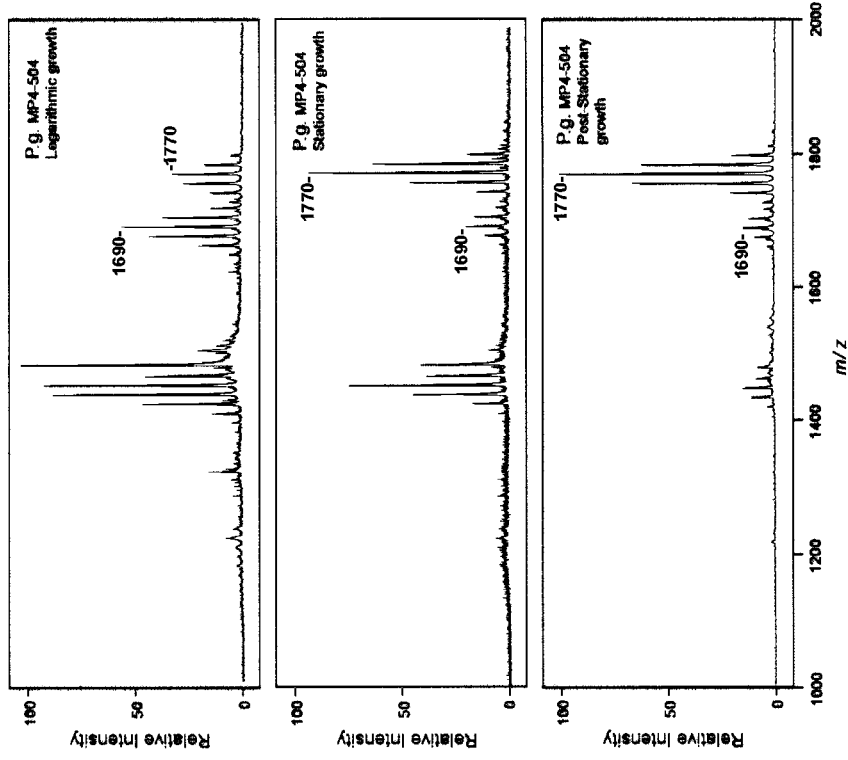


Figure 2.3: *P. gingivalis* growth curve and MALDI-TOF characterization of different growth phases. (A) *P. gingivalis* MP4-504 was grown and harvested during log, stationary and post-stationary growth phases. [red arrows] (B) MALDI-TOF characterization of lipid A during these growth phases suggest a shift towards higher acylated lipid A species the longer batch culture is allowed to grow.

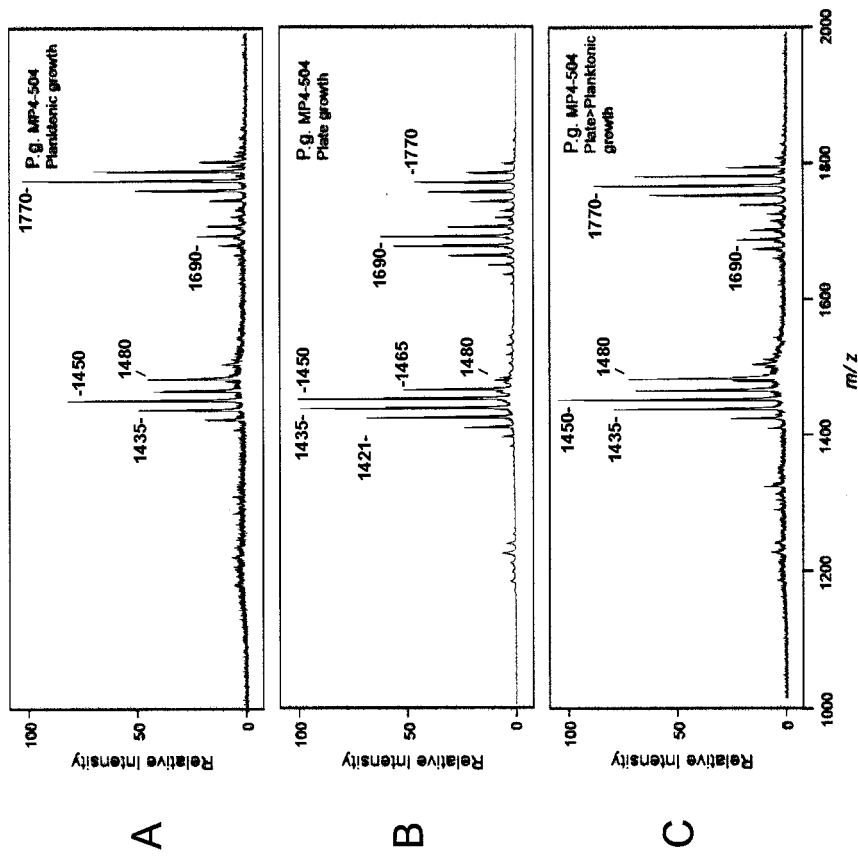


Figure 2.4: MALDI-TOF characterization of *P. gingivalis* MP4-504 grown under different culture conditions (planktonic vs. plate growth). (A) *P. gingivalis* MP4-504 was grown in liquid culture and characterized as described. Notice the presence of a significant peak at m/z 1480. (B) An inoculum from this liquid growth was then used to seed plates made with the same media used for liquid growth. This resulted in a substantial drop in the peak intensity for m/z 1480 as well as the m/z 1770 upper mass ion complex (C) Reappearance of the m/z 1480 peak after an inoculum from the plate growth was used to seed fresh liquid media as well as a return in the pattern for the upper m/z ion peaks.

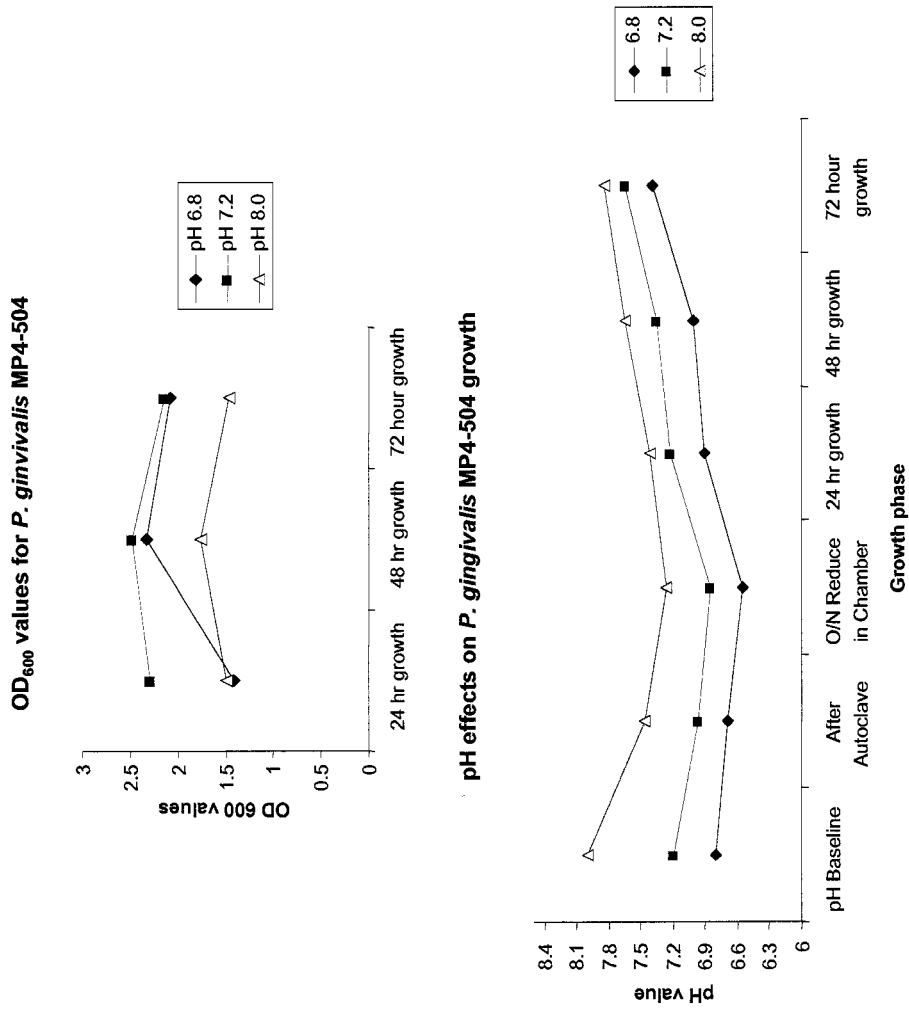


Figure 2.5: pH effects within culture media for *P. gingivalis* during growth. (A) Indicates growth phase using OD₆₀₀ values for each sample with different starting pH media values. (B) Reveals the pH reduction that occurred during normal autoclaving and media reducing procedures as well as the increase in pH values for all samples during *P. gingivalis* growth.

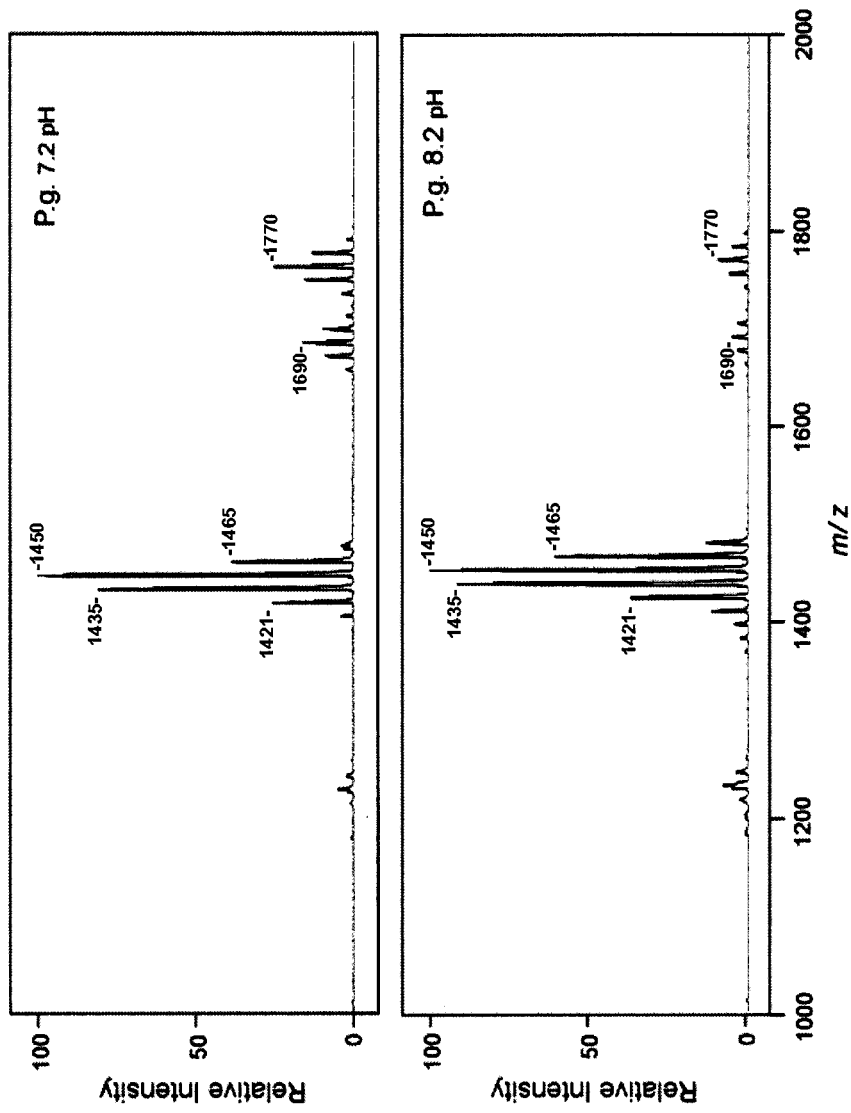


Figure 2.6: MALDI-TOF characterization of *P. gingivalis* MP4-504 grown under different pH conditions. *P. gingivalis* MP4-504 grown in initially higher pH conditions resulted in reduction of both the m/z 1690 as well as the m/z 1770 complex corresponding to a reduction of penta-acylated lipid A at basic pH values.

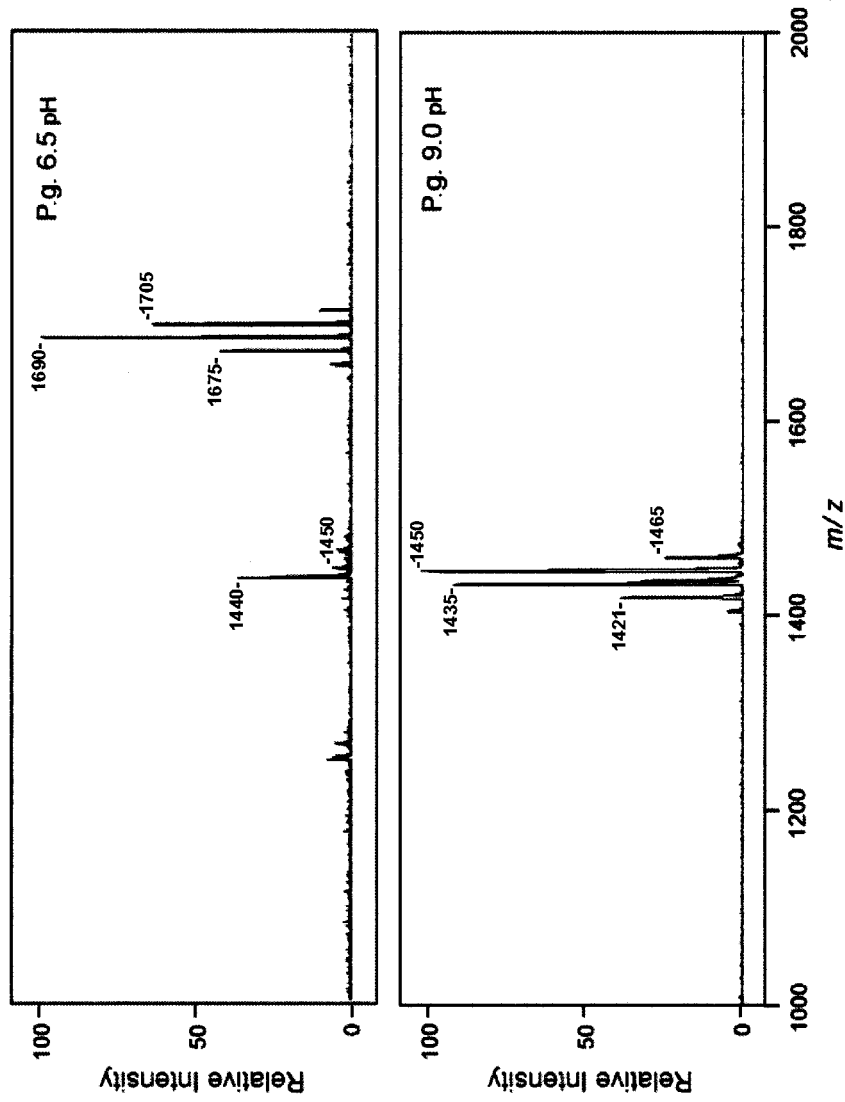


Figure 2.7: MALDI-TOF characterization of *P. gingivalis* 33277 grown under different pH conditions. *P. gingivalis* 33277 grown in acidic conditions (initial pH 6.5) resulted in the increase in relative intensity of the m/z 1690 mass ion cluster with the loss of the m/z 1770 cluster and significant drop of the m/z 1435/1450 mass ion complex. In contrast, *P. gingivalis* 33277 grown in basic conditions (initial pH 9.0) revealed the absence of any penta-acylated lipid A (evidenced by the loss of m/z 1690/1770 mass ion complexes) and the relative increase in tetra-acylated lipid A (m/z 1435/1450).

CHAPTER 3: Selective hydrolysis affect on lipid A structure and biologic function

INTRODUCTION

One of the most interesting findings in the field of oral microbiology is the observation that that LPS and its isolated lipid A component, obtained from Gram-negative oral anaerobic bacteria, do not elicit host responses in a manner consistent with the responses observed with the classic *E. coli* type endotoxin. (Darveau, *et al.*, 1995; Fujiwara, *et al.*, 1990; Mansheim, *et al.*, 1978; Pulendran, *et al.*, 2001) For example, early studies examining endotoxin lethality in mice demonstrated that LPS obtained from Gram-negative oral anaerobic bacteria was significantly reduced in potency when directly compared to enterobacterial LPS (Isogai, *et al.*, 1988; Takada and Galanos, 1987). Specifically, LPS from oral Gram-negative bacteria did not induce a Shwartzman reaction, (Fujiwara, *et al.*, 1990; Mansheim, *et al.*, 1978) a systemic innate immune inflammatory response typically associated with *E. coli* type endotoxin (Takada and Kotani, 1992). Observations such as these have resulted in this type of LPS being classified or designated as having no or very low biological activity.

One explanation for this lowered activity could be due to the number of phosphate groups or altered lengths and positions of the fatty acids present in lipid A from certain oral bacteria compared to enteric *E. coli*. These factors have been proven to be critical in

induction of the Shwartzman reaction in animal based assays. (Takada and Kotani, 1989) Currently, little is known about the overall LPS structure of the 300 to 500 different bacterial species present within the oral cavity; therefore, structural analysis of the lipid A portion of the LPS from different oral bacteria, might render unique insight into the differential host responses towards potential structural differences from such a vast array of species.

In an effort to further characterize lipid A structure of *E. coli* and other Gram-negative bacteria, researchers have used mild alkaline hydrolysis techniques to selectively cleave specific fatty acids from the core lipid A structure. For example, ammonium hydroxide has been used to selectively cleave or deacylate ester linked fatty acids usually found at the C3 and C3' positions on the core β -1,6-D-glucosamine. (Silipo, *et al.*, 2002a)

Therefore, chemical reagents such as ammonium hydroxide (NH₄OH) as well as others, were used to selectively hydrolyze ester or amide linked fatty acids from the lipid A in a systematic approach to determine the chemical lability of specific fatty acids on the lipid A core disaccharide, and to clarify the appearance of specific lipid A structures found in extracted preparations.

Results from these experiments described within this Chapter indicate that innate immune host cell activation towards oral bacteria is dependent on specific structures within the lipid A molecule. Specifically, chemical removal of ester-linked fatty acids or dephosphorylation of the core lipid A molecule resulted in significant reduction in

hTLR4 utilization and E-selectin expression. This effect was seen regardless of the bacteria used or its activation pattern before alteration. Furthermore, the experiments performed indicated that partial fatty acid deacylation also resulted in the ability to block unaltered lipid A molecules whereas complete ester linked deacylation resulted in loss of agonistic as well as antagonistic activity.

MATERIALS AND METHODS

Selection of bacterial samples

Samples from the Red, Orange as well as the Green complex, as described by Socransky et al. were used for these studies. (Socransky, *et al.*, 1998; Socransky, *et al.*, 1988) Here *P. gingivalis* MP4-504 represented the Red complex. *P. intermedia* ATCC 25611 represented the Orange complex. *C. ochracea* AT #25 represented the Green complex. Samples were grown according to the protocol in Chapter 1. Additional bacteria such as *P. gingivalis* 33277 as well as *B. fragalis* ATCC 25285 were used as controls or for specific features as in the case of *B. fragalis* which lacks a phosphate group in the C4' position of its lipid A structure. A commercially obtained synthetic *E. coli* lipid A disaccharide (Sigma A-3919, Sigma Chemicals USA) was characterized via MALDI-TOF analysis. This core disaccharide is commonly present in enterobacteria as well as the majority of Gram-negative oral bacteria and served as an experimental control.

(Figure 3.1)

De-O-acylation of Lipid A with ammonium hydroxide (NH₄OH)

In an attempt to hydrolyze specific fatty acids from the core lipid A structure, purified lipid A samples were subjected to mild alkaline hydrolysis as described by Silipo et al. (Silipo, *et al.*, 2002a) Routinely, 200 µg of lipid A from various oral bacteria including *P. gingivalis* MP4-504, *P. intermedia* ATCC 25611 and *C. ochracea* #25 were treated with 200ul of 1 M NH₄OH (Fisher Scientific, Fair Lawn, NJ) at room temperature for various lengths of time, with and without the application of heat (70 °C). Samples were dried under liquid nitrogen and analyzed. To ensure complete removal of residual chemical reagents, separate samples were further purified by cold 0.375 M MgCl₂ and ethanol washes before applying to biologic assays. (Darveau, *et al.*, 2004)

De-O, N-acylation of lipid A with sodium hydroxide (NaOH)

Additional isolated lipid A samples were further subjected to modified strong alkaline hydrolysis as first described by Vinogradov et al. Here, lipid A samples from *P. gingivalis* MP4-504, *P. intermedia* ATCC 25611 and *C. ochracea* #25 were treated with 4 M NaOH (Baker Chemical Co, Phillipsburg, NJ) and incubated at 100°C for 2 hours. (Vinogradov, *et al.*, 2000) Samples were then dried under forced air to reduce the overall volume then reconstituted with 500ul of endotoxin free water. pH of the preparations were neutralized with approximately 110 ul of 1 N HCl. Preparations were air dried to a volume of 150 ul. Desalting was accomplished by passing the preparations through a Sephadex G50 spin column (Pharmacia AB, Uppsala, Sweden). Samples were

further purified by cold 0.375 M MgCl₂ and ethanol washes as described above before applying to biologic assays.

De-acylation of fatty acids in secondary substitution.

Hydrazine treatment was employed to remove fatty acids in secondary esterification.(Aussel, *et al.*, 2000) Here, lipid A samples were first treated with NH₄OH to remove fatty acids in primary esterification. Next, 100ul of anhydrous hydrazine (Sigma-Aldrich, St. Louis MO) was applied to 200ug of pretreated lipid A and the sample was heated at 37°C for 90min with stirring. Two volumes of cold (-20°C) acetone (Sigma-Aldrich, St. Louis MO) was used to stop the reaction. Sample was reduced under forced air and remaining residue was reconstituted with endotoxin free water (Gibco, Invitrogen Corp. Grand Island, NY), frozen and lyophilized. Samples were then analyzed via MALDI-TOF.

Host assays: Human E-selectin expression assay (HUVEC) and hTLR4 utilization (HEK)

The method for the E-selectin expression assay within HUVEC cells was described in Chapter 1. hTLR4 utilization was determined using transient transfection of human embryonic kidney (HEK) 293 cells with the TLR4 activation complex (human TLR4 [hTLR4] plus membrane associated CD14 [mCD14]) as described by Darveau *et al.* (Darveau, *et al.*, 2004) As controls, known TLR4 ligands were used to compare dose response activation, via an NF- κ B-dependent reporter plasmid (ELAM-firefly luciferase), of untreated oral bacteria lipid A compared to NH₄OH treated samples. Specifically,

HEK 293 cells were transiently transfected by calcium phosphate precipitation and stimulated with varying dosages of different lipid A or LPS samples in a 96 well plate assay format. Cells were washed twice with media 3hrs after transfection and stimulated 20 to 24 hrs post-transfection. All stimulations were performed using defined stimulation media (Dulbecco's modified Eagles's medium containing 10% human serum for 4 hrs at 37°C using incremental concentrations (varying by factor of 10s) as indicated in the figures. After stimulation, cells were rinsed with phosphate-buffered saline (Biowhittaker, Walkersville, Md.) and lysed with 50µl of passive lysis buffer (Promega, Madison WI). Dual Luciferase reporter assay system (Promega) was used to measure the gene expression in 10µl of each lysate. Data was then expressed as the fold increase in relative light units (RLUs) corresponding to the ratio of ELAM-luciferase to β -actin *Renilla*-luciferase expression as compared to that of a no-stimulation control.

RESULTS

P. gingivalis MP4-504 lipid A NH₄OH treated time course (1-42 hrs)

P. gingivalis MP4-504 lipid A samples were subjected to NH₄OH treatment for 1 hr, 4hrs, 16hrs, 21hrs and 42hrs without the application of heat. MALDI-TOF analysis of lipid A subjected to NH₄OH for 1 hour revealed a marked reduction of the upper mass ions corresponding to mono and di-phosphorylated, penta-acylated lipid A, as well as the presence of a mass ion peak at m/z 1512 and at m/z 1195 that was previously unobserved

in baseline (untreated) samples. **(Figure 3.3)** Chemical calculation reveals that a mass ion value at m/z 1512 equates to a di-phosphorylated tetra-acylated lipid A that contains a C15:0 (3-OH) at the C3' position and the proposed loss of an ester linked C16:0 (3-OH) at the C3 position. This result also indicates that the C16:0 (3-OH) located in the C3 position, is potentially more acid labile. The m/z 1195 mass ion peak corresponds to a mono-phosphorylated, tri-acylated lipid A as first described by Ogawa (Ogawa, 1993) and is diagrammed in **Figure 1.5**. The relative intensity of mono-phosphorylated, tetra-acylated lipid A (m/z 1435/1450) appears to be unaffected. At 16 hours, mono and di-phosphorylated penta-acylated lipid A is eliminated, creating the m/z 1195 and 1512 mass ion as well as a new mass ion at m/z 1272. This new mass ion equates to a di-phosphorylated tri-acylated lipid A. At 21 hours, there appears to be the complete elimination of all tetra and penta-acylated lipid A with the remaining mono and di-phosphorylated, tetra-acylated lipid A at m/z 1195 and 1272 respectively. Application of NH_4OH for 48 hours resulted in relative intensity reduction of m/z 1195 and 1272 and the appearance of a new major mass ion at m/z 954. **(Figure 3.4)** Chemical calculation suggest that this peak represent the loss of the C16:0 fatty acid in secondary substitution on the amide linked C17:0 in the C2' position creating a mono-phosphorylated, di-acylated lipid A.

Analysis of NH_4OH samples treated for 4hrs with and without heat

The next experiments were devised to determine if the fatty acid chains were heat labial in the presence of NH_4OH . Here, lipid A samples were subjected to NH_4OH as

previously described but in addition selected samples were heated at 70°C with intermittent vortexing. Results displayed in **Figure 3.5** indicate that at 4hrs without heat, NH₄OH treatment resulted in partial *O*-deacylation of the lipid A samples evidenced by reduction of the mono and di-phosphorylated penta-acylated lipid A species (m/z 1690 and 1770 mass ions) with the presence of mono and di-phosphorylated tetra-acylated lipid A (m/z 1435/1450 and 1512 mass ions) as well as a predominate mass ion at m/z 1193. This lower mass ion corresponds to a mono-phosphorylated tri-acylated lipid A (as first described by Ogawa) with the additional m/z 1272 mass ion indicating the presence of a di-phosphorylated tri-acylated lipid A. In contrast, additional NH₄OH treated samples (4 hrs), heated to 70°C and subjected to intermittent vortexing, resulted in complete elimination of upper mass ions at m/z 1690 and 1770 as well as a significant reduction of the m/z 1512 mass ion and elimination of the 1435/1450 mass ion complex. (**Figure 3.5**) This result suggests that ester-linked acylation within *P. gingivalis* is heat sensitive in the presence of a base chemical.

Analysis of NH₄OH samples treated for 16hrs without heat

In this experiment, approximately 200ug of TRI-CAROFF extracted lipid A samples from *P. gingivalis* MP4-504, *P. intermedia* ATCC 25611 and *C. ochracea* AT #25 were subjected to 1M NH₄OH and incubated for 16 hrs at room temperature as described by Silipo et al. (Silipo, *et al.*, 2002a) MALDI-TOF analysis of the resulting lipid A profile were compared to baseline lipid A profiles of the respective bacterial samples, with *P. gingivalis* MP4-504 serving as a control. (**Figure 3.6**) Results indicated that selective

cleavage of the ester-linked fatty acids found at the C3 and C3' positions on the core β -1,6-D-glucosamine did occur in both *P. intermedia* ATCC 25611 and *C. ochracea* AT #25 as it occurred in the *P. gingivalis* MP4-504 control. MALDI-TOF analysis of *P. intermedia* ATCC 25611 revealed the absence of pent-acylated lipid A with a di-phosphorylated, tetra-acylated lipid A predominate at m/z 1222.7. With *C. ochracea* AT #25 under these same conditions, we saw similar values (relative intensities) of both a di-phosphorylated tri and tetra-acylated lipid A as well as the absence of the di-phosphorylated penta-acylated lipid A structure as seen in the original, untreated preparation. **(Figure 3.6)**

16hrs NH₄OH samples (N=16) vs. untreated lipid A (N=0) on HUVEC and HEK cells

Untreated as well as NH₄OH treated lipid A samples from *P. gingivalis* MP4-504, *P. intermedia* ATCC 25611 and *C. ochracea* AT #25 were applied to the HUVEC E-selectin assay as described. E-selectin expression in HUVEC cells was abrogated after lipid A treatment with NH₄OH as well as hTRL4 activity in HEK 293 cells. **(Figure 3.7 and Figure 3.8)** In most instances, activation of E-selectin or hTLR4 became saturated at or around a concentration of 1 μ g/ml which is in agreement with other observations. (Coats, *et al.*, 2003; Darveau, *et al.*, 2004) Significant antagonism of E-selectin expression ligands occurred with the NH₄OH treated preparations. **(Figure 3.9)** Here, E-selectin expression by either *E.coli* JM83 or *P. gingivalis* 33277 LPS was reduced by 33% to 66% after co-application with any of the NH₄OH treated samples indicating that E-selectin expression, occurring through presumed TLR4 binding of bacterial LPS, was competitive

in nature. Additional cell viability assays with trypan blue (0.8mM at 1:1 ratio) indicated that lack of cell activation or expression was not due to cell death. It was also interesting to note that *E. coli* LPS preparations were 100 fold more potent than any other oral Gram-negative LPS or lipid A preparations within the HUVEC assay, and due to this observation was routinely used at a concentration of 100 fold less in all reported HUVEC host assays. In the HEK system, *E. coli* LPS was 5-20 fold more potent in terms of hTLR4 expression fold increase than oral bacterial LPS or lipid A. These observations are in agreement with previous reported studies. (Coats, *et al.*, 2003; Darveau, *et al.*, 2004)

Lower molecular weight lipid A or core lipid A affect on host activity

Lipid A samples containing low numbers of fatty acids or phosphate groups were obtained by multiple applications of NH₄OH (labeled N=37) or after NH₄OH treatment for 16 hrs of LPS obtained by the method of Darveau and Hancock (Darveau and Hancock, 1983) which had been further purified by the Folch (Folch, *et al.*, 1957) and Vogel procedure (Hirschfeld, *et al.*, 2000) (Labeled Pg1). Two separate applications of NH₄OH for 18.5 hrs (N=37) yielded a preparation with a predominate mass ion at *m/z* 1193 indicating the presence and isolation of the *P. gingivalis* mono-phosphorylated, tri-acylated lipid A that, in our laboratory, had not yet been accomplished but had been reported by Ogawa (Ogawa, 1993). **(Figure 3.10, A)** In addition, the same isolation event occurred when the Pg1 preparation was subjected to 16hrs of NH₄OH treatment. Minor mass ion peaks observed at *m/z* 1480 and 1440 possibly represent minor

lipoprotein artifacts that are unique to *P. gingivalis* MP4-504 and 33227 respectively, and in the case of m/z 1440, apparently have no associated bio-activity (unpublished data).

Application of the m/z 1193 isolated preparation (labeled N=37) on HUVEC cells revealed the loss of E-selectin antagonism in HUVECs similar to what was observed with the fatty acid depleted core *E. coli* lipid A disaccharide control. **(Figure 3.10, B)**

DISCUSSION

Separation of specific lipid A structural components is critical to elucidate the exact mechanism of host activation and antagonism. Therefore an important goal in accomplishing this task was to create or isolate different components of the lipid A biomolecule and determine their biologic function through host assays. Besides synthetic preparations, other ways to generate unique lipid A molecules are to chemically modify a pre-existing lipid A preparations or devise specific growth condition(s) or environmental changes that naturally generates lipid A heterogeneity. Chapter 2 dealt with specific environmental conditions and resulting lipid A structural changes, therefore, chemical techniques, aimed at systematically creating structural modification of the lipid A molecule were described in this Chapter.

Alkaline hydrolysis has been widely used to chemically cleave specific labile bonds. (Silipo, *et al.*, 2002a; Vinogradov, *et al.*, 2000) Depending on the strength of the

chemical interaction, varying concentrations of base solutions are used. Although significant data exists concerning synthetic preparations of altered lipid A structure (Takada and Kotani, 1989; Takada and Kotani, 1992), little is known about the effects of specific fatty acid removal on biologic function or cell activation.

Role of NH_4OH

Results confirm that ammonium hydroxide (NH_4OH) treatment of lipid A from *P. gingivalis* selectively removed ester-linked fatty acids from the core disaccharide (Figures 3.3, 3.4) and that continued application of NH_4OH on lipid A effectively cleaves the fatty acids in secondary esterification as well. (Figure 3.4) In addition, deacylation was not only time dependent, but temperature dependent as well. (Figure 3.5) These results suggest that specific fatty acids from *P. gingivalis* are labile to *in vitro* manipulation.

Host response to altered fatty acid composition, within *P. gingivalis*, was displayed by reduced E-selectin expression in **Figure 3.2**. Here, preparations of *P. gingivalis* LPS (labeled Pg 1) enriched with mono-phosphorylated, tetra-acylated lipid A, as described by Darveau et al. (Darveau, *et al.*, 2004), were mixed with LPS from oral bacteria and directly placed on HUVECs. After 4 hours of stimulation, costimulation of oral bacterial LPS with altered *P. gingivalis* LPS resulted in dramatic antagonism of E-selectin expression when compared directly to the uncombined LPS samples. This suggests that

loss of fatty acid structure from the core disaccharide, within *P. gingivalis*, reduces the ability of HUVECs to respond appropriately to the presence of stimulatory LPS.

We know from past reports that the lipid A structure required to produce the *highest* or *most potent* bioactivity, include: 1) a β , (1'-6)-linked D-glucosamine disaccharide backbone; 2) phosphorylation at positions 1 and 4' on the disaccharide unit; 3) an appropriate number of 3-acyloxyacyl groups (Shimizu, *et al.*, 1987); 4) the position of these 3-acyloxyacyl groups on either the reducing GlcN I or distal GlcN II residues of the disaccharide (Takada and Kotani, 1992); and 5) an appropriate number (usually 6) of hydrophobic fatty acid chains with a suitable carbon length (C₁₂-C₁₈) (Kumazawa, *et al.*, 1988; Nakatsuka, *et al.*, 1989) with or without hydroxylation. Therefore, the findings presented here are in agreement with other reports stating that alteration in the basic lipid A structure, such as changing the number, position, or length of the primary or secondary acyl groups, subtraction of phosphate or monosaccharide groups results in dramatic alteration of biologic effects (Loppnow, *et al.*, 1989; Schumann, *et al.*, 1996; Takada and Kotani, 1992). What is most interesting about these reported results was the ability of synthetically derived lipid A (containing four fatty acids and one phosphate group), when mixed with unaltered full structure LPS, resulted in antagonism of LPS stimulation.

We therefore predicted that any biologically prepared stimulatory LPS, whether it was from *P. gingivalis* or any oral Gram-negative bacterium, could be reduced to a non-stimulatory-potentially antagonistic preparation by removing specific fatty acids or

phosphate groups from their respective lipid A core disaccharide. After 16hrs of NH₄OH treatment, MALDI-TOF analysis revealed the presence of lower mass ion peaks (consistent with *O*-deacylation), suggesting that the labile nature of ester-linked fatty acids exists in other oral bacteria as initially revealed in *P. gingivalis*. **(Figure 3.6)** This result then allowed for direct comparison of altered fatty acid composition lipid A from other Gram-negative oral bacteria to *P. gingivalis* in HUVECs. Results from these experiments confirmed the prediction and revealed that elimination or significant reduction of mono or di-phosphorylated penta or hexa-acylated lipid A from oral bacterial lipid A preparations abrogates E-selectin activity in HUVECs. **(Compare Figure 3.6 MALDI-TOF results with Figure 3.7 HUVEC data)** In addition, these same preparations — when used in a co-stimulatory role — antagonized the known E-selectin stimulatory effects of untreated *E. coli* or *P. gingivalis* LPS, similar to the effects seen by the Pg 1 preparation in HUVECs. **(Figure 3.9)**

hTLR4 utilization was then explored using the hTLR4/ membrane (mCD14) construct transfected into HEK293 cells. **(Figure 3.8)** Here, hTLR4 activation was significantly reduced in NH₄OH treated lipid A (N=16) from all oral bacteria samples when compared directly to the parent, untreated lipid A. These experiments confirmed that hTLR4 activation toward oral bacterial LPS/lipid A is dependent on the full, unaltered lipid A structure and reduction or elimination of specific components alters activation. This result is consistent with previously published data on mono-phosphorylated, tetra-acylated enriched *P. gingivalis* LPS (Darveau, *et al.*, 2004).

The ability to reduce lipid A structure to predominately contain mono-phosphorylated, tri-acylated lipid A was explored next. In these experiments, the NH_4OH protocol was altered to produce lipid A enriched for low molecular weight species. For example, it was empirically determined that NH_4OH , when applied for two 18.5 hour applications, resulted in a lipid A preparation with the desired *O*-deacylated product with a predominate mass ion at m/z 1195. **(Figure 3.10, panel A –middle)** This result represents the first time that our laboratory was able to produce and *isolate* this particular mass ion from *P. gingivalis* LPS. This result could also be duplicated by using a previously reduced preparation (Pg 1), which was enriched for mono-phosphorylated, tetra-acylated lipid A, by applying NH_4OH for 16hrs, yielding the same major mass ion at m/z 1195. **(Figure 3.10, panel A –lower)** These results suggested that the major mass ion, originally described by Ogawa (Ogawa, 1993) as the predominate form of lipid A for *P. gingivalis*, was most likely due to the labile nature of the chemical bonds associated with lipid A structure. Next, it was theorized that this form of lipid A would have low or absent bio-logic activity and isolation of the once elusive mono-phosphorylated, tetra-acylated lipid A, therefore allowed for experimental analysis. Results from the HUVEC E-selectin assay suggests that mono-phosphorylated, tri-acylated lipid A reacts similarly to lipid A devoid of any fatty acids (*E. coli* control disaccharide) in that it lacks the ability to antagonize *E. coli* LPS whereas preparations that are enriched for tetra-acylated lipid A (N=16) contain potent antagonistic properties. **(Figure 3.10, panel B)** These results are in agreement and help explain Ogawa's initial findings of low biologic activity associated

with *P. gingivalis* LPS (Ogawa, 1993). In other words, it appears that a “window” of activity exists for lipid A structure, in that high stimulatory activity is associated with full structure lipid A, moderate as well as antagonistic properties are associated with intermediate molecular weight lipid A, and low to absent activity in HUVECs is associated with low molecular weight (mono-phosphorylated, tetra-acylated lipid A) species of lipid A. These results in biologically prepared lipid A samples is similar to what is observed with the structure-activity relationships observed with synthetically derived preparations (Takada and Kotani, 1989; Takada and Kotani, 1992).

Effects of NaOH and anhydrous hydrazine on lipid A structure

MALDI-TOF analysis after NaOH revealed a single major mass ion group centered around m/z 551 which would be consistent with *O,N*-deacylation from the disaccharide backbone. **(Figure 3.11)** This result depicts a monophosphorylated disaccharide with the hypothetical addition of an aminoarabinose group off of the C4' phosphate group. This potential configuration (with the aminoarabinose group addition) has been seen in other pathogenic bacteria, such as *S. typhimurium* which confers polymixin antibiotic resistance to this bacterium (Ernst, *et al.*, 1999). **(Figure 3.11, A)** However, although the proposed structure is almost correct in regards to the formula weight, a CMR blast search, with the amino acid sequence for the 4-amino-4-deoxy-L-arabinose lipid A transferase found in *Salmonella typhimurium*, revealed no direct homologue within the *P. gingivalis* genome (Accession # AAL34393 blasted against protein genome for *P. gingivalis* W83, TIGR CMR database, TIGR institute for genomic research: RESULTS: PG0578 UDP-N-

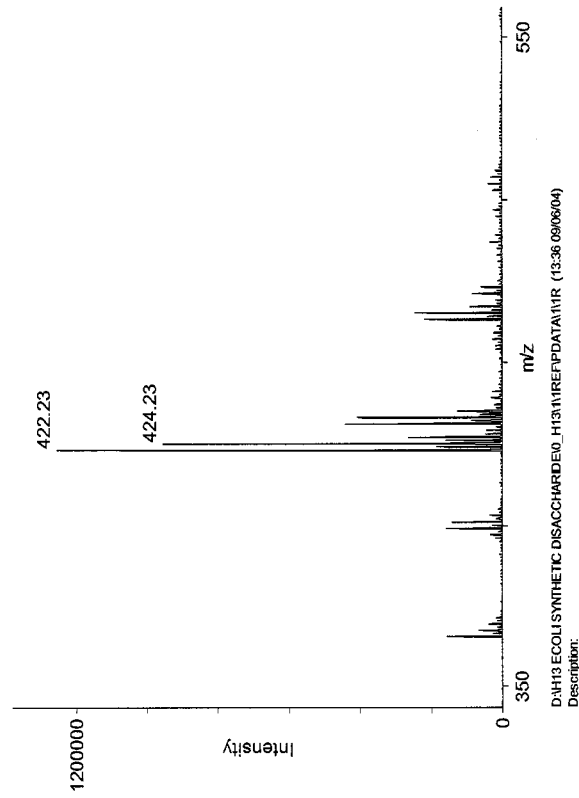
acetylmuramoylalanine-D-glutamate ligase (murD) **Score = 56, Expect = 1.2**; PG0308 electron transport complex **Score = 61, Expect = 1.7**; PG0867 hypothetical protein **Score = 57, Expect = 6.5**) Therefore, this chemical configuration and MALDI-TOF result is highly unlikely and more realistically represents a chemically derived sodium adduct artifact. Likewise, initial attempts at cleaving the ester-linked fatty acid in secondary substitution using anhydrous hydrazine produced a MALDI-TOF result that was unrelated to known lipid A chemical structure (data not shown) leading to the conclusion that the described protocol was unsuccessful and was not further pursued.

Reduced “potency” of oral Gram-negative bacteria

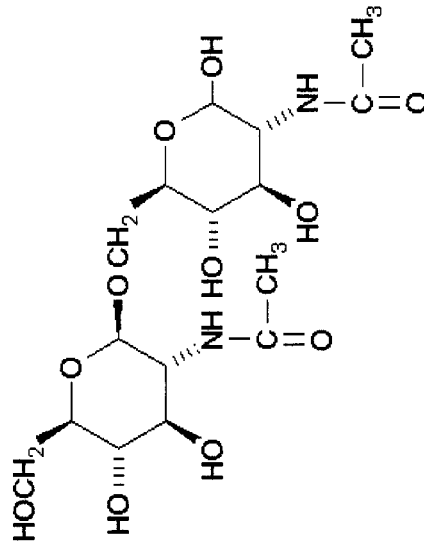
The role of specific structural components of the lipid A molecule within synthetic preparations of *E. coli* is well established. (Takada and Kotani, 1989; Takada and Kotani, 1992; Takahashi, *et al.*, 1987) However, little is known about the host effects towards structural changes within lipid A of oral Gram-negative bacteria. It is currently believed that by nature, oral Gram-negative bacteria are less potent, in terms of host responses towards specific bacterial products than enteric bacteria. (Isogai, *et al.*, 1988; Takada and Galanos, 1987) (Fujiwara, *et al.*, 1990; Mansheim, *et al.*, 1978) (Takada and Kotani, 1992). The evidence presented here supports this belief by demonstrating the potency of *E. coli* LPS in both the HUVEC and HEK assay systems when directly compared to oral Gram-negative LPS or lipid A.

Those bacteria, whose lipid A structure more closely mimics *E. coli* lipid A responded with greater potency than oral bacteria, whose lipid A structure was deficient in phosphate groups or fatty acid composition, indicating a unique range of host activity responses towards different oral bacterial species. (Chapter 1) However, evidence presented in this Chapter indicates that any oral bacteria lipid A, once specific fatty acids are systematically removed, respond similarly in terms of decreasing host responses. **(Figures 3.6-3.10)** This data supports the hypothesis that any oral bacteria, that have evolved ways to alter its individual lipid A structure or neighboring bacteria within a bacterial biofilm or consortium (*via* deacylase activity), might be able to direct specific host responses towards a more favorable outcome for bacterial survival. In addition, this evidence may also suggest a way to use pharmacological methods to chemically decrease or reduce the significance of the destructive inflammatory host response that occurs during chronic type infections with specific oral bacteria.

A



B



FW: 424 Sigma A-3919

Figure 3.1 (A) MALDI-TOF analysis and confirmation of structural formula weight of *E. coli* synthetic disaccharide (Sigma A-3919). (B) Reference compound for the study of 2-amino-6-O-(2-amino-2-deoxy-b-D-glucopyranosyl)-2-deoxy-D-glucopyranose (b-D-GlcN-[1,6]-D-GlcN), the basic structure in *E. coli* Lipid A. This structure is commonly found in both enterobacteria as well as the majority of oral bacteria and provides sites for ester and amide fatty acid linkages which helps to secure the LPS to the outer bacterial membrane. Reference: Rosner, M.R., et al., *J. Biol. Chem.* **254**, 5926 (1979)

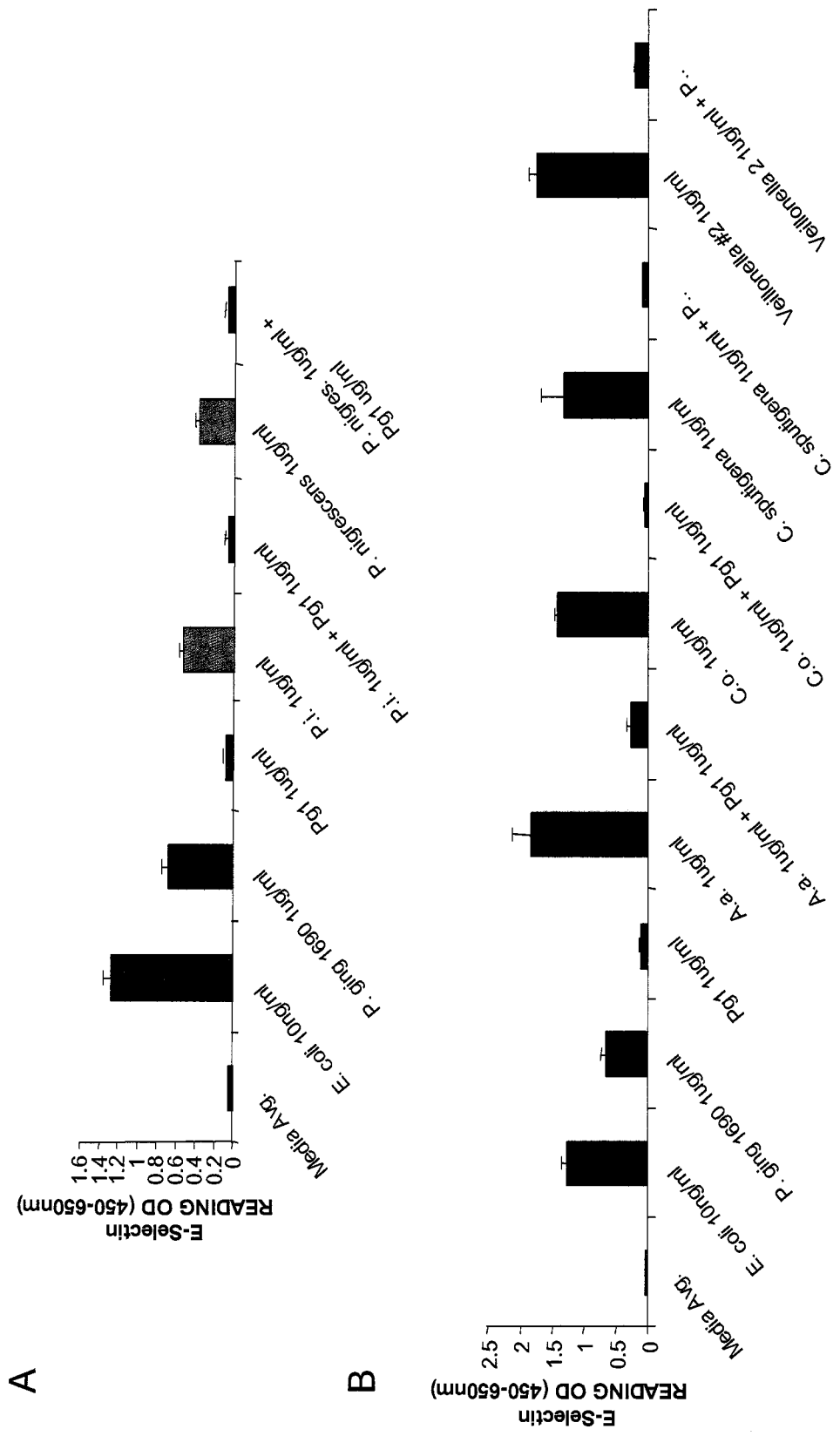


Figure 3.2: HUVEC E-selectin assay using LPS from various oral Gram-negative bacteria alone and combined with Pgl. (A) Orange complex bacteria. (B) Green and Magenta complex bacteria. Results suggest that Pgl preparation antagonizes E-selectin expression from HUVEC cells from all types of oral bacteria.

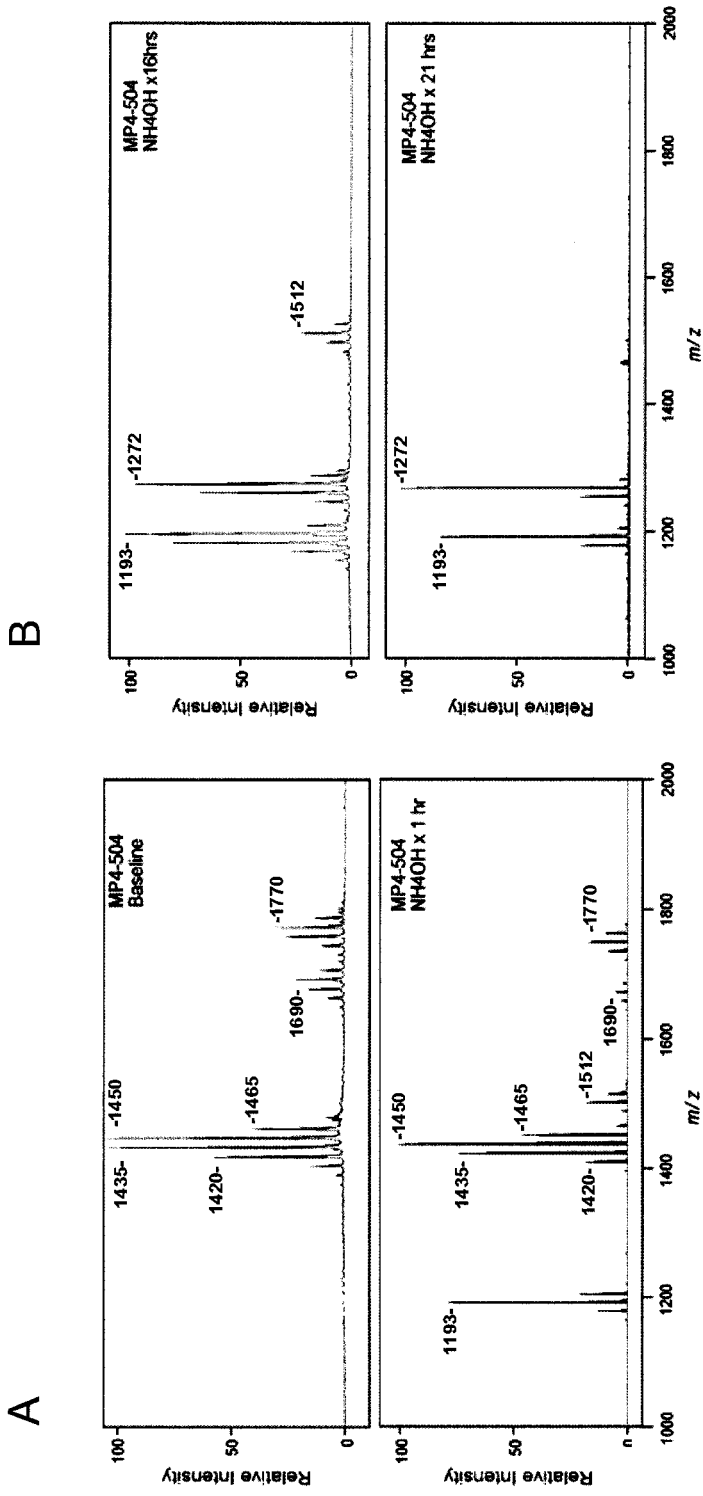


Figure 3.3 MALDI-TOF analysis of *P. gingivalis* MP4-504 lipid A after ammonium hydroxide (NH₄OH) treatment. Panel (A) represents baseline, untreated lipid A and the appearance of the *m/z* 1193 mass ion after 1 hr NH₄OH treatment. Panel (B) reveals continued left shift of mass ion groupings with the elimination of the *m/z* 1690/1770 mass ion complex and the appearance of *m/z* 1273 at 16hrs and complete ester-linked deacylation at 21hrs

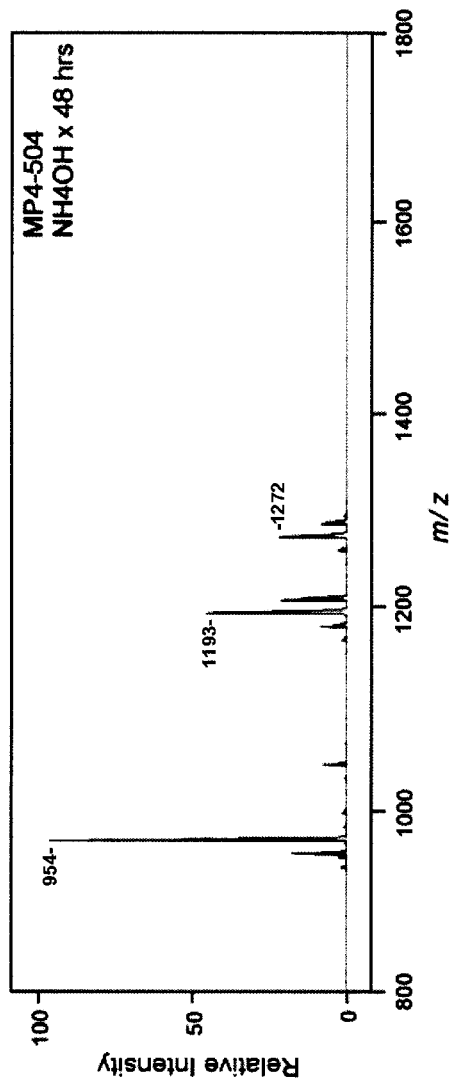


Figure 3.4 MALDI-TOF analysis of *P. gingivalis* MP4-504 lipid A after ammonium hydroxide (NH₄OH) treatment for 48 hrs. Continued application of NH₄OH revealed the presence of a mass ion peak at m/z 954 which indicates the loss of a C16 fatty acid in secondary substitution off of the amide-linked heptadecanoic acid (C17:0) located at the C2' position.

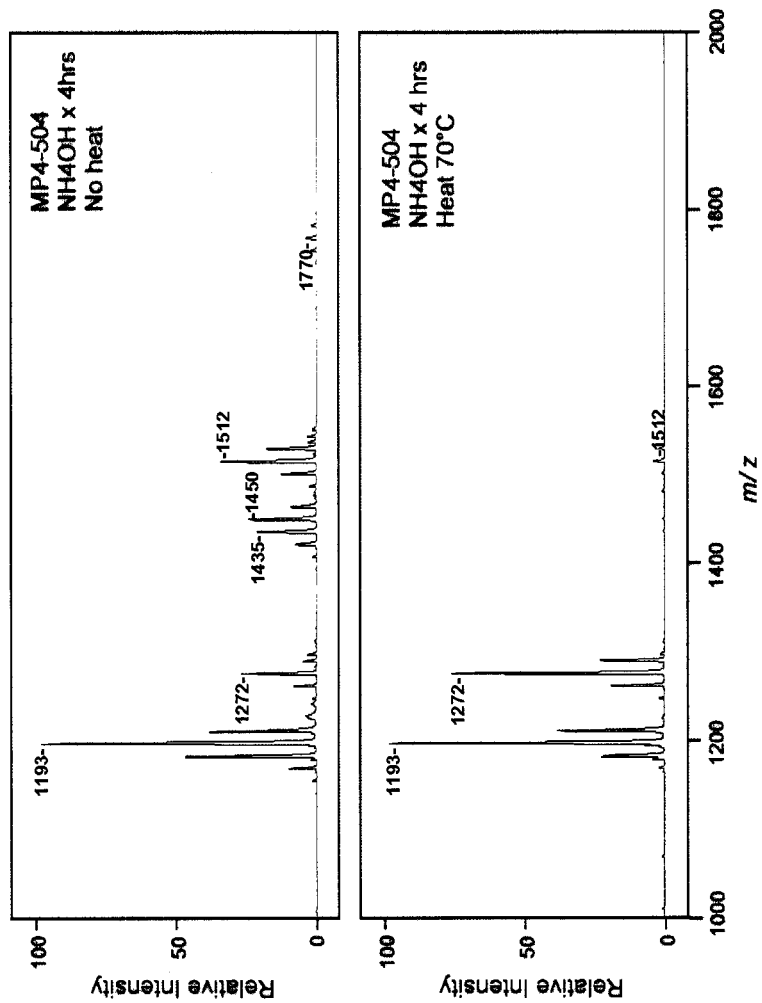


Figure 3.5 Ammonium hydroxide hydrolysis with *P. gingivalis* MP4-504 at 4hrs with and without the application of heat. Results indicate increased *O*-deacylation after NH₄OH treatment in the presence of 70°C heat suggesting that the ester-linked fatty acids are heat sensitive in the presence of a mild base.

MALDI-TOF MS Analysis

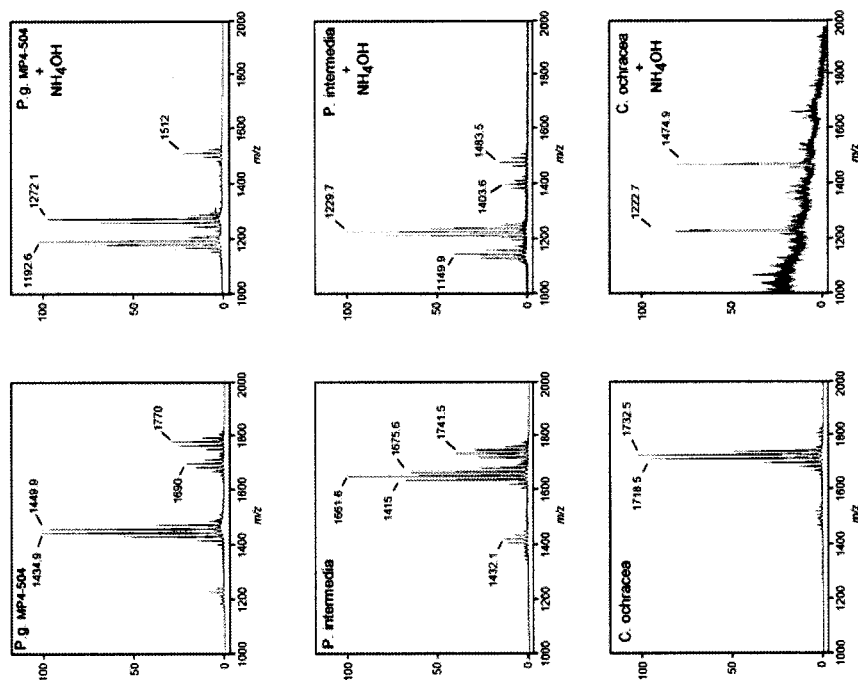


Figure 3.6 Ammonium hydroxide hydrolysis of different Gram-negative lipid A with untreated lipid A preparations on the left and NH₄OH treated samples (16hrs) on the right. MALDI-TOF spectral analysis reveals the presence of additional lower mass ion peaks that are consistent with O-deacylation from the core lipid A structure after the addition of NH₄OH (16 hrs at room temperature) as seen with *P. gingivalis* MP4-504.

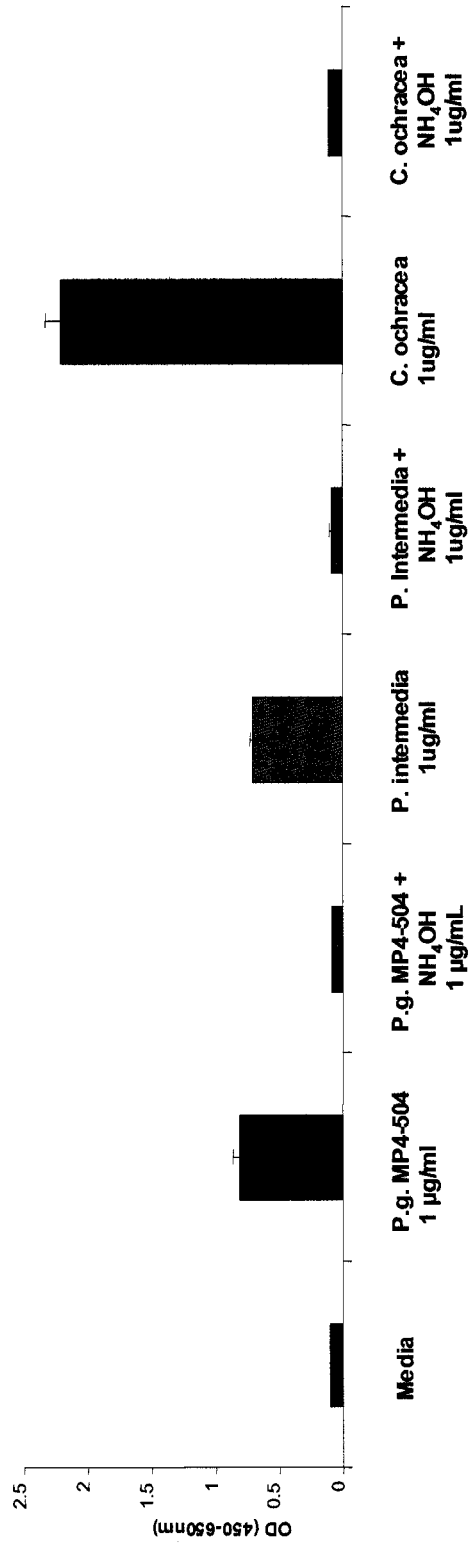


Figure 3.7 Ammonium hydroxide hydrolysis preparations (N=16) of different Gram-negative lipid A results on HUVECs. E-selectin expression was significantly reduced after ammonium hydroxide hydrolysis (16hrs) suggesting that O-deacylation modulates or abrogates expression of E-Selectin from HUVECs when compared to the unaltered forms of lipid A.

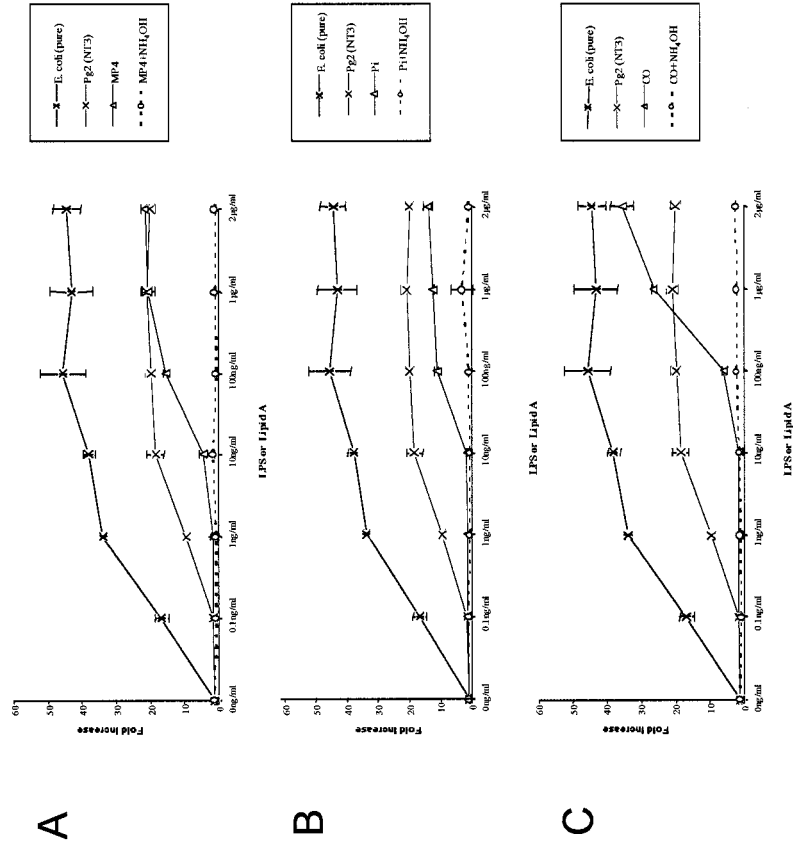


Figure 3.8 hTLR4 activation with ammonium hydroxide hydrolysis preparations (NH₄OH N=16) of different Gram-negative lipid A. HEK 293 cells were transiently transfected with human mCD14 and hTLR4, with human MD-2 together with the NF-κB reporter (ELAM-1 firefly luciferase) and the transfection control (β -actin-Renilla luciferase) as described by Darveau et al. 2004. hTLR4 expression was significantly reduced after ammonium hydroxide hydrolysis (16hrs) suggesting that O-deacylation modulates or abrogates expression through hTLR4 when compared to the unaltered forms of lipid A. (*E. coli* a *P. gingivalis* LPS preparation labeled Pg2 (NT3) were used as positive controls for experiments).

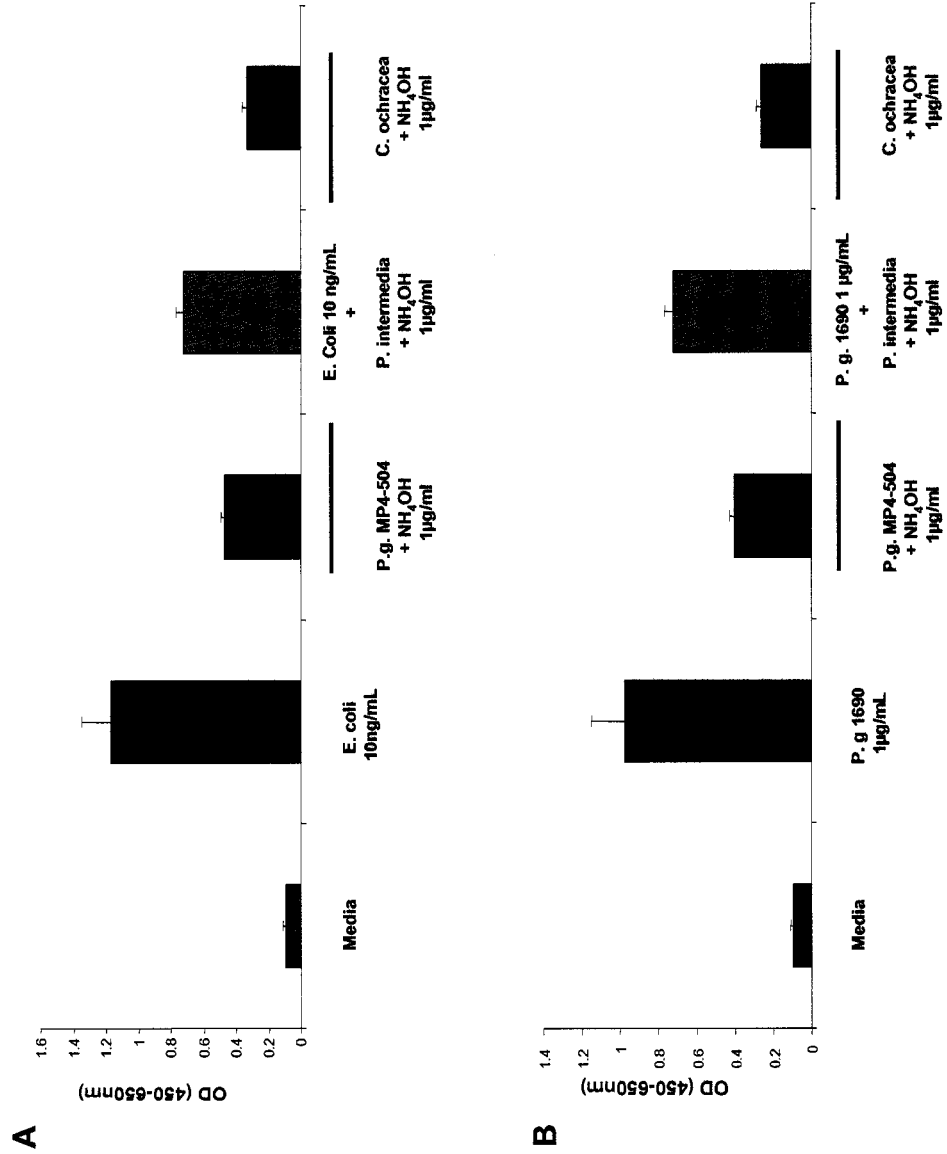


Figure 3.9 HUVEC E-selectin antagonism with ammonium hydroxide hydrolysis preparations combined with known E-Selectin agonists. NH_4OH hydrolyzed lipid A preparations partially blocked E-Selectin expression when combined at a 1:100 ratio with *E. coli* (A) and a 1:1 ratio with a *P. gingivalis* 1690 preparation (B).

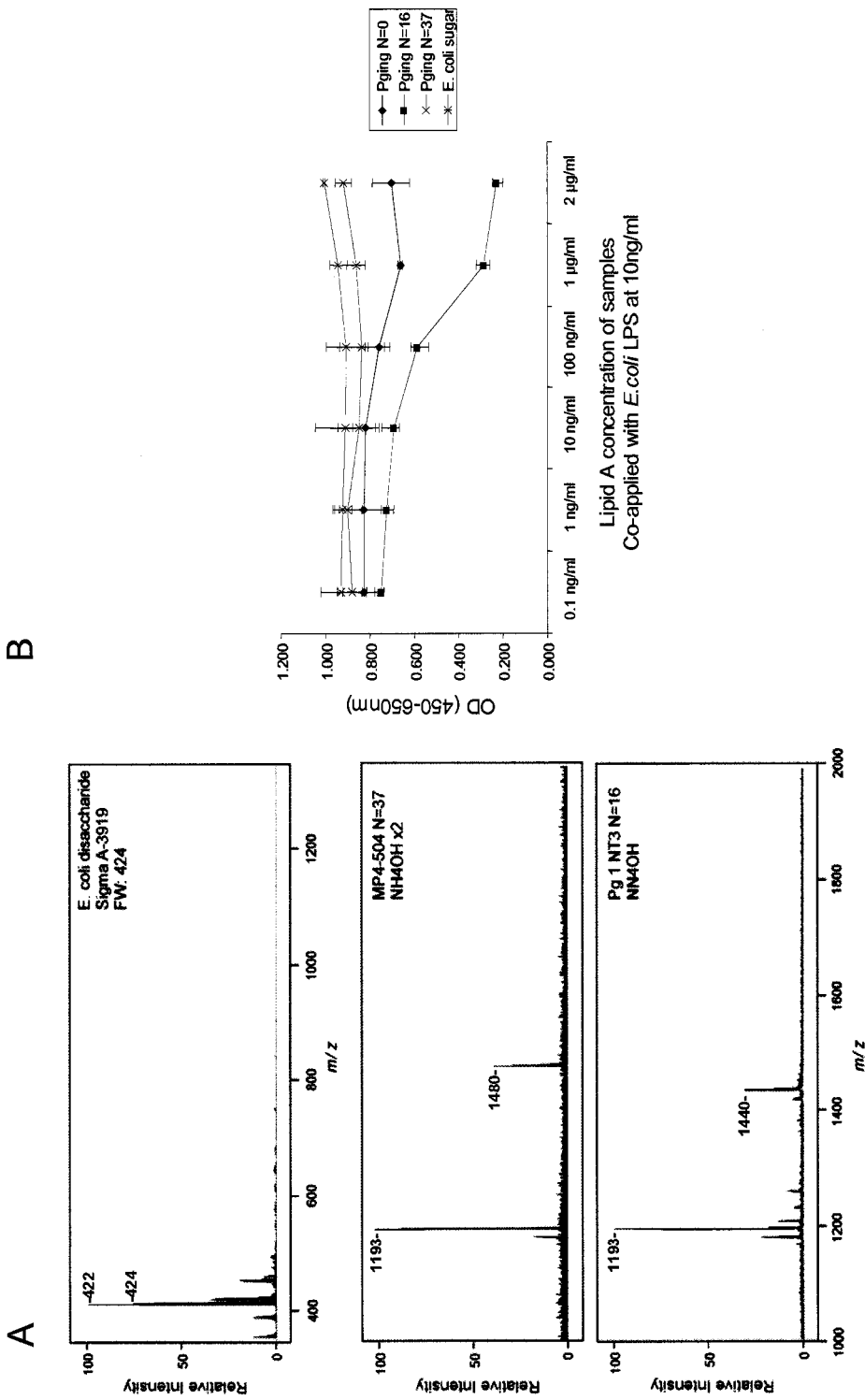


Figure 3.10 Prolonged ammonium hydroxide (NH_4OH) treatment. (A) MALDI-TOF analysis of *E. coli* core disaccharide (control) as well as lipid A from *P. gingivalis* MP4-504 with 2 applications of NH_4OH for 18.5 hrs each (N=37) in addition to 16hrs (NH_4OH) treatment of a *P. gingivalis* 33277 previously reduced preparation (Pg 1 NT3) both yielded similar MALDI-TOF spectral patterns. (B) E-selectin expression in HUVECs of *P. gingivalis* MP4-504 samples demonstrating that lower molecular weight lipid A loses antagonistic properties when applied with an E-selectin activator (*E. coli* LPS) compared to potent antagonism with the N=16 preparation.

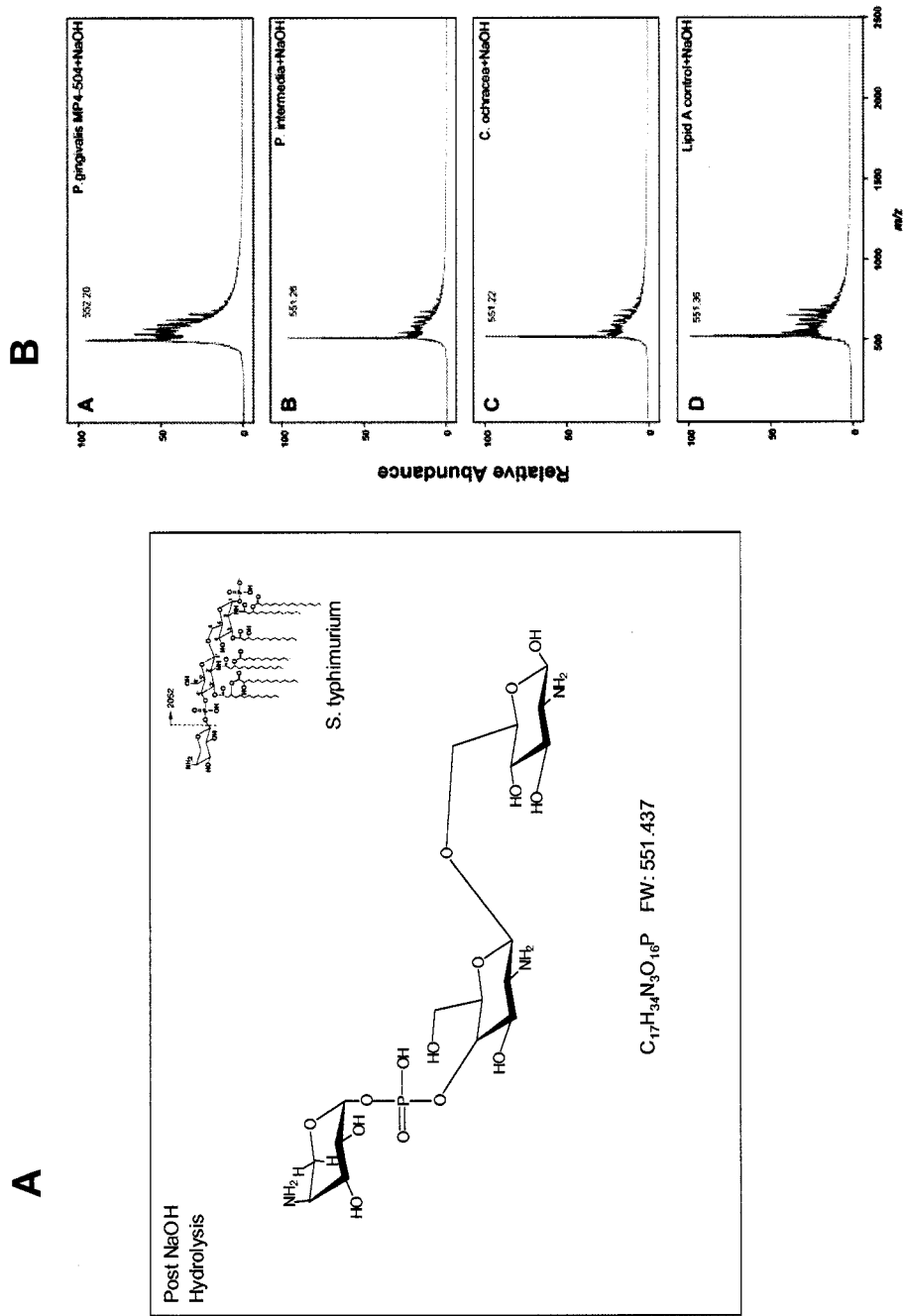


Figure 3.11: Sodium hydroxide hydrolysis of oral Gram-negative lipid A. NaOH hydrolyzed lipid A were created by subjecting purified lipid A preparations to further hydrolysis with 4 M NaOH at 100°C for 2 hrs. (A) Hypothetical chemical configuration of core monophosphorylated lipid A disaccharide with the addition of an aminoarabinose group. (B) MALDI-TOF spectral analysis of NaOH preparations with a major mass ion peak at an expected molecular weight if *O*, *N*-deacylation has occurred.

CHAPTER 4: Extraction techniques affects on LPS and lipid A structure and biologic activation

INTRODUCTION

Lipopolysaccharide (LPS) has long been known as a heat stable, endotoxic component of Gram-negative bacterial outer membranes. Past and current efforts to extract, isolate and then purify individual or whole LPS components are focused on key components within the structure itself. For example, as stated earlier in Chapter 1, LPS consists of three basic components: an outer polysaccharide, a core (inner + outer) oligosaccharide and a lipid (Raetz and Whitfield, 2002). Together, this structure is amphiphilic by nature, with a hydrophobic lipid A portion and a hydrophylic polysaccharide component and is usually isolated from whole bacteria *via* the hot phenol-water method described by Westphal and Jan (Galanos, *et al.*, 1969; Westphal, *et al.*, 1952). Westphal and co-workers showed that by shaking bacterial cells in an “*emulsion of equal volumes of liquid phenol and water at low temperatures (5-10°C)*”, a liquid phase separation occurs resulting in the upper water layer containing all of the residual undegraded lipopolysaccharide. (Westphal and Jann, 1965) Over the years, this method became the method of choice for most researchers by proving to be widely applicable to lipopolysaccharide extraction from the majority of Gram-negative bacteria.

However, lipopolysaccharides extracted from bacterial whole cells in the presence of cold phenol-water solutions, were found to contain varying amounts of firmly attached proteins (Westphal and Jann, 1965). After modification to include the application of heat and dialysis, higher purity was achieved but researchers found that this technique preferentially extracted LPS containing a long, extended O-antigen region (smooth-type LPS)(Darveau and Hancock, 1983) whereas rough-type LPS (short O-antigen region), was obtained when bacterial cells were extracted using petroleum ether, chloroform and phenol. (Galanos, *et al.*, 1969) This led to modifications of each technique in an attempt to limit the exclusion of different types of LPS or the application of one technique over the other to obtain adequate yields. (Darveau and Hancock, 1983)

To address this problem, an alternative isolation technique was developed (Darveau and Hancock, 1983) utilizing mechanical cell disruption, *via* a high pressure cell, to break apart whole bacteria, then application of proteinase treatment to remove resistant proteins in addition to the application of DNase/RNase treatments to remove residual genomic DNA or RNA contamination. Ultra-centrifugation as well as the application of cold $MgCl_2$ allowed for precipitation of the LPS from the residual cellular debris. This technique was found to extract high amounts of both smooth and rough types of LPS from both *Pseudomonas* and *Salmonella* strains with a high degree of purity.

A more recently described LPS extraction technique (Yi and Hackett, 2000), eliminates the need for mechanical cell disruption by the use of a commercially available reagent.

Here, the TRI-extraction technique incorporates the use of phenol and guanidinium thiocyanate in an aqueous solution. The guanidinium thiocyanate allows for cell membrane disruption without mechanical means or the need for high temperatures. This procedure was shown to be successful in LPS isolation from a variety of Gram-negative bacteria with minimal degradation and has the advantage of eliminating time consuming steps necessary for the other procedures. However, little is currently known about the purity of these preparations.

Although naturally occurring LPS heterogeneity does in fact occur, it must be noted here that certain conditions and procedures utilized during routine LPS extractions techniques (i.e. excessive temperatures or length of time preparations are subjected to phenol or alkaline based chemicals) (Caroff, *et al.*, 2002; Okuda, *et al.*, 1975; Tsang, *et al.*, 1974) can *potentially* add to the heterogeneity observed in LPS preparations. These LPS alterations could significantly affect the overall response seen in different host innate defense assays. For example, the basic structure of lipid A for *P. gingivalis* 381 was described by Ogawa as a monophosphorylated tri-acylated disaccharide with a negative ion FAB MS-MS mass ion located at m/z 1195. (Ogawa, 1993) Later, Kumada *et al.* reported additional *P. gingivalis* lipid A moieties (within a clinical isolate) to include lipid A species containing four to five fatty acid chains with a negative ion FAB MS-MS mass ion(s) located at m/z 1435, 1449, 1690 and 1770 respectively (Kumada, *et al.*, 1995). Furthermore in a recent publication from our laboratory, after LPS purification and lipid A isolation, all lipid A moieties were found (as previously reported) with the

exception of the Ogawa tri-acylated, monophosphorylated lipid A (m/z 1995). The significance of this observation was not understood at the time. (Darveau, *et al.*, 2004). Examination of the lipid A isolation techniques revealed that isolation of the lipid A from the LPS molecule was accomplished by either application of 0.6% acetic acid at 105°C for 2.5 hrs (Ogawa, 1993) or 1.0 % acetic acid at 100°C for 1.5 hrs (Kumada, *et al.*, 1995), whereas the Darveau group describes using 10mM sodium acetate in the presence of 1% SDS at 100°C for 1 hr. (Darveau, *et al.*, 2004) It is interesting to note that the lipid A initially described by Ogawa was shown to be of low potency whereas the different lipid A species described by Darveau had differential host responses dependent on the host receptor involved. Chapter 3 discussed the deacylation effects of increased temperature or prolonged time in the presence of chemical bases on the fatty acid chains attached to the core lipid A molecule; therefore, it was hypothesized that prolonged application of chemical hydrolysis techniques, especially in the presence of heat, could affect the overall configuration of the post-extracted lipid A and might explain the discrepancy between reported structures and host responses for *P. gingivalis* LPS/lipid A.

In our laboratory, we have shown the ability to successfully cleave the lipid A portion of LPS from *Porphyromonas gingivalis* 33277 and then characterize the biochemical structure of this lipid A via MALDI-TOF and GC analysis. However, what has yet to be determined conclusively (especially in oral bacteria) is if specific extraction techniques, those that are commonly employed to separate and then purify LPS and lipid A from whole bacterial cells, are significantly disrupting the biologic structure itself. Initial

MALDI-TOF analysis suggest that specific oral bacteria, when subjected to different extraction techniques, displayed altered lipid A patterns that were reproducible and correlated with the type of extraction technique performed. Therefore, to explore the possibility of a specific extraction technique playing a role in producing the heterogeneity observed in LPS preparations, a series of experiments were devised to test three different LPS extraction procedures to determine the purity as well as the overall effect of chemical and physical *in vitro* manipulation on the chemical structure of specific Gram-negative bacteria LPS.

MATERIALS AND METHODS

Hydrolysis comparison

Porphyromonas gingivalis MP4-504 was used for these experiments. LPS was extracted via the TRI-extraction technique as previously described in Chapter 1. 5mg of crude LPS samples were then subjected to hydrolysis using either the procedures described by Ogawa (0.6% acetic acid at 105°C for 2.5 hrs) or Kumada (1.0 % acetic acid at 100°C for 1.5 hrs). (Kumada, *et al.*, 1995; Ogawa, 1993) Control samples were subjected to 10mM sodium acetate in the presence of 1% SDS at 100°C for 1 hr. After hydrolysis, all samples were neutralized, frozen and lyophilized. Samples were then subjected to acidified and non-acidified ethanol washes, dried, reconstituted with endotoxin free water and refrozen. After lyophilization, samples were analyzed *via* MALDI-TOF.

Extraction comparison

Porphyromonas gingivalis 33277 or MP4-504, *Prevotella intermedia* 25611 and *E. coli*

JM83 were chosen for these experiments. Lyophilized, whole cell samples from each bacterial species were subjected to crude LPS extraction using one of three techniques:

(1) Hot phenol and water method (PHENOL) as described by Westphal and Jann.

(Westphal and Jann, 1965) Briefly, 500mg of lyophilized bacteria is suspended in 20 mls of distilled water, to which an equal volume of 90% phenol is added. This mixture is heated to 70°C and vortexed for 1hr. After cooling on ice and centrifugation, the upper aqueous layer is removed, saved and the lower phenol layer is extracted twice again with 20mls of water. These three extractions are then pooled together. This sample is then dialyzed against multiple changes of distilled water for 3 days (removing the residual phenol). Samples were frozen, lyophilized and reconstituted in 10mM Tris pH adjusted to 8 and sonicated to resuspend. After centrifugation, MgCl₂ is added to a final concentration of 25mM and ultracentrifuged at 200,000 x g for 2 hrs to pellet the LPS.

This pellet is then resuspended in 10mM Tris (pH 8) to which 200ug/ml DNase (DNase I, Sigma chemicals, USA) and 50 ug/ml RNase (RNase A, Sigma chemicals, USA) is added and incubated at 37°C for 2hrs with continuous shaking. Two hundred ug/ml of proteinase E (Proteinase E, Sigma chemicals, USA) is added and the solution is incubated at 37°C overnight with continuous shaking. The sample is centrifuged again and the pellet is resuspended in MgCl₂ to a final concentration of 25mM and ultracentrifuged at 200,000 x g for 2 hrs to pellet the LPS. This process is repeated again and then the final

pellet is suspended in deionized water, frozen and lyophilized. (2) Cold MgCl₂ Darveau and Hancock method (D/H) as described by Darveau and Hancock. (Darveau and Hancock, 1983) In this method, 500 mg of lyophilized cells are resuspended in a solution containing 10mM Tris buffer (pH8), 2mM MgCl₂, 100 ug/ml DNase (DNase I, Sigma chemicals, USA), and 25 ug/ml RNase (RNase A, Sigma chemicals, USA). This solution is passed twice through a French pressure cell (Amicon Corp). 100 ug/ml DNase (DNase I, Sigma chemicals, USA), and 25 ug/ml RNase (RNase A, Sigma chemicals, USA) is added again and incubated at 37°C for 2 hrs. To this, 5 mls of 0.5 M tetrasodium EDTA (ED4SS, Sigma chemicals, USA), 2.5 mls of 20 % SDS in Tris buffer and an additional 2.5 mls of Tris buffer was added. The resulting solution was vortexed, and centrifuged at 50,000 x g for 30 min at 20°C. After discarding the pellet, 200 ug/ml of proteinase E (Proteinase E, Sigma chemicals, USA) is added and the solution is incubated at 37°C overnight with continuous shaking. This solution is then centrifuged at 1000 rpm for 10 min, with the resulting pellet discarded. Two volumes of 0.375 M MgCl₂ in 95% ethanol are added and the solution is cooled to 0°C. The solution is kept cold and centrifuged at 12 k x g for 15 min at 0°C. The precipitate that forms is resuspended in 0.5 M tetrasodium EDTA (ED4SS, Sigma chemicals, USA), 2.5 mls of 20 % SDS in TRIS buffer and an additional 17.5 mls of Tris buffer were added and sonication was performed to complete pellet suspension. The pH was neutralized with 4 N HCl and incubated at 85°C for 30 min. After cooling the solution was pH adjusted to 9.5 with 4 N NaOH, to which 25 ug/ml of proteinase E (Proteinase E, Sigma chemicals, USA) is added and incubated overnight at room temperature with continuous shaking.

Afterwards, two volumes of 0.375 M $MgCl_2$ in 95% ethanol was added and the cold precipitation as described was repeated. The resulting pellet was resuspended in 15 mls Tris, sonicated and centrifuged at 1000 rpm for 10 min. Pellet was resuspended in 25mM $MgCl_2$ and ultracentrifuged at 200 k x g for 2 hrs at 20°C. This process was repeated and the final pellet was resuspended in deionized water, frozen and lyophilized. Additional samples of the Darveau and Hancock (D/H) extracted LPS were further subjected to purification using the Vogel (Manthey and Vogel, 1994) and Folch (Folch, *et al.*, 1957) purification techniques. These samples were subjected to repeated extractions with 0.2 triethylamine (TEA, Sigma chemicals, USA), 10 % sodium dexocholate (DOC, Sigma chemicals, USA) in endotoxin free water as well as room temperature water-saturated phenol. The aqueous phases of each extraction were saved and pooled then subjected to 100 % ethanol in the presence of 3 M sodium acetate pH to 5.2, vortexed, and placed at -20°C for 1 hour. Repeated cold ethanol washes were performed and the final pellet was resuspended with 0.2 % TEA. Next, these samples were suspended in 1-2 % (w/v) chloroform/methanol (2:1 ratio), vortexed and centrifuged at 10 k x rpm at 4°C for 5 min. The supernatant was removed and discarded and the chloroform/methanol extraction was repeated. The resulting pellet was suspended in deionized water and frozen. (3) TRI-reagent extraction (TRI) as described by Yi and Hackett. (Yi and Hackett, 2000) In this technique, 50 mg of lyophilized whole cells is extracted with 1 ml of commercially obtained TRI reagent (Tri-Reagent, Molecular Research Center, USA) consisting of guanidinium thiocyanate and phenol (in undisclosed amounts). The mixture is vortexed and sonicated then incubated at room temperature for 30 min. 200 ul/ml of chloroform

(Chloroform-C-2432, Sigma chemicals, USA) is added and the suspension is again vortexed and allowed to incubate at room temperature for 30 minutes. The samples are then centrifuged at 12,000 rpm for 10 min. The aqueous (top) layer is removed and saved and 500 ml of endotoxin free water is added to the lower phase. Again, the sample is vortexed and allowed to incubate at room temperature for 30 min. The process is repeated twice and all aqueous (top) layers are pooled, frozen and lyophilized. To separate lipid A from the total LPS molecule, all samples were then further subjected to a Caroff extraction (CAROFF) as described previously in Chapter 1. (Caroff, *et al.*, 1988) Purified lipid A samples were then characterized *via* MALDI-TOF analysis.

SDS-PAGE with silver staining

Following extraction using the various techniques outlined above, LPS samples were analyzed by SDS- polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining as described by Tsai and Frasch. (Tsai and Frasch, 1982) Briefly, 10 ug of sample was used to ensure staining of both the major and minor LPS components (inner-outer core carbohydrates as well as the O-antigen region) of the various bacteria selected. Briefly, samples were boiled after mixing with a loading buffer consisting of 1M Tris (pH 6.8), 10% SDS, glycerol and 0.5% bromophenol blue for 5 min at 95°C. Samples were applied to a 4-15% polyacrylamide gel (Biorad laboratories Inc., USA) and run at 80 volts at room temperature for 2 hrs or until the loading buffer was approximately 1 cm from bottom of gel. Gels were fixed overnight in 40% EtOH and 5% acetic acid at room temperature with continuous shaking. The gels were oxidized using 0.7% periodic acid

dissolved in the fixation solution. The polyacrylamide gels were then washed repeatedly, with distilled water and then 100 ml of a silver staining solution containing 0.1 NaOH, NH₄OH, 20% silver nitrate, was applied for 10 min. Gel was washed again and developed in a solution containing citric acid and 37% formaldehyde. After developing, gels were then fixed overnight, dried and stored until photographed.

Total Protein staining

Colloidal gold staining of purified LPS preparation extracted as described above was used to determine residual protein within samples. Here, a commercially available total protein staining kit was used. (Colloidal Gold Total Protein Stain, Bio-Rad Laboratories, Hercules, CA) Briefly, proteins are bound to a nitrocellulose membrane by electrophoretic transfer. The membrane was then transferred to an incubation vessel and washed for 20 min with a 1x TTBS solution and twice repeated as indicated by the manufacturer. Next 100mls of double distilled water was used to repeatedly rinse the membrane to ensure removal of all salts that could interfere with the colloidal gold staining. After rinsing, the colloidal gold stain was applied to the membrane and was allowed to incubate for 60-120 min. Increased staining time was dependent on amount of background observed while staining. Upon completion of staining, membranes were removed and repeatedly rinsed with double distilled water, allowed to dry overnight and then scanned.

RESULTS

Hydrolysis comparison

Hydrolysis of the lipid A portion from TRI-extracted LPS from *P. gingivalis* MP4-504 using 0.6% acetic acid, heated to 105°C for 2.5 hrs then cooled and neutralized with 1 N HCl, as described by Ogawa, (Ogawa, 1993; Ogawa, *et al.*, 1994) resulted in a lipid A preparation containing mono-phosphorylated, tetra-acylated lipid A with *m/z* 1420, 1435, 1450, 1465 mass ions. (Figure 4.1, B) Hydrolysis using 1.0% acetic acid, heated to 100°C for 1.5 hrs, cooled and neutralized with 1 N HCl, as described by Kumada,(Kumada, *et al.*, 1995) also resulted in a lipid A preparation containing mono-phosphorylated, tetra-acylated lipid A with *m/z* 1420, 1435, 1450, and 1465 mass ions. (Figure 4.1, C) Neither preparation, obtained using the above criteria contained tri-acylated or penta-acylated lipid A forms as evidenced by the apparent absence of upper and lower mass ions as previously described. In contrast, results from hydrolysis using 10 mM sodium acetate in the presence of 1% SDS as described by Caroff,(Caroff, *et al.*, 1988) resulted in a lipid A preparation containing both mono-phosphorylated, tetra-acylated as well as mono and di-phosphorylated penta-acylated lipid A with *m/z* 1420, 1435, 1450, 1465, 1690 and 1770 mass ion peaks. This preparation served as a control for this experiment and has been described in detail in other chapters. (**Figure 4.1, A**)

Extraction comparison

LPS percent (%) yields were calculated from each of the three different extraction techniques for the selected bacteria and are summarized in **Table 4.2**. It is generally known that LPS extraction yields typically results in approximately 10% of the total dry weight of whole bacterial cells (Darveau and Hancock, 1983; Eidhin and Mouton, 1993) which was also observed in this experiment. However, these results also reveal that among the extraction procedures evaluated, the Darveau/Hancock (D/H) had the highest average yield at 8.8 % with the lowest overall yield obtained with the hot phenol-water (PHENOL) technique (1.4%). In regard to specific bacterial strains, *Prevotella intermedia* ATCC 25611 had the highest % yield but had the most variability in LPS % yields among the different extraction techniques whereas *P. gingivalis* 33277 LPS yields were very consistent in average LPS yields and standard deviation. (**Table 4.2**)

Lipid A recovery from the different LPS extraction was also recorded and is summarized in **Table 4.3**. Here, regardless of the LPS extraction approach, each LPS sample was subjected to the same (CAROFF) hydrolysis procedure and analyzed. The results indicated that the TRI-reagent extraction, on average yielded the highest % recovery of lipid A followed by the (D/H) method and finally the hot phenol-water (PHENOL) technique. Again, in regard to specific bacterial strains, higher % yields were obtained from *P. gingivalis*, followed by *E. coli* and finally *P. intermedia*. (**Table 4.3**)

SDS-PAGE with silver staining

Analysis by SDS-gel electrophoresis followed by silver staining (Tsai and Frasch, 1982) was employed to detect and visually characterize LPS samples extracted using the described techniques. **Figure 4.2** reveals that within the *P. gingivalis* 33277 samples, the hot phenol-water (PHENOL) and the Darveau/Hancock (D/H) techniques (lanes 2 and 3) produced dominate overlapping leader bands that migrated to the bottom (10-15 kD) whereas the TRI-reagent (TRI) as well as the Darveau/Hancock/Vogel (D/H/V) techniques (lanes 1 and 4) produced similar but more defined doublets. (**Figure 4.2**) In addition, it was noted that the TRI-reagent (TRI) technique did not produce, to the same extent as the other techniques, additional bands located approximately at 37-50 kD. (Compare lanes 1 to lanes 2, 3 and 4) The *P. intermedia* 25611 samples revealed that the banding patterns were similar for both the TRI-reagent (TRI) and Darveau/Hancock (D/H) techniques (lanes 5 and 7) producing a leading doublet pair at 10-15 kD whereas the hot phenol and water (PHENOL) technique (lane 6) resulted in reduced mobility of the leading doublet, migrating to only 20-25 kD. The Darveau/Hancock/Vogel (D/H/V) technique (lane 8) produced a single, dominate band with almost the complete absence of upper bands suggesting removal of the core carbohydrate and O-antigen regions. The *E.coli* JM83 control revealed that the techniques that employed the use of multiple phenol extractions (i.e. Hot phenol-water (PHENOL) as well as the Vogel repurification of the Darveau/Hancock (D/H) preparation (lanes 10 and 12)) produced the broadest, diffuse bands whereas the TRI-reagent (TRI) as well and the Darveau/Hancock (D/H) without the Vogel repurification (lanes 9 and 11), produced smaller, but still diffuse banding patterns.

Total Protein staining of purified preparations

Figure 4.3 represents colloidal gold staining after LPS samples were extracted from three different Gram-negative bacteria using different extraction and purification techniques.

Within *P. gingivalis* 33277 samples, dark protein bands are evident throughout a wide kD range with the hot phenol-water extraction technique (PHENOL), whereas only minor bands are present below 20 kD for the Darveau/Hancock (D/H) which are then completely removed with the Manthey and Vogel plus Folch repurification (D/H/V).

(Figure 4.3, lanes 2-4) For the TRI-reagent extraction samples (TRI), only a light smear was observed within the 30-37 kD range. **(Figure 4.3, lane 1)** In *P. intermedia* 25611,

again dark protein bands are evident throughout a wide kD range with the (PHENOL) extraction technique and appear very similar to the bands observed within the *P.*

gingivalis samples. **(Figure 4.3, lane 6)** Dark bands are also observed within the (D/H) technique at a similar but slightly higher kD than *P. gingivalis* and not as wide spread as

in the (PHENOL) preparations. **(Figure 4.3, lanes 6 and 7)** Further repurification of this preparation (D/H/V) appears to have removed these dark staining bands from the

samples. **(Figure 4.3, lane 8)**

A small smearing pattern appears at approximately 90-100 kD after the (TRI) extraction technique. In regard to the *E. coli* JM83 samples, the (PHENOL) technique displayed the lowest protein contamination **Figure 4.3, lane 12** whereas the (D/H) had the highest amount of protein banding observed. **(Figure 4.3, lane 13)** A portion of these banding

patterns was still present after (D/H/V) repurification. **(Figure 4.3, lane 14)** The (TRI) extraction sample showed the presence of 2 small bands at approximately 100 kD.

(Figure 4.3, lane 11)

MALDI-TOF analysis

Figure 4.4 reveals the effects on lipid A structure from the various LPS extraction techniques on *E. coli* JM83 samples. Panel A reveals that in *E. coli* JM83, the TRI-reagent extraction (TRI) produces a major mass ion peak at m/z 1798 which corresponds to the reported undegraded lipid A structure of *E. coli* which consists of a diphosphorylated, $\beta(1 \rightarrow 6)$ -interlinked disaccharide that is hexa-acylated. (Zahringer, *et al.*, 1999) and an additional minor peak at m/z 1360 suggesting the loss of a C14:0 (3-OH) and a C14:0. **Panel B** reveals the lipid A structure after hot phenol and water extraction. Here, multiple mass ions are found and correlate as follows: m/z 1798 full lipid A structure; m/z 1570 correlates to a diphosphorylated penta-acylated lipid A with the loss of a C14:0 (3-OH) fatty acid; m/z 1360 corresponds to the loss of an additional C14:0 creating a diphosphorylated, tetra-acylated lipid A and is now the dominate mass ion; m/z 1280 represents the loss of a phosphate group whereas m/z 1133 is still diphosphorylated but has lost an additional C14:0 (3-OH) creating a monophosphorylated, tri-acylated lipid A species. **Panels C and D** represents the lipid A structure after the Darveau and Hancock (D/and Darveau and Hancock followed by the Vogel and Folch repurification techniques (D/H/V), respectively. Here, it is apparent that the same lipid A structures are present with these preparations, as compared to the

(PHENOL) extracted samples, with higher peak values (relative intensity) observed for the m/z 1570, 1280 and 1133 mass ions.

For *P. gingivalis* 33277 samples, the TRI-reagent extraction (TRI) produces a di and monophosphorylated, penta-acylated lipid A with mass ions located at m/z 1770 and 1690, respectively as well as a monophosphorylated, tetra-acylated lipid A moiety that differs in presence or absence of either a C16:0 (3-OH) or C15:0 (3-OH), on the C3 or C3' position, as described previously. **(Figure 4.5, Panel A)** In contrast, the hot phenol-water extraction produced a lipid A preparation without the diphosphorylated, penta-acylated moiety and several unidentified degradation products with mass ion values (m/z) of: 1565, 1383, 1368 as well as 1295, and 1255. Also present in this preparation was the m/z 1435 and 1450 mass ion complex although the relative intensity of these products was low. The Darveau/Hancock (D/H) extraction alone seemed to eliminate any penta-acylated lipid species thereby producing a lipid A preparation consisting of only the m/z 1435 and 1450 mass ion complex which indicated the presence of a monophosphorylated, tetra-acylated lipid A alone, whereas the additional Vogel repurification created not only the 1435/1450 mass ions but the 1195 mass ion as well, indicating the presence of a monophosphorylated, tri-acylated lipid A as described by Ogawa.(Ogawa, 1993) **(Figure 4.5, Panel C)** In addition, this extraction product also contained similar degradation products within the mass ion range of m/z 1360-1398 as was found with the hot phenol-water extraction. **(Figure 4.5, Panel D)** What was interesting in this preparation was the presence of the mono-phosphorylated, penta-

acylated lipid A at m/z 1690. Lastly, the majority of the preparations contained a biologically inactive mass ion located at m/z 1440 that seems to be somewhat unique to *P. gingivalis* 33277 isolate. (Unpublished data)

P. intermedia 25611, when extracted with TRI-reagent procedure (TRI), produces a di and monophosphorylated, penta-acylated lipid A with mass ions located at m/z 1740 and 1660 respectively, as well as a monophosphorylated, tetra-acylated lipid A moiety similar to that observed for *P. gingivalis* and also described previously in Chapter 1. **(Figure 4.6, Panel A)** Again, as was seen in the hot phenol-water extraction (PHENOL) of *P. gingivalis*, the diphosphorylated, penta-acylated lipid A was absent leaving only a monophosphorylated, penta-acylated lipid A species at m/z 1660. Also present was the m/z 1461 mass ion (mono-phosphorylated, tetra-acylated lipid A) as well as a major mass ion at m/z 1222, potentially indicating the presence of a di-phosphorylated, tri-acylated lipid A and an unknown mass ion products at m/z 1369, 1270 and 1062. **(Figure 4.6, Panel B)** The Darveau/Hancock (D/H) extraction alone produced no mono or diphosphorylated pent or tetra-acylated lipid A. **(Figure 4.6, Panel C)** Only unidentified mass ion products at m/z 1369, 1270 and 1062 were observed and additional phenol repurification (D/H/V) resulted in obliteration of any recognizable mass ion with a relatively small peak observed at m/z 1369. **(Figure 4.6, Panel D)**

hTLR4 utilization in HEK 293 cells

To explore the effects of extraction techniques on LPS structure and hTLR4 activation, HEK 293 cells transfected with hTLR4, mCD14 plus human MD-2 were used as described in Chapter 3. Due the potent agonistic affects of *E. coli* LPS on hTLR4 activation, LPS sample from *E. coli* JM83 served as a control but were proportionally used at a reduced concentration compared to the oral LPS samples. Figure 4.7, Panel A shows the potent effects on activation of all *E. coli* LPS samples regardless of the extraction technique with little variation, however, each preparation contained full structure lipid A. **(Review Figure 4.4, Panels A-D)** In contrast, LPS preparations containing no upper lipid A mass ions such as the Darveau/Hancock alone extraction in *P. gingivalis* as well as the Darveau/Hancock and Darveau/Hancock/Vogel extraction technique on *P. intermedia* showed little, if any, activation in HEK cells through hTLR4. **(Compare Figure 4.5, Panel C with Figure 4.6, Panel B) and (Compare Figure 4.6, Panels C and D with Figure 4.7, Panel C) respectively.**

DISCUSSION

Role of acidic conditions and heat during lipid A hydrolysis

Conditions were identified in the previous chapters indicating that pH alteration, temperature and duration of temperature affected the final lipid A product after *in vitro* extraction. It was therefore hypothesized that some of the long standing discrepancies between different research groups, and the reported lipid A structure for *P. gingivalis*,

could be explained, at least in part, by the different extraction procedures utilized. The original mono-phosphorylated, tetra-acylated lipid A, first described by Ogawa as the major lipid A moiety was later reported as only a minor lipid A structural species by Kumada and was only occasionally if rarely found in our preparations utilizing the (CAROFF) hydrolysis technique. (Caroff, *et al.*, 1988; Darveau, *et al.*, 2004; Kumada, *et al.*, 1995; Ogawa, 1993). The reason behind the apparent absence of the mono-phosphorylated, tri-acylated lipid A from the majority of the lipid A preparations, extracted using the (CAROFF) technique, was not clearly understood at the time. (Darveau, *et al.*, 2004)

pH determination of the hydrolysis technique utilizing 0.6% acetic acid revealed a value of 2.85 and the 1.0% acetic acid solution had a pH value of 2.75 whereas the (CAROFF) technique is pH balanced (using sodium acetate) to 4.5 with 1 N HCl in the presence of 1% SDS as summarized in **Table 4.1**. Furthermore, the duration of time in which each LPS sample is subjected to heat is dramatically different from procedure to procedure especially when considering the pH values. Chapter 3 dealt with the affects of temperature in the presence of base solutions and it is apparent from this data that acidic solutions alter the final lipid A product, whereas the duration of time spent in the acidic solutions (1.5 hrs compared to 2.5 hrs) did not seem to create further *O*-deacylation. (**Figure 4.1**) One significant finding was the isolation of the *m/z* 1435/1450 mass ion complex with the apparent absence of upper or lower mass ions using both of the low pH hydrolysis techniques compared to the pH value of 4.5 (CAROFF) which yielded the

higher mass ions at m/z 1690 and 1770 as well as m/z 1435/1450. This result was predicted but what was unexpected was the lack of an m/z 1195 mass ion. This result may indicate that additional time, in the presence of low pH, would be necessary to create the mono-phosphorylated, tri-acylated lipid A as was seen with the ammonium hydroxide procedure or after repeated phenol repurification.

Effects of Extraction Procedures on LPS visualized by SDS-PAGE with silver staining

It has been reported that differences in LPS profiles in SDS-polyacrylamide gels correlate with biochemical differences in LPS. (Hitchcock and Brown, 1983) In fact, the unique banding pattern or “ladder” observed in polyacrylamide gels helped identify certain serologic specificities that led to the serotype classification and agglutination schemes serving as the basis for *Salmonella* identification assays (Rietschel and Westphal, 1999) as mentioned in the Background section. However, it has been widely speculated that some of the observed difference in phenol-water extracted LPS was due to the chemical cleavage of specific chemical linkages (Alaupovic, *et al.*, 1966; Nowotny, 1966; Tsang, *et al.*, 1974) The results presented in this work suggest that different extraction procedures did have an affect on the migration, pattern (single bands vs. doublets vs. smears) as well as the amount of stained bands or content of banding for each of the bacterial samples. In addition, it was observed that additional purifications techniques (i.e. Vogel and Folch procedures) seemed to eliminate specific carbohydrate components, in bacteria such as *P. intermedia*, suggesting that further phenol purification resulted in significant loss of apparent LPS structure in specific bacterial species. This finding is in

agreement with work done on LPS preparations from *Serratia marcescens* where they found that phenol, being slightly acidic, affected acid-sensitive linkage(s) within the lipid moiety and within the core portion of the polysaccharide (PS) especially in the presence of heat. (Tsang, *et al.*, 1974) Additional observations found in work done on *Serratia marcescens* revealed that specific ratios of stained LPS doublets seemed to vary in relation to the fatty acid constituent in lipid A, (Hitchcock and Brown, 1983) and that lipid A heterogeneity was occurring due to fatty acid hydrolysis (up to 11% of the total fatty acid composition) by the presence of heated 45% phenol in an aqueous solution. (Tsang, *et al.*, 1974)

Effects of different LPS extraction techniques on LPS and lipid A yields.

It has been reported that two of the most widely used techniques to extract LPS from the majority of Gram-negative bacteria are the hot phenol-water (PHENOL) technique and the Darveau/Hancock (D/H) technique. (Eidhin and Mouton, 1993) Recently, an additional technique, using a commercial reagent (TRI-reagent) has been used successfully to extract LPS from a variety of bacteria. (Yi and Hackett, 2000) Therefore, these three extraction techniques served as the basis for the experimental procedures. As stated, total yields of LPS from the varying techniques displayed in **Table 4.2** are in agreement with previous publications reporting approximately a 1-10% LPS yield recovery from dried whole cells. (Darveau and Hancock, 1983; Eidhin and Mouton, 1993) However, it was interesting to note that although the Darveau/Hancock procedure (D/H) had the highest overall yield (avg. 8.8%) compared to the TRI-reagent (TRI) extraction

(avg. 3.8) and then the hot phenol-water (PHENOL) with a 1.4% yield average, it also had the highest variation between bacterial samples (\pm SD = 8.69). On the other hand, typical low yields from the PHENOL technique have encouraged researchers to modify this procedure in an attempt to increase overall yields (Folch, *et al.*, 1957; Kropinski, *et al.*, 1982; Schneider, *et al.*, 1982) This has resulted in exclusion of specific types of LPS (Darveau and Hancock, 1983) as well as additional unknown structural effects.

Furthermore, there was significant variability in the ability to extract LPS from *P. intermedia* 25611 using the different techniques. **(Table 4.2)** In our experiments, the (PHENOL) technique resulted in very low LPS amounts whereas the (D/H) resulted in the highest amounts. This might indicate that LPS degradation in *P. intermedia* is very susceptible to chemical denaturation (PHENOL vs D/H) or that something additional to the LPS is present in preparations after the (D/H) procedure.

In regard to lipid A recovery, percent (%) yields after the (CAROFF) technique resulted in highest yields from TRI extracted LPS and lowest from the (PHENOL) technique suggesting that high concentrations or duration of phenol with LPS degrades the molecule regardless of the bacterial sample. Lower yields after the Vogel repurification (repeated phenol repurification) have also been observed with samples first extracted without the use of phenol as in the (D/H) procedure. (unpublished data) In addition, as was observed in LPS extractions from *P. intermedia* 25611, lipid A yields were also lowest from *P. intermedia* samples when compared to the other bacterial samples; and after (PHENOL) LPS extraction, were the lowest overall further confirming the disruptive nature of phenol on specific oral LPS(s). **(Table 4.3)**

Effects of Different Extraction procedures on Lipid A structure

After determining that different lipid A hydrolysis procedures either altered or was selective for specific lipid A structures, we next investigated the role that specific LPS extraction techniques had on lipid A structure. Here, the three described extraction techniques were employed to separate LPS from whole bacterial cells and then these samples were subjected to the same lipid A hydrolysis technique and evaluated by MALDI-TOF analysis. Using *E. coli* JM83 as a standard, we observed that when this bacterium was subjected to repeated phenol exposures, with or without the application of heat, apparent disruption of the m/z 1798 mass ion occurred as evidenced by the reduction of the relative intensity of the m/z 1798 mass ion and the appearance of the additional mass ions at m/z : 1570, 1360, 1280, and 1133. **(Compare Figure 4.4, Panels B and D with Panel A)** Furthermore, repeated phenol repurification, as with the Vogel and Manthey procedure, seemed to increase the loss of lipid A structure as revealed by the relative intensity increase of the m/z : 1570, 1280 and 1133 mass ions. **(Compare Figure 4.4, Panel D to panels B and C)**. This result was predicted and has been initially characterized in phenol extractions in LPS from *Serratia marcescens*. Here, they found that although exposure to phenol for 10min or less resulted in less than 1% of ester-linked fatty acid hydrolysis, exposure for 60 min resulted in up to 11% hydrolysis of the total fatty acid content. In the Darveau/Hancock procedure, after mechanical disruption of the whole cells and proteinase and DNase/RNase treatments, the pellet is solubilized in a solution containing 0.5 M tetrasodium EDTA, 20 % SDS and Tris buffer with a pH of 9.8 which is neutralized with 4N HCl and incubated at 85°C for 30 min. Following this, the

pH of the solution is then adjusted to 9.5 with 4N NaOH and incubated overnight with shaking. The rationale for the alkaline conditions is to assist in removal of SDS-resistant proteins potentially contained in the preparation. However, in Chapter 3, experimental evidence revealed that exposure to alkaline conditions results in ester-linked fatty acid hydrolysis (*O*-deacylation) and additional experiments with NaOH resulted in both ester and amide-linked fatty acid hydrolysis. Therefore, it was predicted that this procedure would have the tendency to selectively cleave the ester-linked fatty acids with the potential for amide-linked hydrolysis as well. Results from this experiment, as evidenced in **Figure 4.4, Panel C**, confirmed this prediction and suggests that application of alkaline conditions for extended periods may result in fatty acid hydrolysis or dephosphorylation similar to that observed and reported with the application of phenol and heat. (Tsang, *et al.*, 1974)

Similar effects were observed in *P. gingivalis* 33277 and *P. intermedia* 25611 lipid A. Specifically, hot phenol applied to *P. gingivalis* and *P. intermedia* removes a phosphate group from the core disaccharide as seen by the reduction of 80 mass units from the highest observed peak in **Panel A** in both **Figure(s) 4.5 and 4.6** resulting in the a mass ion of *m/z* 1690 and 1660 for *P. gingivalis* and *P. intermedia* respectively. (**Compare Panel(s) B to A in both Figures 4.5 and 4.6**) In addition, the hot phenol technique resulted in increased unidentified degradation products in both bacteria. Furthermore, in *P. intermedia*, results revealed the presence of a mass ion located at approximately *m/z* 1222 which would indicate the presence of a tri-acylated lipid A that was not observed in

any other extraction procedure. In regard to the Darveau/Hancock procedure, for *P. gingivalis*, only the m/z 1435/1450 was observed suggesting that this procedure potentially selects for these particular lipid A products as has been reported. (Darveau, *et al.*, 2004) As predicted, further phenol purification resulted in increase fatty acid hydrolysis and is shown with the appearance of the m/z 1195 peak suggesting the presence of a monophosphorylated, tetra-acylated lipid A as well as the presence of some additional unknown mass ions. In *P. intermedia*, the Darveau/Hancock procedure resulted in multiple unidentified peaks and with the additional purification (D/H/V), the lipid A was completely disrupted revealing the labile nature of the chemical linkages within *P. intermedia* lipid A. **(Compare 4.6, Panel(s) C and D with B and A)**

Effects of Different Extraction Procedures on hTLR4 utilization in HEK 293 cells

We then wanted to explore the role potential alterations in LPS from these different extraction techniques had on hTLR4 utilization. Initial pilot experiments with and without the use of membrane bound CD14 (mCD14) or soluble CD14 (sCD14) revealed the necessity for mCD14 to be co-transfected for use in this HEK system for the detection of oral bacterial LPS whereas in the *E. coli* LPS samples, either was sufficient. (Data not shown) Therefore, after HEK 293 were transfected with both hTLR4, mCD14 as well as human MD-2, cells were exposed to incremental concentrations of the different LPS samples. As expected, in *E. coli* JM83, all samples maintained activity through hTLR4 and were potent at reduced concentrations compared to the oral LPS samples. **(Figure 4.7, Panel A)** However, activation was lost in *P. gingivalis* samples prepared with

Darveau/ Hancock (D/H) as well as *P. intermedia* samples prepared with Darveau Hancock with (D/H/V) or with out (D/H) additional phenol purification. **(Figure 4.7, Panel(s) B and C)** Here, the lipid A MALDI-TOF analysis reveals the absence of any full structure lipid A in these preparations. **(Figure 4.5, Panel C and Figure 4.6, Panel(s) C and D)** Furthermore, the results indicate that the loss of one phosphate group, compared to the loss of a phosphate group plus a fatty acid from the core disaccharide was not sufficient to lose hTLR4 activation in these cells. This is evidenced by the preparations that maintained a full structure lipid A (minus a phosphate group) possessing hTLR4 activation whereas those preparations completely void of any full structure lipid A had no hTLR4 activity. **(Example: compare Figure 4.5, Panel C to Panel(s) A, B and D with Figure 4.7 activation patterns)** This suggests that to have activation through hTLR4, in cells co-transfected with mCD14 and human MD-2, full structure of the lipid A molecule (or full structure minus a phosphate group) is needed or is sufficient for activation through hTLR4 even in the presence of hydrolysis products. However, loss of these essential structures eliminates activation. This observation is in full agreement with previous reports identifying critical lipid A components in synthetic lipid A preparations. (Takada and Kotani, 1989; Takada and Kotani, 1992)

Total Protein staining of purified preparations

Although it has been shown that the bacterial lipoproteins or other endotoxin associated proteins seem to utilize different hTLRs other than hTLR4, the Colloidal Gold stain was used to determine the presence of potential contaminants that could interfere with these

hTLR4 utilization studies as well as documenting the residual protein content found within these purified LPS preparations. **(Figure 4.3)** What was intriguing about these results was the considerable variability of residual protein found after different extraction procedures were employed. For example, high residual protein content was found during (PHENOL) extraction of *P. gingivalis* 33277 and *P. intermedia* 25611 but not for the rough strain *E. coli* JM83. On the other hand, the highly purified (D/H/V) preparation showed residual protein in the *E. coli* JM83 but were observed to be one of the purest preparations for both of the oral bacterial samples. The (TRI) extraction appeared to be the most consistent, in regard to low levels or absence of residual protein; however, it appears that only the highly purified (D/H/V) preparations seemed to be completely free of protein contamination. Considering all of this, the application of phenol during extraction of LPS and further repurification seems advantageous however, it is apparent that this purification comes at quite a cost to the overall LPS structure.

Extraction degradation and chemical labile features within lipid A

The first described use of an 88% or 95% concentration of phenol was by Palmer and Gerlough (Palmer and Gerlough, 1940) who showed the capability of this organic solvent to dissociate LPS from bacterial cells. (Tsang, *et al.*, 1974) Since then many modifications have occurred including the application of heat and dialysis (Westphal and Jann, 1965) which improved the overall reduction of retained protein contamination over low temperature phenol applications. Later, with the application of petroleum ether to a mixture of chloroform and phenol, preferential extraction of “rough” LPS resulted as

compared to the “smooth” type of LPS seen with the hot phenol and water technique. In the 1980’s, the Darveau and Hancock (D/H) method, which did not incorporate the use of phenol during LPS extraction, seemed to improve the overall effectiveness of extracting both smooth and rough forms of LPS from a variety of organisms as well as having the potential to increase LPS yields in *P. aeruginosa* bacterial samples. (Darveau and Hancock, 1983)

However, some researchers found that within oral bacteria such as the *Bacteriodes*, *Porphyromonas* or *Prevotella* species, the use of these techniques had significantly low LPS yields or due to the presence of SDS, reacted poorly with several LPS-specific monoclonal antibodies. (Eidhin and Mouton, 1993) Furthermore, with the recent ability to transfect and express specific LPS-specific receptors and co-associated molecules within cells, the exact stimulatory mechanisms are now becoming apparent, however, it is also apparent that any additional protein contamination within LPS extraction preparations could affect specific hTLRs involved within the LPS signaling cascade. Results from these experiments suggest that specific extraction techniques do, in fact, contain variable inherent abilities to extract different amounts LPS from whole dried bacterial cells with and without the presence of contaminating proteins.

In addition, these results show that due to the potential chemical labile nature of glycosidic and ester-linked bonds present within the LPS as well as the lipid A molecule, certain chemicals or pH conditions employed, in addition to specific extraction

procedures (presence of heat, vortexing, sonication, ect.) can either significantly alter or selectively extract specific forms of the LPS as well as the lipid A component.

Furthermore, these results confirm that structural differences within LPS preparations from different bacteria as well as within preparations from the same bacteria has shown to be critical in hTLR4 utilization. Efforts to purify LPS preparation such as in the Manthey and Vogel protocol, which consists of a modified phenol re-extraction of LPS to eliminate trace endotoxin protein contamination, is beneficial in removing residual protein but was also shown to be highly disruptive to the lipid A molecule in all of the bacterial samples tested.

Currently, there is no LPS extraction technique that can be applied universally, extracting high yields with equal amounts of rough and smooth LPS forms, without structural degradation or limitation due to incompatibilities with chemical additives and antibody-based assays. Therefore, during LPS extraction it would be prudent to try to reduce the effects of these agents to enable a delicate balance between purity and structural integrity. From observations such as these, it has become widely recommended that any LPS or lipid A preparation (regardless of how prepared or obtained) should be examined for content *via* mass spectrometry, thin layer chromatography (TLC)(Caroff, *et al.*, 2002), or SDS-PAGE with additional residual total protein determination *before* host assays are performed. Furthermore, although some have advocated the use of species specific extraction techniques, it must be realized that specific procedures might disrupt the

original form of LPS molecules resulting in altered biologic function thereby negating any beneficial effect of high yields or overall preparation purity.

Table 4.1 pH values of three different hydrolysis techniques used for lipid A isolation from oral bacterial LPS.

	Ogawa hydrolysis	Kumada hydrolysis	Caroff hydrolysis
pH	2.85 ^a	2.75 ^b	4.5 ^c
Temperature	105°C	100°C	100°C
Time	2.5 hrs	1.5 hrs	1 hr

^a: pH value calculated from a 0.6% acetic acid solution in H₂O

^b: pH value calculated from a 1.0% acetic acid solution in H₂O

^c: pH value calculated from a 10mM sodium acetate solution in the presence of 1% SDS pH adjusted with 1N HCL

Table 4.2 LPS % yield for *P. gingivalis* ATCC33277, *P. intermedia* ATCC 25611, and *E. coli* JM83 using three different LPS extraction techniques. Darveau/Hancock extraction technique yielded the largest variability in terms of total amount for these three bacterial strains.

	<i>P. gingivalis</i> 33277	<i>P. intermedia</i> 25611	<i>E. coli</i> JM83	
	LPS % yield	LPS % yield	LPS % yield	Avg (\pm SD)
TRI-REAGENT ^a	2.4	4.4	4.6	3.8 (1.21)
PHENOL-WATER ^b	2.1	0.47	1.5	1.4 (0.82)
DARVEAU/HANCOCK ^c	1.7	18.5	6.2	8.8 (8.69)
Avg (\pm SD)	2.1 (0.35)	7.8 (9.48)	4.1 (2.39)	

^a: % yield based on calculations starting from dry weight of whole cells (50mgs); samples were purified by cold MgCl₂ plus ETOH washes before weighing

^b: % yield based on calculations starting from dry weight of whole cells (500mgs)

^c: % yield based on calculations starting from dry weight of whole cells (1000mgs); samples were purified by cold MgCl₂ plus ETOH washes before weighing.

Table 4.3 Lipid A % yield for *P. gingivalis* ATCC33277, *P. intermedia* ATCC 25611, and *E. coli* JM83 from purified LPS obtained by using three different LPS extraction techniques.

	<i>P. gingivalis</i> 33277	<i>P. intermedia</i> 25611	<i>E. coli</i> JM83	
	Lipid A % yield	Lipid A % yield	Lipid A % yield	Avg (\pm SD)
TRI-REAGENT ^a	58	50	78	62.0 (14.22)
PHENOL-WATER ^b	36	17	25	26.0 (9.53)
DARVEAU/HANCOCK ^c	64	42	25	43.6 (19.55)
Avg (\pm SD)	52.7 (14.74)	36.3 (17.21)	42.7 (30.60)	

^a: % yield based on calculations starting from purified LPS of 1.2 mg, 2.2 mg and 2.3 mg respectively

^b: % yield based on calculations starting from purified LPS of 5.0 mg, 1.2 mg and 6.0mg respectively

^c: % yield based on calculations starting from purified LPS of 2.2 mg, 6.0 mg and 5.5 mg respectively

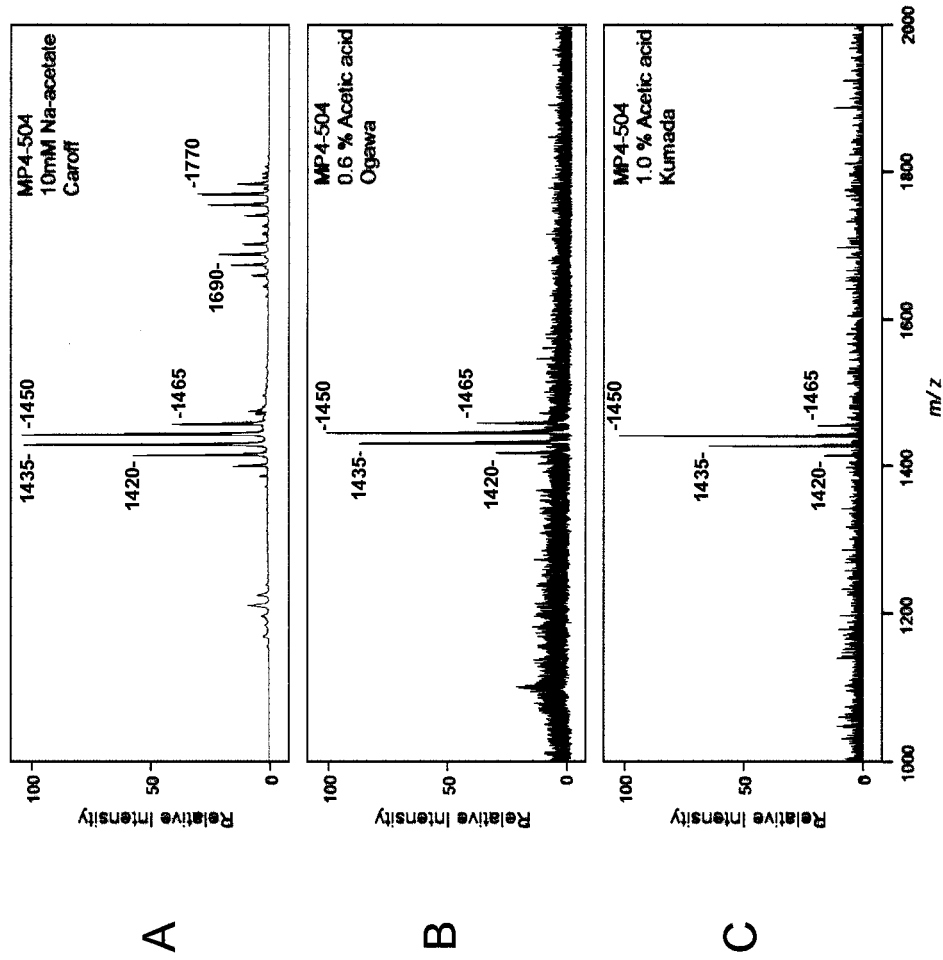


Figure 4.1 MALDI-TOF analysis of *P. gingivalis* MP4-504 using different hydrolysis techniques. Panel (A) represents baseline, lipid A extracted using 10 mM sodium acetate in the presence of 1% SDS as described by Caroff et al. Panel (B) and (C) represents the lipid A product after hydrolysis using 0.6 % acetic acid heated at 105°C for 2.5 hrs as described by Ogawa et al. or 1.0 % acetic acid heated to 100°C for 1.5 hrs as described by Kumada et al.

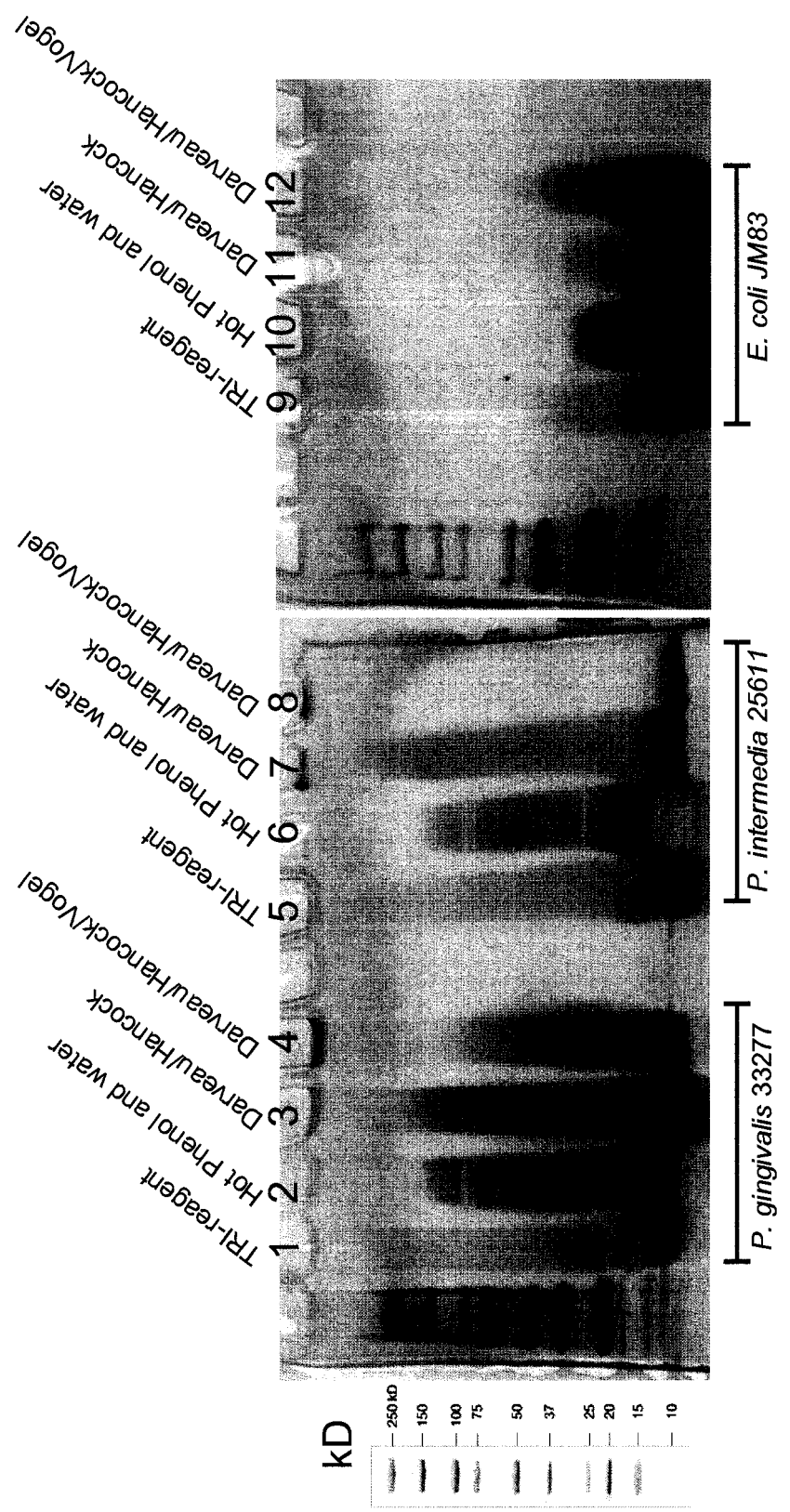


Figure 4.2 SDS-PAGE with preferential staining of LPS with the silver stain technique. LPS was extracted with either the TRI-reagent, Hot Phenol and Water, Darveau/Hancock or Darveau/Hancock/Vogel technique. The various gels represent the extraction effects on LPS structure from *P. gingivalis* 33277, *P. intermedia* 25611 or *E. coli* JM83.

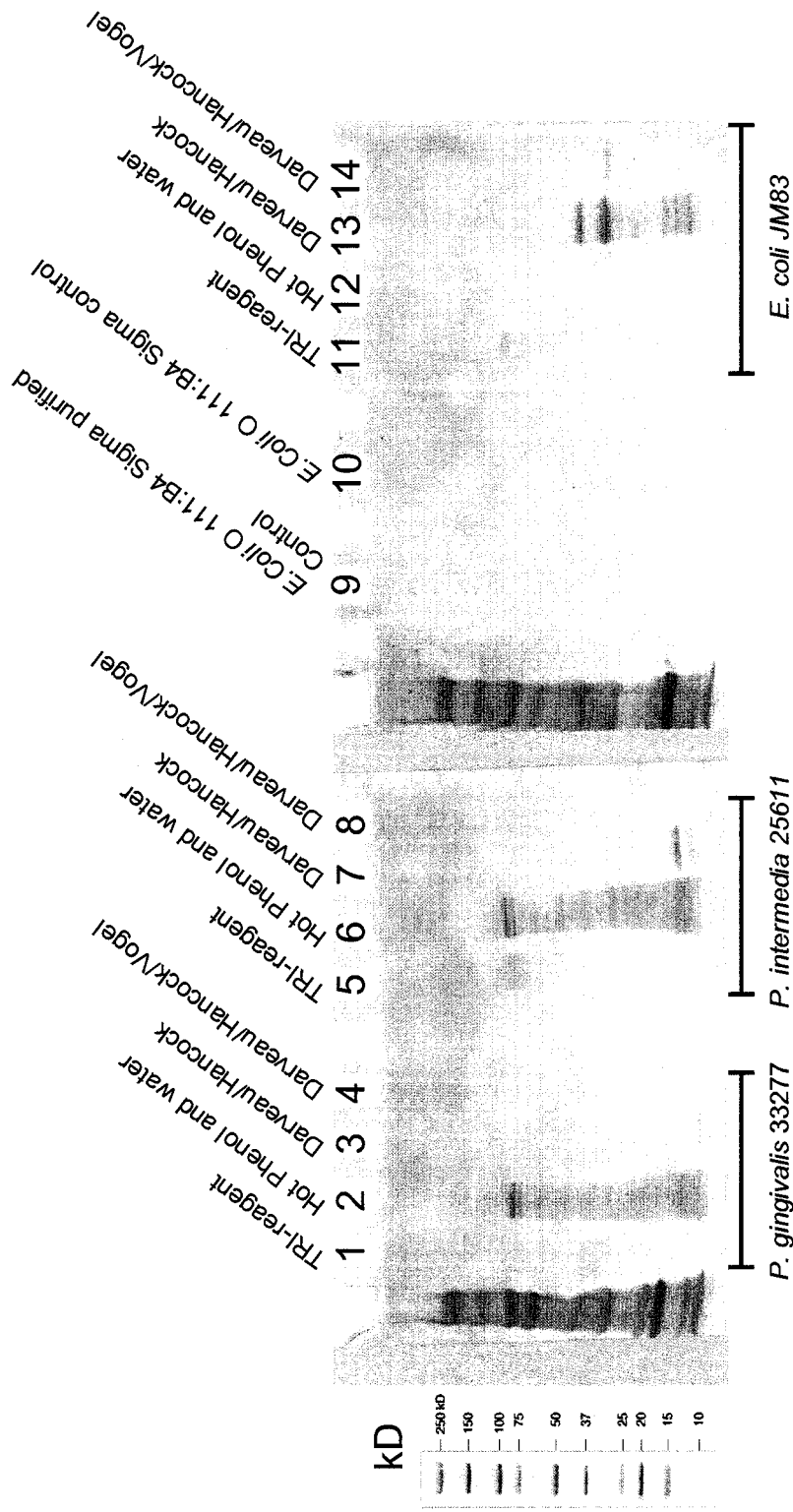


Figure 4.3 Colloidal Gold Total Protein staining of LPS preparations in which protein bands stain dark red. LPS was extracted with either the TRL-reagent, Hot Phenol and Water, Darveau/Hancock or Darveau/Hancock/Vogel technique. Individual lanes indicate presence or absence of residual protein other than LPS remaining in purified LPS preparations. For both *P. gingivalis* and *P. intermedia*, the hot Phenol-water technique had the highest amount of residual protein (lanes 2 and 6) whereas in *E. coli*, the Darveau/Hancock without the Vogel and Folch procedure contained the most (lane 13). Lane 9 and 10 serve as LPS controls obtained commercially (lane 10) and repurified. (lane 9)

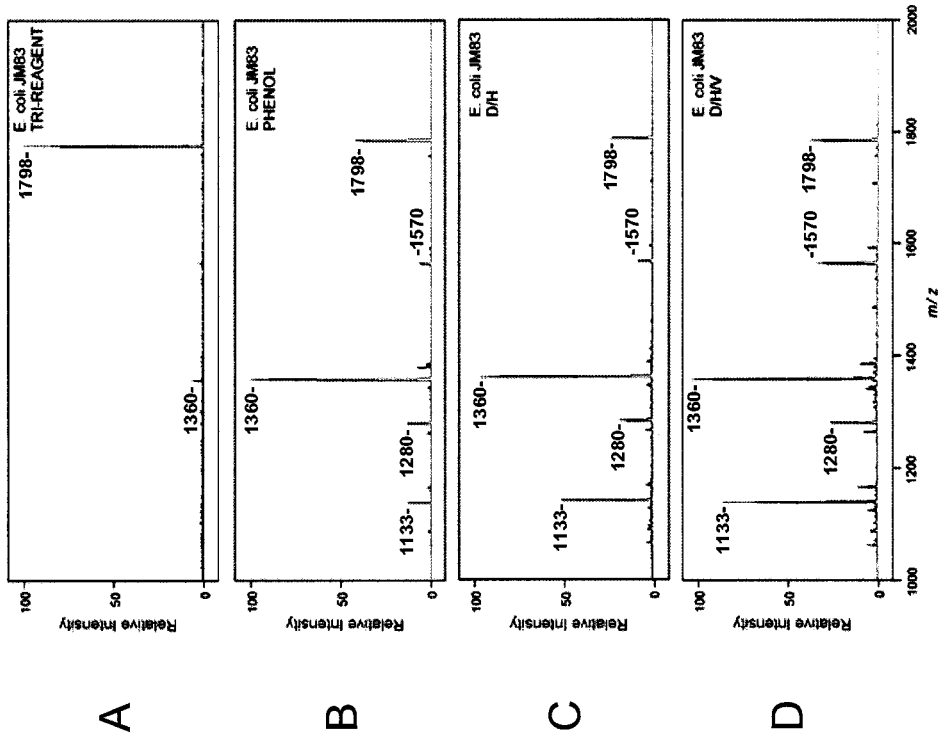


Figure 4.4 MALDI-TOF analysis of *E. coli* JM83 lipid A using different LPS extraction procedures. Panel (A) represents lipid A results after TRI-reagent was used to extract the LPS. Panel (B) represents the lipid A after the hot Phenol-water procedure was used to extract the LPS. Panels (C) represents the lipid A product after the Darveau and Hancock procedure and (D) represents the lipid A product after the Darveau and Hancock procedure followed by repurification using the Vogel and Folch techniques.

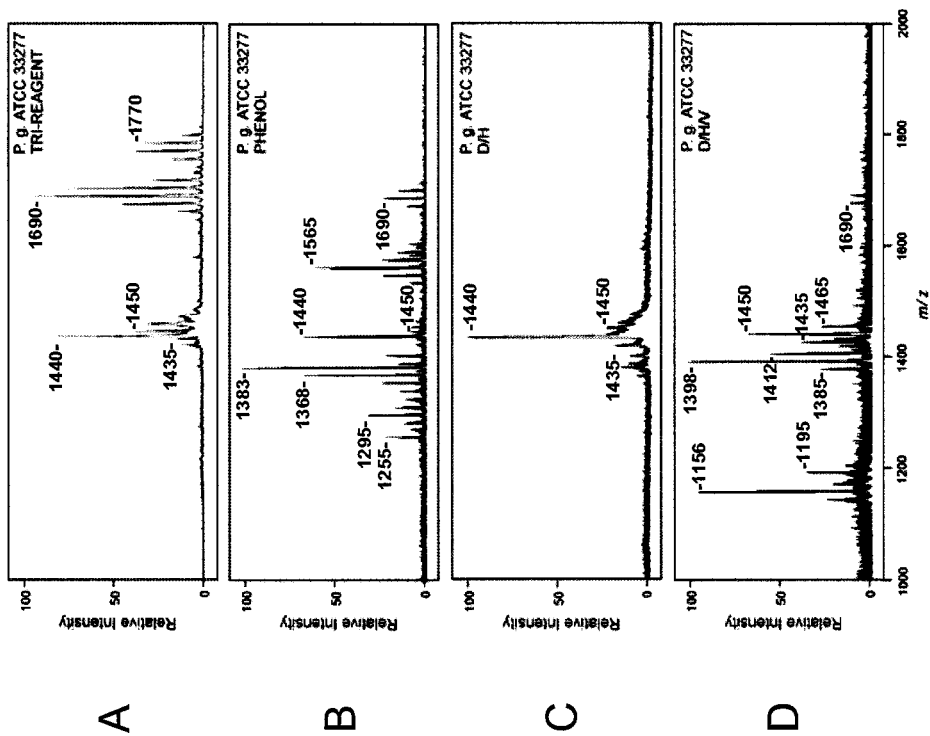


Figure 4.5 MALDI-TOF analysis of *P. gingivalis* 33277 lipid A using different LPS extraction procedures. Panel (A) represents lipid A results after TRI-reagent was used to extract the LPS. Panel (B) represents the lipid A after the hot Phenol-water procedure was used to extract the LPS. Panels (C) represents the lipid A product after the Darveau and Hancock procedure and (D) represents the lipid A product after the Darveau and Hancock procedure followed by repurification using the Vogel and Folch techniques.

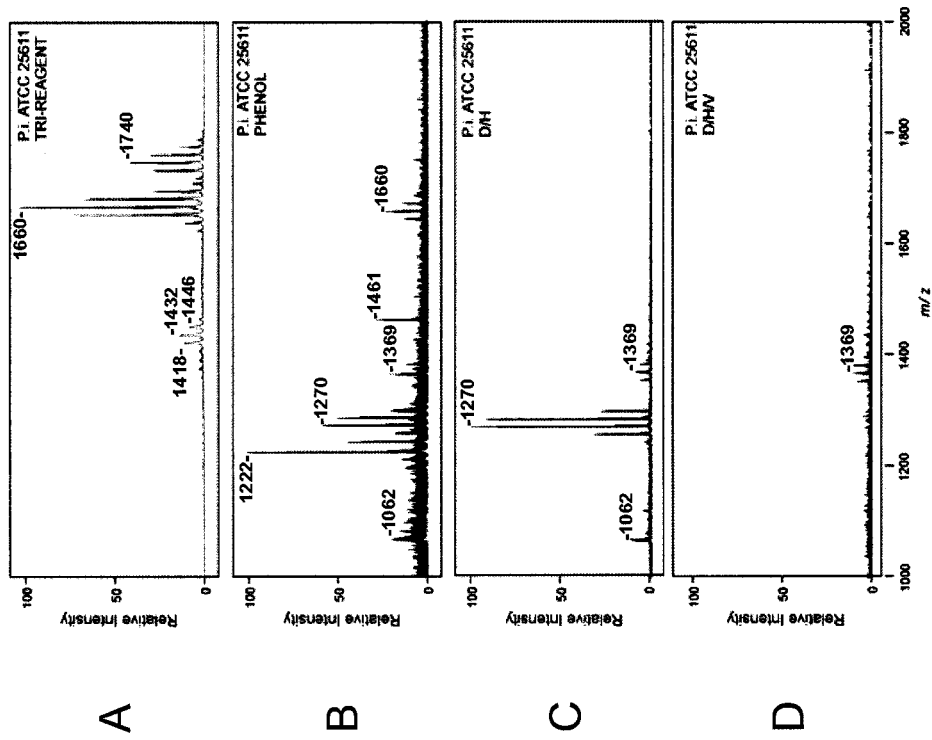


Figure 4.6 MALDI-TOF analysis of *P. intermedia* 25611 lipid A using different LPS extraction procedures. Panel (A) represents lipid A results after TRI-reagent was used to extract the LPS. Panel (B) represents the lipid A after the hot Phenol-water procedure was used to extract the LPS. Panels (C) represents the lipid A product after the Darveau and Hancock procedure and (D) represents the lipid A product after the Darveau and Hancock procedure followed by repurification using the Vogel and Folch techniques.

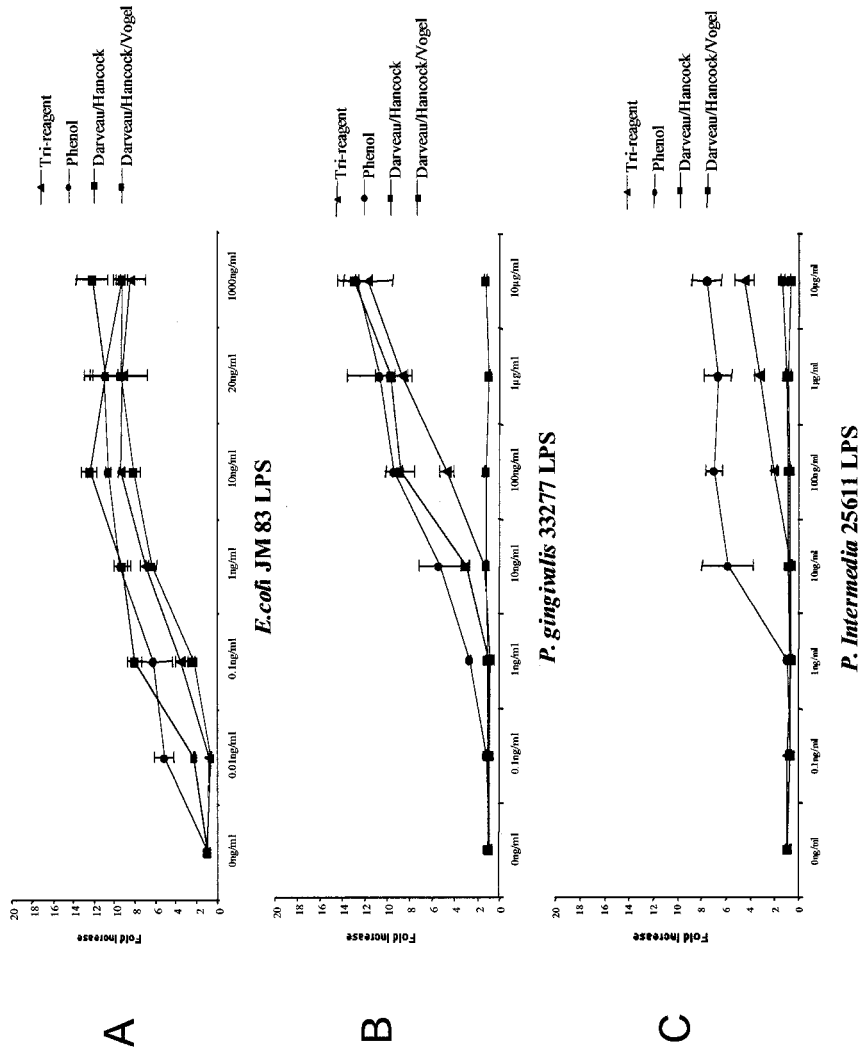


Figure 4.7 hTLR4 activation with LPS samples from three different bacteria using different LPS extraction and purification procedures. HEK 293 cells were transiently transfected with human mCD14, hTLR4E and human MD-2 together with the NF- κ B reporter (ELAM-1 firefly luciferase) and the transfection control (β -actin-Renilla luciferase) as previously described. Results indicate that specific bacterial samples are resistant to extraction procedures (Panel A) compared to other bacterial samples (Panel B and C). In addition, exposure to extreme basic or acidic conditions in *P. gingivalis* (D/H) or phenol repurification in *P. intermedia* (D/H/V) significantly reduced or eliminated activation of LPS through hTLR4 in the presence of mCD14 and MD-2. (Panels B and C)

CONCLUSIONS

The aim of this project was to investigate the role of natural LPS heterogeneity as well as experimental structural modification, with special emphasis on the lipid A portion, within innate host defense system assays. To do so, specific experiments were carried out to test the hypothesis that lipid A heterogeneity exists both naturally and experimentally derived within specific Gram-negative bacteria, which can affect activation or alter levels of innate immune modulators from host cells. The four specific aims outlined in the introduction were met. First, we determined that there was a high degree of endogenous lipid A heterogeneity within oral Gram-negative bacteria and that this heterogeneity correlated with E-selectin expression. This lipid A heterogeneity was limited to only a few select bacteria which also correlated with known pathogenic features possessed by these species. Secondly, a series of experiments were designed to identify specific environmental conditions that would regulate or explain the heterogeneity observed in specific bacterial species. Here, specific phases of growth, growth within different media states (liquid vs. solid) or altered pH conditions played a role in the relative abundance of specific lipid A moieties within *P. gingivalis*. These results indicated that certain environmental conditions, such as those potentially seen *in vivo* by the bacterium, might affect or change the structural components of the lipid A and partially explain the heterogeneity observed within these samples. Third, selective hydrolysis of ester-linked fatty acids within the lipid A molecule was accomplished and was shown to have a dramatic effect on host activation and response. These results indicate that any bacterium

with the ability to modify its own LPS structure would have an enormous impact on its ability to survive and grow by evading host responses. Finally, the conditions resulting in *in vitro* lipid A structural heterogeneity (experimentally induced) were identified for specific oral and enteric bacteria. The ability of specific LPS extraction reagents or experimental conditions to partially degrade or select for specific lipid A moieties became apparent and was correlated with specific hTLR utilization. What factors that remain to be determined is the degree of overall LPS modifications that occur, and the extent of bacteria that are susceptible to these reagents and/or identified procedural conditions.

It has long been understood that *P. gingivalis* is a significant periodontal pathogen and has been associated with multiple forms of periodontal disease. (Socransky and Haffajee, 2002; Socransky, *et al.*, 1998; Socransky, *et al.*, 1988) What is novel about these findings is the extent of the lipid A variability possessed by this bacterium, regardless of the strain or isolate tested, when compared to other Gram-negative oral or enteric bacteria. Furthermore this variability seemed to extend into other members of the genus *Bacteroides*, and when present, resulted in a lower E-selectin expression (2 fold or greater) within HUVECs. (Chapter 1) Within *P. gingivalis*, this lipid A variability was linked to environmental conditions; the most remarkable result being the loss of mono or diphosphorylated, penta-acylated lipid A in the presence of alkaline environment. Alkaline conditions have been reported to occur in the mouth in response to inflammation.(Bickel and Cimasoni, 1985) Here, it was observed that as clinical

inflammation increased, a pH change from 6.9 to 8.6 occurred within the gingival sulcus. Our results showed that when *P. gingivalis* was grown in culture medium with an initial pH of 9.0, lipid A structure was significantly changed compared to results found with neutral or acidic media. (Chapter 2) This same finding occurred when purified lipid A was subjected to a mild base (NH₄OH) as described in the Materials and Methods section of Chapter 3. In these experiments, application of base chemicals selectively de-acylated ester-linked fatty acids in a predictable and sequential manner that mirrored the effect seen in the alkaline media experiments. Furthermore, any oral bacterial lipid A, when subjected to NH₄OH, lost ester-linked fatty acids from the core lipid A disaccharide in the same manner as observed in *P. gingivalis*. This deacylation effect revealed the universal labile nature of this ester-linkage within oral bacteria. Once deacylation had occurred, hTLR4 utilization as well as E-selectin expression was significantly reduced. (Chapter 3) Furthermore, these deacylated lipid A preparations were then shown to possess the ability to antagonize E-selectin expression from unaltered oral LPS. Significant deacylation, resulting in a tri-acylated lipid A product resulted in loss of all biologic activity which has been previously confirmed. (Ogawa, *et al.*, 2000; Ogawa, *et al.*, 1994)

Identifying and distinguishing natural lipid A heterogeneity from experimentally induced heterogeneity remains one of the major analytical challenges when dealing with LPS preparations. (Yi and Hackett, 2000) Successful cleavage of lipid A from LPS has allowed for structural characterization of a number of oral Gram-negative bacteria.

However, the biologic ramification of the natural or artificially induced heterogeneity contained within these different bacterial species LPS preparations is not currently understood. Experimental procedures and results outlined in (Chapter 4) revealed that there are significant differences in the methods used to extract and purify LPS and that these experimental conditions have a dramatic effect on the LPS structure as a whole as well as its individual components. Increasingly acidic conditions during lipid A hydrolysis resulted in the elimination of mono and diphosphorylated, penta-acylated lipid A from *P. gingivalis*. This observation helps explain the discrepancies within the reported structure for *P. gingivalis* lipid A from research groups using acetic acid hydrolysis (Kumada, *et al.*, 1995; Ogawa, 1993), compared to those using the less acidic hydrolysis technique described by Caroff (Caroff, *et al.*, 1988). It may also help explain differences in their reported biologic activities. (Darveau, *et al.*, 2004) Different extraction procedures also resulted in removal of specific carbohydrate components of LPS as evidence by altered migration patterns during SDS PAGE procedures. In regard to purification, certain extraction procedures were superior to others in eliminating residual protein contamination which is of major concern when applying these preparations to host cell assays.(Hirschfeld, *et al.*, 2000)

An issue sharing equal importance with removal of residual protein contamination is the ability of experimental conditions/procedures to separate LPS/lipid A from whole bacterial cells without structural damage or alteration. LPS extraction procedures revealed a wide range of effects within different oral as well as enteric bacterial samples.

Those procedures, such as the application of heated phenol or the addition of base chemicals during extraction procedures that resulted in the removal of critical lipid A components (such as phosphate groups or fatty acids) were also shown to have a lowered or abolished hTLR4 activation. These results are in agreement with other endotoxin degradation studies with phenol (Takada and Kotani, 1989; Takada and Kotani, 1992; Tsang, *et al.*, 1974) as well as the structure/activity relationships studies done with synthetic lipid A. (Takada and Kotani, 1989; Takada and Kotani, 1992)

Further examination of this observation led to the question of which extraction procedure might be best for oral Gram-negative LPS extraction and purification. **Figure C.1 and C.2** displays the silver-stained LPS migration patterns as well as the residual protein contamination after the (TRI) reagent extraction procedure and purification. After extraction and purification of a number of oral and enteric Gram-negative bacteria it became quite evident that each LPS extraction procedure or technique has advantages as well as disadvantages when working with specific bacterial species. However, it is apparent that within oral bacteria, the application of phenol is best performed at room temperature and the application of base or acid chemicals, especially in the presence of heat, should be avoided to reduce any structural alterations in the LPS.

A secondary purpose of this investigation was to attempt to define the relationship between lipid A structure and host cell function. It has been reported that a relationship exists between specific oral bacteria and inflammatory disease states of epithelial tissues

within the oral environment.(Socransky and Haffajee, 2002; Socransky, *et al.*, 1998; Socransky, *et al.*, 1988) Bacterial classification systems, based on association to these specific inflammatory disease states of the periodontium have grouped certain bacterial species into pathogenic and non pathogenic microbial complexes. However, what is evidenced within this work is the effect of different oral lipid A moieties on host functions that arose naturally as well as from chemical manipulation of the lipid A molecule itself. What is interesting about these observations is that the possibility of lipid A heterogeneity arising from an altered environmental conditions or through inherent lability of chemical bonds within critical antigenic structures may suggest potential targets. These targets, if exploited, may result in pharmacological methods to chemically decrease or reduce the significance of the destructive inflammatory host response, especially those that occur during chronic type infections with specific oral bacteria. Furthermore, if specific bacteria, such as *P. gingivalis* have evolved ways (either by random chance through the inability of chemical linkages to withstand environmental conditions or purposely through a phosphatase or deacylase-type enzyme activity encoded within its bacterial genome) to alter its individual lipid A structure, then this suggest a novel and exciting way these bacterial species might direct specific host responses towards a more favorable outcome for bacterial survival. **(Figure C.3)** Lastly, it is interesting to speculate that a deacylase, under the direct control of a bacterium, might also be able to affect the LPS products from neighboring bacteria. This may then result in significant control of local environments, such as those reported within dental

biofilms (Socransky and Haffajee, 2002), and well as the corresponding host responses towards pathogenic as well as commensal bacteria.

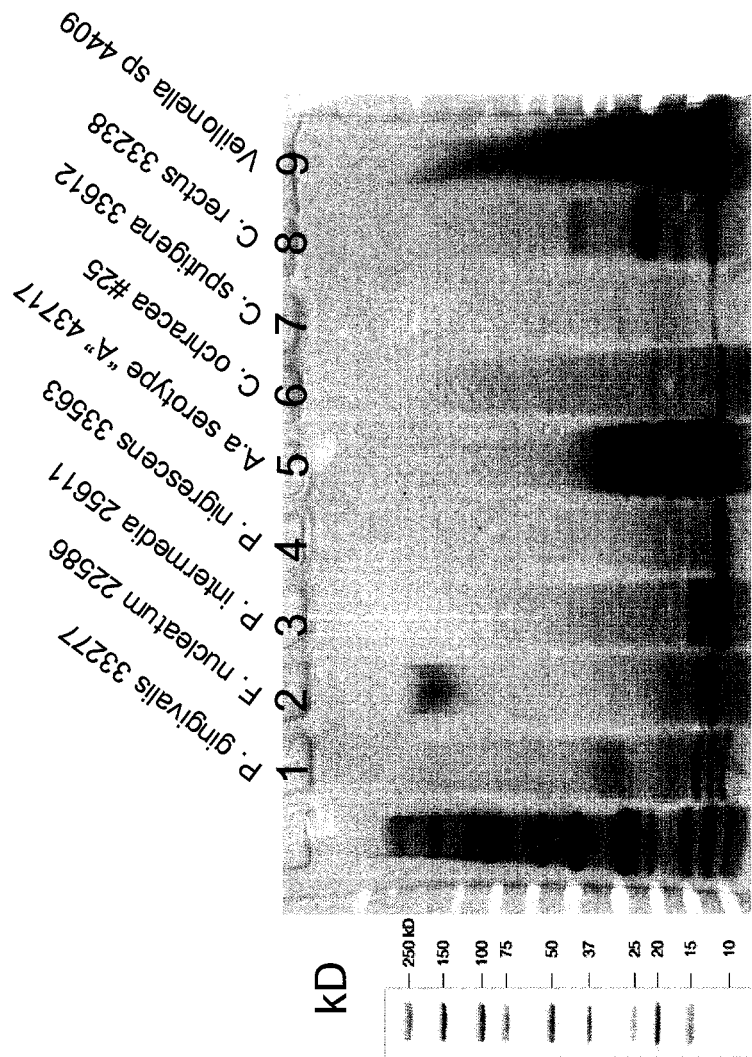


Figure C.1 SDS-PAGE with preferential staining of LPS with the silver stain technique. LPS(s) were extracted with the TRI-reagent technique as described in the text. 10µg of LPS were loaded into these wells and subjected to electrophoresis at 80V for 1-2 hrs. This gel reveals the unique migration patterns resulting from the different LPS constituents possessed by these oral bacteria species.

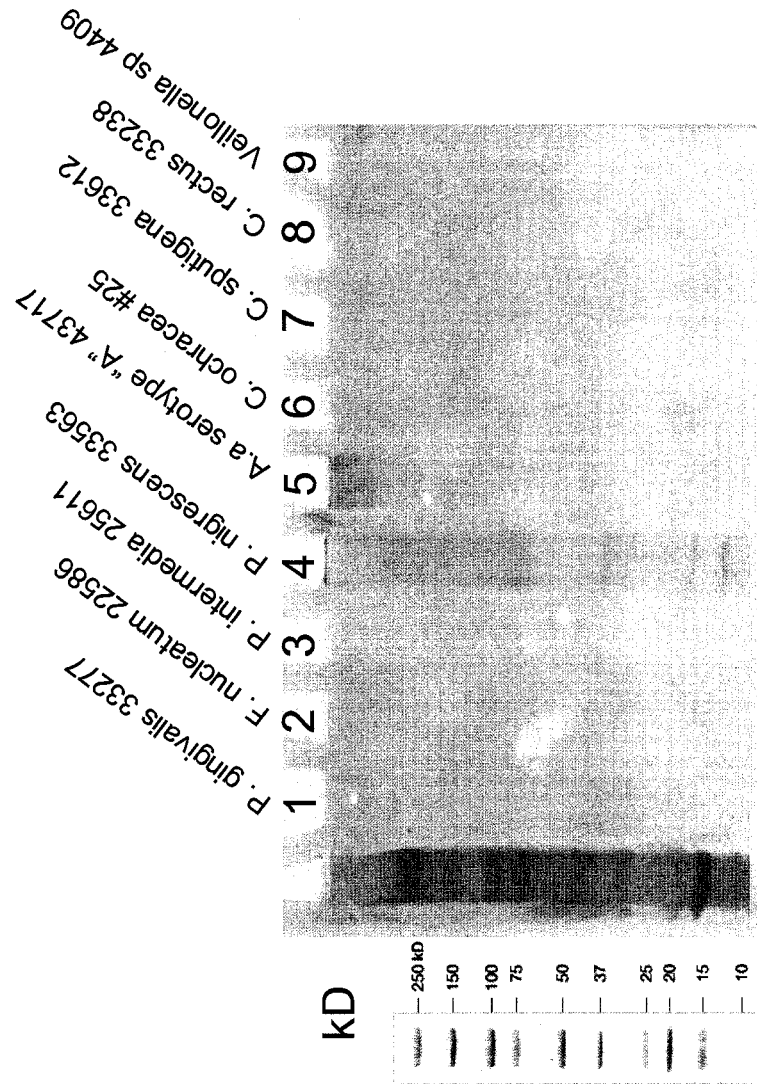


Figure C.2 Colloidal Gold Total Protein staining of LPS preparations in which protein bands stain dark red. LPS was extracted with the TRI-reagent technique. Individual lanes indication presence or absence of residual protein other than LPS remaining in purified LPS preparations. The majority of the lanes are free of contaminating protein except for *P. nigrescens* 33563 (lane 4) which correlated with the inability to obtain a clear MALDI-ToF spectra from the lipid A preparations. It was observed in *P. gingivalis* 33277 (lane 1) as well as *A. actinomycetemcomitans* serotype "A" 43717 (lane 5) the presence of a smearing pattern located at approximately 30-37 kD range and above 250 kD respectively.

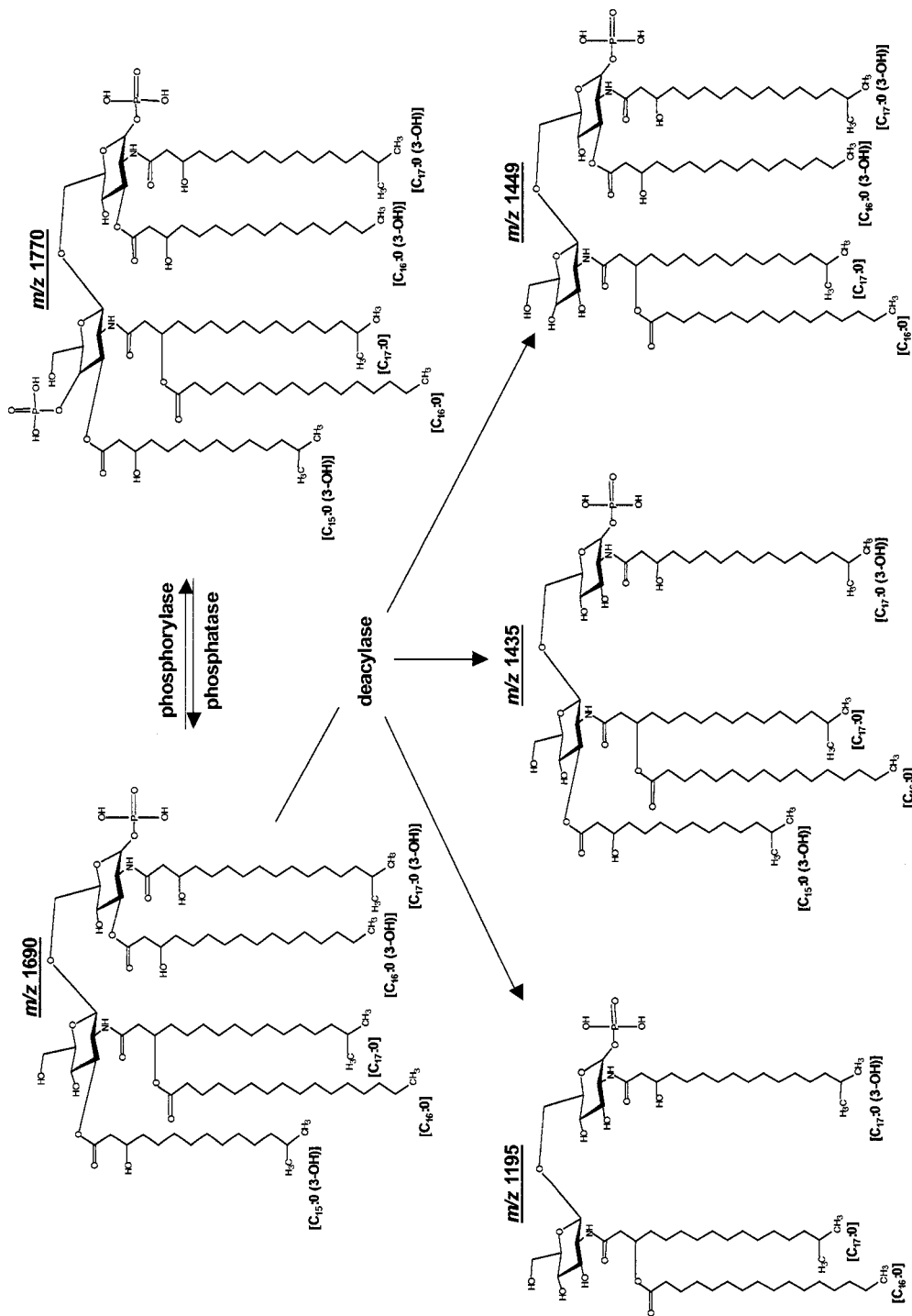


Figure C.3: Chemical structure and associated MALDI-TOF mass ion values (m/z) of lipid A isoforms reported for *Porphyromonas gingivalis* as well as the proposed natural phosphatase and deacylase activity affects.

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VITA

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