The Lipid A from Vibrio fischeri Lipopolysaccharide

A UNIQUE STRUCTURE BEARING A PHOSPHOGLYCEROL MOIETY*

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Vibrio fischeri, a bioluminescent marine bacterium, exists in an exclusive symbiotic relationship with the Hawaiian bobtail squid, Euprymna scolopes, whose light organ it colonizes. Previously, it has been shown that the lipopolysaccharide (LPS) or free lipid A of V. fischeri can trigger morphological changes in the juvenile squid's light organ that occur upon colonization. To investigate the structural features that might be responsible for this phenomenon, the lipid A from V. fischeri ES114 LPS was isolated and characterized by multistage mass spectrometry (MS®). A microheterogeneous mixture of mono- and diphosphorylated diglucosamine disaccharides was observed with variable states of acylation ranging from tetra- to octaacylated forms. All lipid A species, however, contained a set of conserved primary acyl chains consisting of an N-linked C14:0(3-0H) at the 2-position, an unusual N-linked C14:1(3-0H) at the 2’-position, and two O-linked C12:0(3-0H) fatty acids at the 3- and 3’-positions. The fatty acids found in secondary acylation were considerably more variable, with either a C12:0 or C16:1 at the 2-position, C14:0 or C14:0(3-0H) at the 2’-position, and C12:0 or no substituent at the 3’-position. Most surprising was the presence of an unusual set of modifications at the secondary acylation site of the 3-position consisting of phosphoglycerol (GroP), lysophosphatidic acid (GroP bearing C12:0, C16:0, or C16:1), or phosphatidic acid (GroP bearing either C16:0 + C12:0 or C16:0 + C16:1). Given their unusual nature, it is possible that these features of the V. fischeri lipid A may underlie the ability of E. scolopes to recognize its symbiotic partner.

The bioluminescent bacterium Vibrio fischeri exists in a symbiotic relationship with the Hawaiian bobtail squid, Euprymna scolopes. Colonization of the juvenile squid’s light organ by V. fischeri begins within hours of hatching (1). From the complex bacterial community present in seawater, V. fischeri, which represents less than 1% of the bacterial population, is exclusively recruited by E. scolopes in a multistep winnowing process (2). As the colonization of the juvenile squid’s light organ progresses, a series of developmental changes occur in the tissues. The most dramatic of these morphogenetic events is the loss of a superficial ciliated field of cells important for the initial recruitment of V. fischeri. This process occurs over the ~96–120 h following initial colonization by the symbiont (2) and does not occur without interactions with the symbiont in the deep crypt regions of the organ. Once the colonization is established, the squid continues to maintain a population of V. fischeri in its light organ, with cycles of daily flushing and repopulation by residual bacteria. The selection process that leads to the exclusive symbiotic relationship between V. fischeri and E. scolopes involves the interaction of bacterial surface components with host tissues. Previously, we showed that bacterial lipopolysaccharide (LPS) and lipid A can induce early stage apoptosis in the cells of the ciliated field of the juvenile squid’s light organ (3). However, the effect was not species-specific, suggesting that a conserved portion of the lipid A may be the responsible component of the LPS structure (3). In later stages of the colonization process, bacterial peptidoglycan acts synergistically with LPS to induce most, if not all, of light organ morphogenesis (4).

How the developmental signals of bacterial LPS and peptidoglycan, which are presented by the symbionts in the deep crypts of the light organ, are conveyed to the superficial tissue remains unknown, but one piece of the puzzle has recently been discovered. At hatching, the light organ has high levels of nitric oxide (NO). Following symbiont colonization of the crypts, the levels of both NO and the enzyme that catalyzes its production, nitric-oxide synthase, are attenuated (5). Recent studies of the system revealed that LPS and peptidoglycan work synergistically to turn down the NO/nitric-oxide synthase of the organ. In addition, this attenuation is critical for inducing the onset of the apoptotic program (6). These data provide evidence that the LPS and peptidoglycan do not work by direct interactions with the superficial epithelium (i.e. by transport through the tissues to responsive sites) but rather exert their influence by intermediary molecules, including NO. Whether LPS and/or peptidoglycan are required for the other developmental events of the light organ remains to be determined.

To further explore the role(s) of V. fischeri LPS in the host-bacterium interaction, we have initiated studies aimed at characterizing structural features of the LPS. Typical of Gram-negative bacteria, the LPS of V. fischeri consists of three domains: a membrane-associated lipid A, a core oligosaccharide, and an O-antigen moiety. However, when analyzed by SDS-PAGE, the...
LPS of *V. fischeri* showed only two bands rather than the ladder-like banding pattern typical of LPS with large O-antigen polysaccharides composed of multiple repeat units (7). Mass spectrometric analysis suggested that the higher molecular weight LPS species bore a single O-antigen repeat unit, whereas the lower molecular weight forms contained only core sugars (7). The details of these carbohydrate structures are currently being investigated.

Focusing on the lipid A region of the LPS, we recently generated a series of *V. fischeri* mutants deficient in the secondary acyltransferases HtrB and MsbB (8). In pathogenic interactions, the acyl chains added to lipid A by secondary acyltransferases can be critical for colonization and virulence, and thus we were interested in assessing their importance in this symbiotic relationship. In *V. fischeri*, we identified one *msbB* gene and two paralogs of *htrB*, designated *htrB1* and *htrB2*. Mutations in *msbB* and *htrB2* did not impair symbiotic colonization, whereas the *htrB1* mutant displayed a slight symbiosis-specific defect (8). Preliminary biochemical analyses of these mutants, as well as transgenic *Escherichia coli* carrying the *V. fischeri* *htrB1*, *htrB2*, and *msbB* genes, suggested that this MsbB adds a C12:0 fatty acid to lipid A, whereas HtrB1 and HtrB2 may decorate the lipid A with C14:0(3-OH) or C14:0. Deciphering the precise role of these proteins was difficult because the structure of the wild-type lipid A had not yet been elucidated.

In the present study, we report on the characterization of the lipid A from a wild-type strain of *V. fischeri*, strain ES114. Along the lines of recent studies by our group (9, 10) and others (11–17) on the detailed analysis of lipid A acylation patterns, we have employed multistage mass spectrometry (MS) to fully investigate the lipid A from *V. fischeri*. Using MS2 methods in conjunction with selective chemical treatments, a structural model for the *V. fischeri* lipid A has emerged emphasizing microheterogeneity and a novel modification not previously associated with lipid A structures.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—*V. fischeri* ES114, a wild-type strain isolated from *E. scolopes* (18) was used in these studies. *V. fischeri* was grown in LBS (19) or SWT medium (18) made with Instant Ocean (Aquarium Systems, Mentor, OH).

**Isolation of LPS**—Bacterial cultures were centrifuged, and cell pellets were washed once with phosphate-buffered saline (PBS). Proteinase K-digested, phenol-extracted LPS was then extracted from the cells using a modified hot phenol/water method as described previously (20).

**Isolation of Lipid A**—LPS from *V. fischeri* ES114 (~2 mg) was hydrolyzed in 1 ml of 1% acetic acid at 100 °C for 2 h. The sample was centrifuged at 4 °C for 30 min, the supernatant was removed, and the lipid A pellet was washed with water (200 μl) and then dried under a stream of nitrogen. This crude lipid A pellet was then further purified by partitioning in CHCl3/CH3OH/H2O (10:5:6, v/v/v) where the bottom organic layer and interface were saved and evaporated to dryness under a stream of nitrogen.

**Dephosphorylation of Lipid A**—To remove phosphate moieties from the LPS, ~0.5 mg of *V. fischeri* LPS was treated with 50 μl of 48% aqueous HF for 48 h at 4 °C. HF was evaporated from the sample under a stream of nitrogen in a polypropylene desiccator attached to a water aspirator, with an in-line NaOH trap. The sample was then redissolved in 40 μl of deionized H2O and evaporated to dryness in a Savant SpeedVac concentrator (Thermo Scientific, Waltham, MA). The dephosphorylated LPS was then hydrolyzed in 1% acetic acid and processed as described above to obtain dephosphorylated lipid A.

**Time Course of O-Deacylation of Lipid A**—Small aliquots (~50 μg) of the *V. fischeri* lipid A sample were treated with 120 μl of a solution consisting of CHCl3, MeOH, 0.6 N NaOH (2:3:1, v/v/v) to remove O-acyl groups with reaction times ranging from 1 min to 4 h (21). Reactions were quenched with 636 μl of CHCl3, MeOH, 0.1 N HCl (220 μl, 210 μl, 206 μl). After vortexing and centrifuging, the upper aqueous layers were removed, and the lower organic layers containing the O-deacylated lipid A samples were evaporated to dryness under a stream of nitrogen.

**Gas Chromatography/Mass Spectrometry (GC-MS) Analysis of Fatty Acids Derived from Lipid A**—Approximately 50 μg of the *V. fischeri* lipid A sample were treated with 0.5 ml of 10% (w/w) BF3-methanol (Supelco, Inc., Bellefonte, PA) and heated at 100 °C for 6 h. After cooling to room temperature, the sample was partitioned between 0.5 ml of saturated NaCl and 0.5 ml of HPLC grade hexanes (Aldrich). The aqueous layer was extracted a second time with 0.5 ml of hexanes, and the combined organic layers were back-extracted with 0.3 ml of H2O. The organic layer was then evaporated to dryness under a stream of nitrogen. The fatty acid methyl esters (FAMEs) were dissolved in hexanes and analyzed by GC-MS in the electron impact mode using a Varian Saturn 2100T ion trap MS/MS interfaced with a Varian 3900 GC (Agilent Technologies, Santa Clara, CA). The injections were made with a 1:20 split, with the injector temperature set at 200 °C and the ion trap temperature at 195 °C. FAMEs were separated on a 30 m × 0.25-mm BPX70 column with a 0.25-μm film thickness (SGE, Inc., Austin, TX) using helium as the carrier gas (constant column flow, 1.0 ml/min). The initial oven temperature was 90 °C for 3.5 min, followed by a temperature gradient from 90 to 220 °C at 4 °C/min. A commercially available mixture of bacterial acid methyl esters (Matreya, LLC, Pleasant Gap, PA) was used as a GC-MS standard solution. For GC-MS/MS analysis, the instrument was run in the chemical ionization mode using MeOH as the reagent gas, with the isolation width parameter set to 2.0 for the selection of precursor ions.

**GC-MS Analysis of Acylglycerols and Diacylglycerols Released from Lipid A**—Approximately 1.0 mg of *V. fischeri* LPS was dephosphorylated by HF treatment as described above. To solubilize released acylglycerols and diacylglycerols, the dried sample was extracted three times with 100 μl of CHCl3/MeOH (1:1, v/v). This extract was then evaporated to dryness under a stream of nitrogen, and the components were peracetylated by treatment with 100 μl of acetic anhydride, pyridine (1:1, v/v) at 86 °C for 30 min (22). After cooling to room temperature, the
reaction mixture was evaporated to dryness under a stream of nitrogen. For comparison, a commercially available phospholipid standard, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (Matreya, LLC, Pleasant Gap, PA) and an LPS from *Salmonella typhimurium* Ra mutant (Sigma-Aldrich) were subjected to the same dephosphorylation and peracetylation steps as the *V. fischeri* LPS. Two commercially available diacylglycerols, 1,2-dimyristoyl-sn-glycero and 1,2-dipalmitoyl-sn-glycerol (Avanti Polar Lipids, Inc., Alabaster, AL) were also peracetylated for use as standards. The samples were dissolved in hexanes and analyzed on the GC-MS instrument described above operating in chemical ionization mode outfitted with a 30 m × 0.25-mm FactorFour VF-5ht column, 0.1-μm film thickness (Agilent Technologies, Santa Clara, CA). In this experiment, the initial oven temperature was 150 °C for 3.0 min, followed by a temperature gradient from 150 to 350 °C at 5 °C/min.

MALDI-TOF Mass Spectrometry—Lipid A samples were analyzed by negative ion matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) on a Voyager DESTR Plus instrument equipped with a 337-nm nitrogen laser or on a 4800 TOF/TOF instrument with a 355-nm Nd:YAG laser (both instruments from AB Sciex, Foster City, CA). Spectra were obtained in linear mode using delayed extraction conditions (Voyager DESTR) or in reflectron mode (4800 TOF/TOF). Samples were dissolved in CHCl₃/CH₃OH (3:1, v/v) at a concentration of ~0.1–0.5 μg/μl, mixed 1:1 with a saturated solution of 6-chloro-3-mercaptobenzothiazole in CHCl₃/CH₃OH (3:1, v/v), spotted on a stainless steel target, and allowed to air-dry. Approximately 100 laser shots were recorded for each sample. The spectra were processed with a base-line correction and either Gaussian smoothing or noise correction (0.7). Mass calibration was done externally with a mixture consisting of angiotensin II, renin substrate tetra-decapeptide, and insulin chain B (oxidized) (all from Sigma-Aldrich).

Nanoelectrospray MS/MS and MSⁿ Mass Spectrometry—The *V. fischeri* lipid A sample, dissolved in CHCl₃/CH₃OH (1:2, v/v) at a concentration of ~0.1–0.5 μg/μl, was analyzed by static or dynamic nanoelectrospray using either a LCQXDc quadrupole ion trap or an LTQ XL linear ion trap mass spectrometer (both instruments from Thermo Scientific, San Jose, CA). In the dynamic nanoelectrospray mode, the needle voltage was typically set to 1.6–2.2 kV, the temperature of the heated capillary was at 140–180 °C, and the flow rate was 0.3–0.5 μl/min. In static nanoelectrospray mode, lower needle voltages of 1.0–1.5 kV were typically used. Ions were isolated for collision-induced dissociation with the isolation width parameter typically set to 3.

vMALDI™ Linear Ion Trap-TOF for MS/MS and MSⁿ Mass Spectrometry—As for the MALDI experiments described above, lipid A samples were dissolved in CHCl₃/CH₃OH (3:1, v/v), mixed 1:1 with saturated 6-chloro-3-mercaptobenzothiazole matrix solution in CHCl₃/CH₃OH (3:1, v/v), and then spotted on a 96-position stainless steel target plate. Spectra were acquired in both negative ion and positive ion modes on an LTQ linear ion trap outfitted with a vMALDI™ ion source (Thermo Scientific, San Jose, CA). The intermediate vacuum (170 millitorr) MALDI ion source uses a 337-nm Si nitrogen laser with a frequency of 20 Hz and energy of ~250 μJ/pulse. In MS mode, data were acquired using the high mass range option to observe the full profile of *V. fischeri* lipid A samples (typically ranging from *m/z* 700 to 3000). For MSⁿ spectra, the following parameters were used: precursor ion isolation width of 3, normalized collision energy of 40% (percentage of RF amplitude used to fragment ions), activation Q of 0.25, and activation time of 30 ms. Spectra were recorded using the automatic gain control and spectrum filter tools.

RESULTS

The Fatty Acid Composition of *V. fischeri* Lipid A—To identify fatty acids present in the *V. fischeri* lipid A, a portion of the lipid A released from the LPS by mild acid hydrolysis was trans-esterified with BF₃-MeOH to generate FAMEs. As shown in Fig. 1, the major fatty acids present in the sample were dodecanedioic acid (C12:0), tetradecanedioic acid (C14:0), hexadecanedioic acid (C16:0), cis-hexadecanedioic acid (C16:1), 3-hydroxydodecanedioic acid (C12:0(3-OH)), and 3-hydroxytetradecanedioic acid (C14:0(3-OH)). Other minor constituents included trans-hexadecanedioic acid (C16:1), octadecanedioic acid (C18:0), and cis- and trans-octadecanedioic acids (C18:1).

In addition to these common fatty acids, an unknown constituent eluted at 26.07 min, shortly after the C14:0(3-OH) peak (Fig. 1). The electron impact-MS fragmentation pattern of this species was compared with the spectra of the FAMEs from C14:0(3-OH) (supplemental Fig. S1) and 3-hydroxy-(5Z)-tetradecanedioic acid (23), which supported the presence of a hydroxyl group on the 3-position with a C4-C5 double bond (see inset of supplemental Fig. S1B). When analyzed by GC-MS under chemical ionization conditions, the unknown species gave an intense (M + H)⁺ ion at *m/z* 257, which, when selected for MS/MS analysis, produced a distinctive fragment ion at *m/z* 165 (C₁₂H₂₁), consistent with an ion series that is characteristic of unsaturated fatty acids (data not shown) (24). Together, these data supported the assignment of this peak as a C14:1(3-OH) fatty acid.
Using standard methods, the disaccharide core of the *V. fischeri* lipid A was shown by GC-MS analysis of alditol acetates to be composed of glucosamine (GlcN) (data not shown). To obtain a mass profile of the *V. fischeri* lipid A sample, the mixture was analyzed by MALDI MS in the negative ion mode. As seen in Fig. 2, the lipid A preparation was found to be highly heterogeneous, producing a group of major peaks in the \( m/z \) 1800–2500 range that could be assigned as hexa- to octaacylated forms, with additional species appearing at lower masses. In the high mass range, at least 18 major species were present, consisting of nine pairs of ions separated by 16 Da. These species could be further grouped into three major corresponding ion series separated by 182 Da (dodecanoic acid, C12:0). Within these series were subspecies differing by 80 Da (HPO3), 54 Da (C4H6), and 2 Da (H2). Thus, all of the major high mass species could be readily interrelated.

To study the architecture and compositions of the core structures, the *V. fischeri* lipid A was analyzed by MS\(^n\), using both MALDI and electrospray ionization (ESI) methods. Fig. 3 shows two negative ion nanoelectrospray spectra of the *V. fischeri* lipid A mixture with ESI conditions optimized to produce either doubly and triply charged diphosphorylated lipid A forms (Fig. 3A) or singly charged monophosphorylated lipid A forms (Fig. 3B). Most of the singly charged species appearing in Fig. 3B were also observed in the corresponding \( m/z \) region of the MALDI MS (Fig. 2).

To investigate the structural components of these molecular ion species in more detail, the singly charged tetraacylated species at \( m/z \) 1278.1 (see Fig. 3B) was selected for MS\(^n\) analysis, as shown in Fig. 4. Characteristic of monophosphorylated lipid A structures with a free reducing terminus, the species at \( m/z \) 1278.1 readily underwent a cross-ring cleavage of the \({0,2A2}\)-type (25) to give the ion at \( m/z \) 992.6 (Fig. 4A). This 285-Da loss, which was found to be most abundant in species with no substituent at the 3-position, established the presence of an N-linked C14:0(3-OH) fatty acid at the 2-position of the diglucosamine disaccharide backbone. The species at \( m/z \) 1278.1 could also lose a C12:0(3-OH) fatty acid as either a free acid (\( m/z \) 1061.8) or a ketene (\( m/z \) 1079.7). This potential for dual fragmentation mechanisms for a fatty acid suggests that the fatty acid is located at the 3'-position, adjacent to a phosphate group at the 4'-position that can initiate a charge-driven fragmentation process (12).

When the cross-ring fragment at \( m/z \) 992.6 was selected for further fragmentation (MS\(^3\)), it also underwent a loss of the C12:0(3-OH) fatty acid as a free acid (\( m/z \) 776.5) or a ketene (\( m/z \) 794.5) (Fig. 4B). Further fragmentation of the species at \( m/z \) 776.5 resulted in the dominant loss of 60 Da (C2H4O2) to give the ion at \( m/z \) 716.5, in addition to the loss of a C14:0 fatty acid to give the ion at \( m/z \) 488.3 (Fig. 4D) and an ion at \( m/z \) 282.1 corresponding to the loss of the entire acyloxyacyl group at the 2'-position as a ketene (\( m/z \) 434.4 Da). This established that the N-linked fatty acid at the 2'-position in this core structure was the unusual C14:1(3-OH) fatty acid discovered in the GC-MS studies.

A similar set of MS\(^n\) experiments was also carried out on the species at \( m/z \) 1294.1 (see Fig. 3B), which is 16 Da higher in mass...
than the species at \( m/z \) 1278.1. From the species at \( m/z \) 1294.1, main fragment ions were seen at \( m/z \) 792.5, 1008.5, 1077.5, 1095.5, and 1275.5. When further fragmented at MS³ or MS⁴ stages, the ion at \( m/z \) 792.5 lost 60 Da (C₂H₄O₂) to give a base peak at \( m/z \) 732.5 but also gave the ion at \( m/z \) 548.2, as seen in Fig. 4C, this time arising from the loss of a C₁₄:0(3-OH) fatty acid in acyloxyacyl linkage to the \( N \)-linked fatty acid at the 2'-position (data not shown). Thus, the tetraacylated species at \( m/z \) 1278.1 and 1294.1 were found to differ only at the secondary acylation site on the 2'-position, bearing either a C₁₄:0 or a C₁₄:0(3-OH) fatty acid at that location, respectively.

The two pentaacylated species at \( m/z \) 1476.1 and 1491.9 (see Fig. 3B) were investigated by MS² studies and found to be related to their corresponding tetraacylated species by the addition of a C₁₂:0(3-OH) fatty acid at the 3-position. When selected for MS/MS, they both gave prominent ions for the loss of C₁₂:0(3-OH) as a free acid and as a ketene but no intense \( \alpha,\beta \) cross-ring fragments (data not shown). Selecting either one of those species for further fragmentation (MS³) produced a second pair of C₁₂:0(3-OH) losses, confirming the presence of a second C₁₂:0(3-OH) fatty acid on the structures. Once this fatty acid was eliminated from the 3-position to give a C₃-C₄ double bond, intense fragment ions arising from the loss of 327 Da from the parent ions (\( \alpha,\beta \) cross-ring fragments) were present in the MS⁴ spectra. These results established the four fatty acids at the primary acylation sites on the \( V. \) fischeri lipid A and also showed microheterogeneity at the secondary acylation site on the 2'-position (Fig. 5).
In addition to these core structures, there were substantial peaks that corresponded to tetraacylated and pentaacylated anhydro species in the ESI spectrum of the *V. fischeri* lipid A mixture (see Fig. 3B). All of these species ([m/z] 1259.9, 1275.9, 1458.1, and 1474.1) were thoroughly investigated by MS^n. The MS/MS spectra of the two tetraacylated anhydro species ([m/z] 1259.9 and 1275.9) did not contain the abundant 0,2A2 cross-ring fragments characteristic of their corresponding intact species ([m/z] 1278.1 and 1294.1, respectively). Detailed MS^n analyses revealed that this was due to a preexisting C3-C4 double bond in the reducing terminal glucosamine ring, as shown for the species at [m/z] 1259.9 in supplemental Fig. S2. The pentaacylated anhydro species at [m/z] 1458.1 and 1474.1, however, did not have a C3-C4 double bond. Rather, these two species were shown by MS^n analysis to bear a C12:1 fatty acid at the 3-position. Fragmentation evidence supporting this interpretation is presented for [m/z] 1458.1 in supplemental Fig. S3, and the full set of tetraacyl and pentaacyl structures identified in these studies is given in supplemental Fig. S4. As suggested by the structures, these low mass anhydro species appeared to arise from eliminations involving either the substituent on the secondary acylation site on the 3-position ([m/z] 1458.1 and 1474.1) or the entire group at the 3-position ([m/z] 1259.9 and 1275.9).

**Detection of *V. fischeri* Lipid A Components Bearing an Unknown 154-Da Moiety**—In the next cluster of singly charged ions in the [m/z] 1600–1730 range (see Fig. 3B), a shift in mass of 182 or 236 Da from the pentaacylated lipid A species suggested the presence of a set of hexaacylated lipid A species. For example, the species at [m/z] 1658.2 and 1712.3 could be related to the pentaacylated species at [m/z] 1476.1 by the addition of a C12:0 (+182 Da) or a C16:1 (+236 Da) fatty acid. However, the major species at [m/z] 1630.1 and 1646.1 appeared to be related to the species at [m/z] 1476.1 and 1491.9, respectively, by the addition of a 154-Da moiety (or to the anhydro species at [m/z] 1458.1 and 1474.1 by the addition of 172 Da). This mass increment could in principle have arisen from the addition of a decanoic acid (C10:0) to these structures, but such a fatty acid was not detected in the GC-MS analysis (discussed above).

To examine these structures more closely, the lipid A species at [m/z] 1630.1 and 1646.1 were selected for MS/MS analysis and produced two very distinctive spectra as shown in Fig. 6. Unlike the spectra for the tetraacylated and pentaacylated species described above, these spectra consisted of two main peaks: one arising from a loss of 172 Da from the parent ion and another from the loss of 370 Da. The latter loss could be assigned as sequential or concomitant losses of the 172-Da moiety plus a
Evidence for Phosphoglycerol, Lyso phosphatidic Acids, and Phosphatidic Acids on V. fischeri Lipid A—The last cluster of singly charged ions seen by nanoelectrospray in the m/z 1780–1900 range (see Fig. 3B) all appeared to be related to the species at m/z 1630.1 and 1646.1 by the addition of common fatty acids, and thus they would be expected to also contain the purported GroP moiety. When selected for MS/MS analysis, the species at m/z 1866.1 gave the fragmentation spectrum shown in Fig. 7, which was clearly related to the MS/MS spectra of the species at m/z 1630.1 and 1646.1 (Fig. 6) but was a bit more complex, with four major fragments instead of two. Again, the species at m/z 1866.1 readily lost 172- and 370-Da (172 + 198 Da) moieties to give ions at m/z 1694.1 and 1496.1, respectively. However, the species at m/z 1866.1 could also generate the two anhydro species at m/z 1458.0 and 1259.9 through losses of 408.1 Da (172 + 236 Da) and 606.2 Da (172 + 198 + 236 Da), respectively.

To understand these latter neutral losses, the four major fragments were separately subjected to MSn analyses. When the fragment ion at m/z 1694.1 was selected for MS3 analysis (1866.1 → 1694.1), the familiar pattern of a dominant loss of 198 Da (to give m/z 1496.0) was again observed (supplemental Fig. S5A), suggesting that a C12:1 fatty acid was being lost from the 3-position of the parent ion. The small 0.2A3 ion at m/z 1172.7 (loss of 521.4 Da) indicated the presence of the C16:1 fatty acid in acyloxyacyl linkage on the N-linked C14:0(3-OH) fatty acid at the 2-position. When selected for MS4 fragmentation (supplemental Fig. S5B), the ion at m/z 1496.0 (1866.1 → 1694.1 → 1496.0) readily lost the C16:1 fatty acid almost exclusively as a free acid to give m/z 1241.7 and also gave rise to abundant fragment ions at m/z 1279.8 and 1297.9 that would correspond to the loss of the C12:0(3-OH) fatty acid at the 3′-position as either a free acid or a ketene. A very similar spectrum was obtained when the same ion at m/z 1496.1 was selected from the MS/MS spectrum for further fragmentation (1866.1 → 1496.1) (data not shown). Most notably, neither of these species (m/z 1694.1 or 1496.1) produced the anhydro fragments at m/z 1458.0 and 1259.9 that were present in the MS/MS spectrum (see Fig. 7), suggesting that they arise from a different fragmentation pathway. To confirm the structures of the anhydro fragments, MS3 and MS4 spectra were generated from the ions at m/z 1458.0 (supplemental Fig. S5, C and D) and 1259.9 (data not shown). Their fragmentation patterns corresponded to the spectra of the previously described anhydro species (see supplemental Fig. S3, A and B).

Clearly, fragmentation of the hexaacylated parent ion at m/z 1866.1 appeared to arise from two unrelated fragmentation pathways (m/z 1866.1 → 1694.1 → 1496.1 and m/z 1866.1 → 1458.0 → 1259.9), suggesting isobaric structures. The insets in Fig. 7 show the two proposed isoforms, with isoform A having the C16:1 fatty acid in acyloxyacyl linkage on the 2-position and isoform B having the C16:1 fatty acid linked to the GroP to form a lysophosphatidic acid moiety. Fragmentation of isoform B leads directly to the pentaacyl anhydro structure because the C16:1 fatty acid is linked to the GroP. The same situation was evident for the species 16 Da higher in mass at m/z 1882.1 (see Fig. 3B), which was also found to exist in two corresponding isoforms involving the C16:1 fatty acid substituent (data not shown). Furthermore, the hexaacylated species at m/z 1812.1

C12:0(3-OH) fatty acid (−198 Da). When selected for MS3 analysis, the ion at m/z 1457.9 (see Fig. 6A) gave rise to the ion at m/z 1259.8 as its major fragment (−198 Da), similar to the loss seen from the anhydro species at m/z 1458 when it was selected for MS/MS analysis (see supplemental Fig. S3A). Likewise, when the fragment ion at m/z 1259.8 in Fig. 6A was selected for MS3 analysis, it gave a fragmentation pattern similar to that found in the MS/MS spectrum of the anhydro species at m/z 1259.9 (see supplemental Fig. S2A). Similarly, fragment ions from the parent ion at m/z 1646.1 (see Fig. 6B) were also shown by MSn studies to be the corresponding anhydro structures arising from that species (data not shown). This indicated that the species at m/z 1630.1 and 1646.1 were related to their respective pentaacylated anhydro structures by the addition of 172 Da to the secondary acylation site on the 3-position, which would correspond to a 154- Da residue plus the missing 18 Da, presumably H2O.

After searching the LPS and lipid A literature, phosphoglycerol (GroP) emerged as a likely candidate that was most consistent with this 154- Da incremental mass (C12H26O7P = 154.0031 Da) as well as some unexplained fragments seen earlier in some of the MS3 spectra. As shown in Fig. 6, GroP would be attached at the 3-OH group of the fatty acid at the 3-position. When the GroP moiety was included as a possible modification, compositions for all of the major V. fischeri lipid A species could now be proposed (Table 1). However, further experiments were needed to confirm the existence and location of GroP on the V. fischeri lipid A, and these are described below.
and 1828.1 (see Fig. 3B) corresponded to structures bearing a C12:0 fatty acid instead of a C16:1 fatty acid in either of the acylation sites mentioned above (data not shown). Thus, these results indicated another site of fatty acid microheterogeneity at the secondary acylation site on the 2-position, in addition to acylation variability on the phosphoglycerol moiety.

V. fischeri lipid A constituents above 2000 Da in molecular mass were either analyzed as doubly or triply charged ions by ESI (Fig. 3A) or as singly charged ions on a vMALDI linear ion trap mass spectrometer (supplemental Fig. S6). The singly charged, heptaacylated species ionized using vMALDI MS/MS produced spectra similar to the MS/MS spectrum shown in Fig. 7, except that no direct loss of 172 Da was evident. Instead, the major peaks corresponded to the elimination of the GroP moiety bearing either one or two fatty acids. For example, MS/MS analysis of the monophosphorylated, heptaacylated species at m/z 2104.5 and 2120.6 gave MS/MS spectra with a loss of 410 Da from the parent ions, which could be assigned as the com-
Table 1: Proposed compositions of V. fischeri lipid A species

| (M-H)^- | (M-H)^- | C14:0 | C14:1 | C12:0 | C12:1 | C16:0 | C16:1 | C14:0 | C12:0 | HexN | Phos | GroP | anhydro (-18 Da) |
|---------|---------|-------|-------|-------|-------|-------|-------|-------|-------|------|------|------|-----------------|
|         | obs     | calc  | 3-(OH) | 3-(OH) | 3-(OH) | 3-(OH) | 3-(OH) | 3-(OH) | 3-(OH) |      |      |      |                 |
| ?       | 2564.64 | 2564.74 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 2 | 1 |
|         | 2546.66 | 2548.75 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 2 | 1 |
| octanoylated | 2436.53 | 2436.62 | 2 | 1 | 2 | 1 | 2 | 2 | 2 | 1 |
|         | 2420.51 | 2420.63 | 1 | 1 | 2 | 1 | 2 | 2 | 2 | 1 |
|         | 2382.54 | 2382.57 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 2 | 1 |
|         | 2366.54 | 2366.58 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 2 | 1 |
|         | 2328.50 | 2328.53 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 2 | 1 |
|         | 2312.51 | 2312.53 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 2 | 1 |
|         | 2302.56 | 2302.61 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 2 | 1 |
|         | 2286.53 | 2286.61 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 2 | 1 |
|         | 2248.48 | 2248.56 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 2 | 1 |
|         | 2232.51 | 2232.57 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 2 | 1 |
| heptanoylated | 2200.38 | 2200.41 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 2184.38 | 2184.41 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 2146.35 | 2146.36 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 2130.35 | 2130.37 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 2144.34 | 2144.35 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 2128.33 | 2128.35 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 2120.40 | 2120.44 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 2104.40 | 2104.45 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 2118.36 | 2118.43 | 2 | 1 | 2 | 2 | 1 | 2 | 2 | 1 |
|         | 2102.36 | 2102.43 | 1 | 1 | 2 | 2 | 1 | 2 | 2 | 1 |
| hexanoylated | 2066.38 | 2066.39 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 2050.38 | 2050.40 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 2064.36 | 2064.38 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 2048.36 | 2048.38 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1962.16 | 1962.18 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1946.17 | 1946.18 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1882.20 | 1882.21 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1866.20 | 1866.22 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1828.16 | 1828.16 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1812.16 | 1812.17 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1754.11 | 1754.13 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1738.10 | 1738.13 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1674.11 | 1674.16 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1658.10 | 1658.17 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
| pentanoylated | 1725.98 | 1725.96 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1709.98 | 1709.97 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1645.99 | 1646.00 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1629.99 | 1630.00 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1571.94 | 1571.96 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1555.94 | 1555.97 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1553.92 | 1553.95 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1537.93 | 1537.96 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1491.98 | 1491.99 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1475.98 | 1476.00 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1473.95 | 1473.98 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
|         | 1457.95 | 1457.99 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 |
| tetranoylated | 1373.77 | 1373.80 | 2 | 1 | 1 | 1 | 1 | 2 | 2 | 1 |
|         | 1357.78 | 1357.80 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 |
|         | 1293.81 | 1293.83 | 2 | 1 | 1 | 1 | 1 | 2 | 2 | 1 |
|         | 1277.81 | 1277.84 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 |
|         | 1275.76 | 1275.82 | 2 | 1 | 1 | 1 | 1 | 2 | 2 | 1 |
|         | 1259.72 | 1259.83 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 |
bined loss of 172 + 238 Da (GroP + C16:0). As shown in supplemental Fig. S7, other fragment ions at m/z 1457.8 and m/z 1473.6 could be assigned as the combined loss of 172 + 238 + 236 Da from the parent ions (loss of GroP + C16:0 + C16:1). These latter fragments suggested that the GroP moiety in the V. fischeri lipid A structures could be fully acylated with fatty acids at both the sn-1- and sn-2-positions (i.e. phosphatidic acid structures).

When the higher molecular weight species were selected for MS/MS analysis as doubly or triply charged ions, spectra were obtained that yielded corroborating evidence for the assignment of the moieties released from the 3-position. For example, when the heptaacylated monophosphorylated lipid A species at m/z 2048.6 and 2064.6 (or diphosphorylated lipid A species at m/z 2128.7 and 2144.9), proposed to contain one C12:0 fatty acid more than the hexaacylated monophosphorylated lipid A species at m/z 1866.1 and 1882.1, were individually selected for MS/MS analysis as multiply charged ions, all gave base peaks arising from the loss of 408 Da and smaller peaks arising from the loss of 354 Da. These losses are consistent with the masses predicted for loss of GroP (172 Da) plus either C16:1 (236 Da) or C12:0 (182 Da) and indicate heterogeneity in the acylation of the GroP moiety, as shown for the species at m/z 2048.6 (supplemental Fig. S8) and 2144.9 (Fig. 8).

In all of these MS/MS spectra, complementary low mass fragment ions at m/z 407.3, (172 + 236 − H)\(^−\), and m/z 353.3, (172 + 182 − H)\(^−\), were also present. When the fragment ion at m/z 407.3 was subjected to MS\(^3\) analysis, it eliminated a C16:1

![MS/MS spectrum of m/z 1866.1](image)
fatty acid to give m/z 153.1, as shown in Fig. 8C. Further fragmentation of this unsaturated species gave peaks at m/z 79.0 and 97.0, corresponding to the free phosphate ions (PO₃⁻) and (H₂PO₄⁻), respectively (Fig. 8D). Thus, the species at m/z 407.3 was broken down to its essential elements and shown to fragment as a lysophosphatidic acid (26).

Analysis of Fully and Partially Dephosphorylated Lipid A Species—To reduce some of the complexity of the mixture, a portion of the V. fischeri LPS sample was treated with aqueous HF to remove phosphate esters. As shown in Table 2, both fully and partially dephosphorylated lipid A species were observed after HF treatment, although the fully dephosphorylated species could only be observed by MS as sodiated species, (M + Na)⁺, in the positive ion mode (supplemental Fig. S9).

Positive ion MSⁿ analyses of the fully dephosphorylated lipid A species provided a means to effectively probe fatty acid microheterogeneity at all of the secondary acylation sites without the added complexity of the GroP moiety and its associated fatty acids. Specifically, analyses of fully dephosphorylated species that remained hexa- or heptaacylated helped to establish the alternate locations for the C12:0 fatty acid(s) in secondary acylation. As shown in Fig. 9, the MS/MS spectrum of the fully dephosphorylated species at m/z 714.3(3⁻) carried six fatty acids, had as its base peak an ion for the loss of the C12:0(3-OH) fatty acid from the 3-position as a free acid (−216 Da). When this fragment ion at m/z 1386.0 was selected for MS³ fragmentation, abundant 0.4A₂ cross-ring fragments were observed that were consistent with two isobaric structures: one containing a C12:0 fatty acid in secondary acylation on the 2-position (isoform A, 0.4A₂ ion at m/z 759.6) and another ion at m/z 960.7 and one with a C12:0 fatty acid in secondary acylation on the 3'-position (isoform B, 0.4A₂ ion at m/z 1058.8 and...
acyloxyacyl cleavage ion at \( m/z \) 987.7). The isoform B structure indicated a third location for C12:0 on the \( V. \) fischeri lipid A. The relative abundances of the fragment ions provided a semi-quantitative estimation of the two isoforms.

In heptaacylated species bearing two C12:0 fatty acids (\( m/z \) 1784.3 and 1800.3), both of the secondary acylation sites at the 2- and 3’-positions were occupied (data not shown). Heptaacylated structures with C16:1 and C12:0 secondary acyl chains (\( m/z \) 1838.3 and 1854.3) contained those fatty acids on the 2- and 3’-positions, respectively (data not shown). Thus, the acylation options at all secondary acylation sites could be unambiguously assigned by MS\(^a\) analyses.

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### TABLE 2

Proposed compositions of HF-treated \( V. \) fischeri lipid A species.

All values except where indicated were measured on the LTQ XL mass spectrometer using the normal mass range.

| (M-H)\(^a\) | (M+Na)\(^a\) | C14:0 | C14:1 | C12:0 | C16:0 | C16:1 | C14:0 | C12:0 | HexN | GroP |
|-------------|-------------|-------|-------|-------|-------|-------|-------|-------|------|------|
| obs calc    | obs calc    | (3-OH) | (3-OH) | (3-OH) |       |       |       |       |      |      |

| Partially dephosphorylated species |
|-----------------------------------|
| 2222.18\(^a\)                     | 2222.64                     | 2     | 1     | 2     | 1     | 1     | 2     | 1     |
| 2206.27\(^a\)                     | 2206.65                     | 1     | 1     | 2     | 1     | 1     | 2     | 1     |
| 2220.18\(^a\)                     | 2220.63                     | 2     | 1     | 2     | 2     | 1     | 2     | 1     |
| 2204.18\(^a\)                     | 2204.63                     | 1     | 1     | 2     | 2     | 1     | 2     | 1     |
| 2168.27\(^a\)                     | 2168.60                     | 1     | 1     | 2     | 2     | 1     | 2     | 1     |
| 2152.36\(^a\)                     | 2152.60                     | 2     | 1     | 2     | 1     | 1     | 2     | 1     |
| 2166.36\(^a\)                     | 2166.58                     | 2     | 1     | 2     | 1     | 1     | 2     | 1     |
| 2150.27\(^a\)                     | 2150.58                     | 1     | 1     | 2     | 2     | 2     |       |       |

| Fully dephosphorylated species |
|--------------------------------|
| 1854.33 1854.41                | 2     | 1     | 2     | 1     | 1     | 1     | 2     |
| 1838.33 1838.41                | 1     | 1     | 2     | 1     | 1     | 1     | 2     |
| 1800.33 1800.36                | 2     | 1     | 2     | 2     |       |       |       |
| 1784.33 1784.37                | 1     | 1     | 2     | 2     |       |       |       |
| 1672.25 1672.24                | 2     | 1     | 2     | 1     |       |       |       |
| 1656.25 1656.25                | 1     | 1     | 2     | 1     |       |       |       |
| 1618.17 1618.19                | 2     | 1     | 2     |       |       |       |       |
| 1602.17 1602.20                | 1     | 1     | 2     |       |       |       |       |
| 1436.00 1436.03                | 2     | 1     | 2     |       |       |       |       |
| 1420.08 1420.03                | 1     | 1     | 2     |       |       |       |       |
| 1237.92 1237.86                | 2     | 1     | 1     |       |       |       |       |
| 1221.92 1221.87                | 1     | 1     | 1     |       |       |       |       |

\(^a\) Values measured on the LTQ XL mass spectrometer using the high mass range.

\(^b\) Values measured on the vMALDI-linear ion trap-TOF mass spectrometer using the high mass range.
of the fully dephosphorylated species. Additionally, there was no evidence for a C16:0 fatty acid in secondary acylation at the 2-, 2′-, or 3′-positions, confirming that this fatty acid was only found on the GroP.

To connect secondary acylation patterns with modifications on the GroP moiety, MS³ analyses of the partially dephosphorylated species still bearing the GroP moiety and any associated fatty acids were carried out. In the positive ion mode, such species produced abundant glycosidic bond fragments (Y-type ions) at the MS/MS stage as well as cross-ring fragments and fragments associated with the GroP linkages. For example, in the MS/MS spectrum of the hexaacylated species at m/z 1826.3, both isoforms gave the same Y₁ and ⁰⁴A₂ ions at m/z 1016.7 and 892.8, respectively (Fig. 10A). However, the fragment ions at m/z 1456.1 and 1219.9 arising from cleavage of the entire moieties linked to the 3′-position distinguished iso-
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**FIGURE 10.** Positive ion MS$^n$ analyses of the partially dephosphorylated species at m/z 1826.3. A, MS/MS analysis of m/z 1826.3. B, MS$^3$ analysis of m/z 1826.3 → 1516.1.
forms A and B, respectively. Additionally, positive ion mode fragmentation produced cleavages on either side of the phosphate of the phosphoglycerol moieties that were informative. For example, the fragment ion at m/z 1516.1 represents the loss of 310.2 Da from the parent ion, apparently arising from release of the glycerol moiety bearing the C16:1 fatty acid in isoform B (see Fig. 10A, inset). When the fragment ion at m/z 1516.1 was selected for further fragmentation, it produced the MS<sup>2</sup> spectrum shown in Fig. 10B. The base peak at m/z 1220.0 represents the loss of 296.1 Da from the parent ion and is consistent with the loss of a phosphorylated fatty acid, C12:0(3-phosphate). Additionally, the fragment ion at m/z 1418.2 in Fig. 10B arises from the direct loss of 98 Da (−H<sub>2</sub>PO<sub>4</sub>) from the parent ion, which was not seen in the MS/MS spectrum. Supplemental Table S1 summarizes the key fragment ions observed in these experiments that support the assigned isobaric structures. In cases involving microheterogeneity in placement of the C12:0 fatty acid (at the 2- or the 3'-position or on the GroP), fragments supporting all isomers are indicated.

When analyzed in the negative ion mode, the partially dephosphorylated species fragmented in a fashion very different from that observed in the positive ion mode. All negative ion MS/MS spectra had the loss of 198 Da as their base peak, as shown for the hexaacylated species at m/z 1802.2 in supplemental Fig. S10A. However, the more interesting fragments in these spectra were the low mass fragments arising from the GroP substituent on the 3-position. In supplemental Fig. S10A, the fragment ion at m/z 605.4 corresponds to the mass of the entire branch on the 3-position of isoform B. When this ion was taken through further stages of fragmentation, it first eliminated C12:1 (−198 Da) to give m/z 407.3 (supplemental Fig. S10B) and then lost a C16:1 fatty acid as a free acid (−254 Da) to give an ion at m/z 152.9, corresponding to the mass of the remaining unsaturated GroP moiety (supplemental Fig. S10C). In cases where the partially dephosphorylated lipid A species contained a phosphatidic acid moiety (with two acyl chains), low mass fragments corresponding to this released moiety were observed. For example, the negative ion MS/MS spectrum of the heptaacylated species at m/z 2024.4 (see Table 2 for composition) gave a low mass fragment at m/z 843.3, corresponding to a C12:0(3-OH) fatty acid bearing GroP with two O-linked fatty acids (C16:0 + C16:1) (data not shown). As for the example discussed above, further fragmentation of m/z 843.3 supported the direct linkage of a phosphatidic acid moiety to the C12:0(3-OH) fatty acid of the lipid A.

**Release of O-Linked Phosphoglycerol, Lyso phosphatidic Acid, and Phosphatidic Acid from V. fischeri Lipid A**—The unusual linkage position of the GroP moiety on a secondary acylation site was further investigated by chemical treatment of the V. _fischeri_ lipid A or LPS. First, the _V. fischeri_ lipid A sample was subjected to O-deacylation by mild base treatment to release all of the O-linked moieties on the structures. In a time-dependent fashion, the major intact species at m/z 2184.9, 2200.6, 2383.2, and 2437.3 were rapidly converted to the partially O-deacylated species at m/z 1358.7, 1374.5, 1556.7, and 1611.0, respectively, by the loss of 826 Da (supplemental Fig. S11). This 826-Da moiety is consistent with the combined loss of 154 + 198 + 236 + 238 Da from the intact structures, corresponding to the chemical removal of the labile primary fatty acid at the 3-position bearing its phosphatidic acid substituent in phosphoester linkage. Thus, these results supported the linkage of the GroP moiety to the C12:0(3-OH) fatty acid at the 3-position rather than at an alternative phosphorylation site, such as the 1- or the 4'-positions of the diglucosamine backbone, from which it would not have been expected to be released.

Conversely, treatment of the sample with aqueous HF would be expected to cleave the phosphoester bonds themselves, potentially liberating free acylglycerols and diacylglycerols. To test this hypothesis, the _V. fischeri_ LPS was HF-treated, and then the reaction mixture was extracted with CHCl<sub>3</sub>/MeOH (1:1) to recover released acylglycerols and diacylglycerols, which were then peracetylated. When analyzed by GC-MS, the peracetylated sample contained a mixture of acylglycerols and diacylglycerols (supplemental Fig. S12). The acylglycerols had C12:0, C16:0, or C16:1 fatty acids, and the diacylglycerols were found to be of two fatty acid compositions: containing C12:0 + C16:0 or containing C16:1 + C16:0 fatty acids. A pair of peaks was observed for both compositions, suggesting heterogeneity, although this was not investigated further. In the negative control sample ( _S. typhimurium_ Ra LPS) and in the reaction blank, no acylglycerols or diacylglycerols were detected. Therefore, this experiment provided additional support for the presence of lysophosphatidic acids and phosphatidic acids on the _V. fischeri_ lipid A structures and revealed the compositions of the phosphatidic acids present. In conjunction with the MS<sup>2</sup> data, these results described the _V. fischeri_ lipid A mixture as a complex ensemble of structures with microheterogeneity at all four of the secondary acylation sites, one of which is variably modified with GroP moieties rather than a fatty acid (Fig. 11 and supplemental Fig. S13).

**DISCUSSION**

Many features of the _V. fischeri_ lipid A distinguish it as unusual, containing both a high level of microheterogeneity as well as novel modifications. The most unusual feature of the _V. fischeri_ lipid A was the suite of phosphoglycerol moieties that were found at the secondary acylation site on the 3-position, including GroP, lysophosphatidic acids, and phosphatidic acids. The presence of these moieties was established by complementary GC-MS and MS<sup>2</sup> experiments that showed release of free acylglycerols and diacylglycerols from the structures as well as gas phase fragment ions that preserved the direct linkage of these structural pieces to a C12:0(3-OH) fatty acid of the lipid A via GroP.

There are precedents in the literature for the incorporation of GroP on the O-antigen region of LPS (27–30) but no previous reports of such modifications on lipid A. To our knowledge, no lysophosphatidic acids or phosphatidic acids have ever been detected on LPS before. Also, this would appear to be the first observation of a phosphate substituent incorporated at a secondary acylation site on a lipid A structure. The published structures of some unusual phosphoglycerolipids from _Deinococcus radiodurans_ (31, 32) and two thermophiles (33) have a 1,2-diacyl-sn-glycero-3-phosphate moiety linked to a glyceryl alkyamine that bears a slight resemblance to our proposed
Lipid A from *V. fischeri* Lipopolysaccharide

![Diagram of lipid A from *V. fischeri* Lipopolysaccharide]  

**FIGURE 11.** Composite picture of the major intact structures present in the *V. fischeri* lipid A mixture. In this view, the 3-hydroxy fatty acids are shown with the expected R-configuration. The stereochemical configuration of the GroP moiety was not established in this study but is shown as sn-glycerol-3-phosphate by analogy to bacterial phospholipids. The R4 and R5 fatty acid chains were not distinguished. As in Fig. 5, the asterisk denotes the tentative assignment of the location of the double bond in the C14:1(3-OH) fatty acid.

V. *fischeri* lipid A linkage, although the significance of this observation is unclear.

Another unusual feature of the *V. fischeri* lipid A was the unsaturated fatty acid, C14:1(3-OH), found at the primary acylation site on the 2'-position. We have tentatively assigned the site of unsaturation in this fatty acid as a C4-C5 double bond based on the electron impact fragmentation pattern of the FAME. The fact that an unusual fatty acid occupies one of the primary acylation positions in the *V. fischeri* lipid A sets it apart from the lipid A structures of many other Gram-negative organisms, whose primary acylation patterns are generally symmetrical due to the lipid A biosynthetic pathway, which is highly conserved in Gram-negative organisms (34, 35). Additionally, the published fatty acid compositions of the lipid A moieties of other *Vibrio* species, such as *Vibrio cholerae* (36–38) and *Vibrio vulnificus* (39), contain only common fatty acids.

When compared with many published structures, the *V. fischeri* lipid A also exhibited considerable acylation variability at its secondary acylation sites bearing fatty acid substituents. The secondary acylation site on the 2'-position was found to bear either a C14:0 or a C14:0(3-OH) fatty acid, and the two forms were found in roughly a 1:1 ratio. The existence of a hydroxylated fatty acid in secondary acylation is somewhat unusual, although other examples have been reported (40–43). In addition to this heterogeneity, there was also variability at the secondary acylation site on the 3'-position, primarily in higher molecular weight structures. Although no other fatty acid was found at that location, this alternate site for incorporation of a C12:0 fatty acid created additional microheterogeneity in the mixture.

These secondary acylation patterns are consistent with observations made in our recent report characterizing htrB and msbB mutants of *V. fischeri* (8). In that study, we found an msbB gene and two paralogs of *htrB*, designated *htrB1* and *htrB2*, and studied their roles in *E. coli* and *V. fischeri* mutants. Our data suggested that HtrB1 may be adding a C14:0(3-OH) fatty acid and HtrB2 may be adding a C14:0 fatty acid to the lipid A. This interpretation is consistent with the microheterogeneity at the HtrB acylation site (2'-position) observed in the present study. Additionally, MsbB was proposed to add a C12:0 fatty acid to the lipid A, and here we have found that a C12:0 fatty acid can exist in secondary acylation at the 3'-position of the *V. fischeri* lipid A.

In addition to the microheterogeneity at the 2-, 2'-, and 3'-secondary acylation sites, acylation variability on the GroP moiety added yet another layer of heterogeneity to the *V. fischeri* lipid A mixture. In certain isobaric species, a C16:1 fatty acid could be found at two different locations (in secondary acylation on the 2-position or on the GroP), and a C12:0 fatty acid could be found at three different locations (in secondary acylation on the 2- or 3'-positions or on the GroP). Although the isobaric structures for the more abundant hexaacylated and heptaacylated species were established by MS² studies, the octaacylated forms were described as fully as possible using limited fragmentation data and other available information.

Including the GroP moiety, the *V. fischeri* lipid A has the capability of carrying a total of nine fatty acids composed of seven regular acylation sites and two fatty acids at the sn-1 and sn-2 positions of GroP. Although minor peaks consistent with nonaacylated forms were occasionally seen by MALDI MS (e.g. m/z 2548.7 and 2564.6 seen in Fig. 2, with proposed compositions given in Table 1), the largest structures readily detected in the mixture were octaacylated species. Although C18:0 and C18:1 fatty acids were observed in the CG-MS analysis (Fig. 1), they were highly variable in their abundance and were not found on any of the major lipid A species that were investigated in this study.

A composite picture of the *V. fischeri* lipid A structure has emerged that features an ensemble of hexaacylated to octa-
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Ylated structures bearing GroP, lysophosphatidic acid, or phosphatidic acid modifications. The biosynthetic pathways involved in the phosphorylation of the V. fischeri lipid A with GroP are unknown, as are the biological implications of such a modification. Clearly, some significant alterations in lipid A conformation would be expected to occur in order to accommodate a bulky lysophosphatidic acid or phosphatidic acid group. Given that recognizing lipid A has been discussed as the most sensitive and specific mechanism by which animals detect Gram-negative bacteria (44), it is tempting to speculate that this novel feature of the V. fischeri lipid A might play a role in the selection processes that lead to the exclusive symbiotic relationship between V. fischeri and E. scolopes. Rather than trigging rejection, the unusual V. fischeri lipid A may be one of the key structural features that prompts E. scolopes to recognize its symbiotic partner.

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