Global and targeted lipid analysis of *Gemmata obscuriglobus* reveals the presence of lipopolysaccharide, a signature of the classical Gram-negative outer membrane.

**Running title: Gemmata obscuriglobus contains lipopolysaccharide**

Rajendra Mahat\(^1\)*&$, Corrine Seebart\(^2\)*$, Franco Basile\(^1\)#, and Naomi L. Ward\(^2\)#

\(^1\)Department of Chemistry and \(^2\)Department of Molecular Biology, University of Wyoming, Laramie, WY

*Equal contributors

&Present address: Sinclair Wyoming Refining Company, Sinclair, WY

*Correspondence should be addressed to: basile@uwyo.edu (mass spectrometry), nlward@uwyo.edu (other data)
Abstract

Planctomycete bacteria possess many unusual cellular properties, contributing to a cell plan long considered to be unique among the Bacteria. However, data from recent studies are more consistent with a modified Gram-negative cell plan. A key feature of the Gram-negative plan is the presence of an outer membrane (OM), for which lipopolysaccharide (LPS) is a signature molecule. Despite genomic evidence for an OM in Planctomycetes, no biochemical verification has been reported. We attempted to detect and characterize LPS in the Planctomycete *Gemmata obscuriglobus*. We obtained direct evidence for LPS and lipid A using electrophoresis and differential staining. Gas chromatography-mass spectrometry (GC-MS) compositional analysis of LPS extracts identified eight different 3-hydroxy fatty acids (3-HOFAs), 2-keto 3-deoxy-D-manno-octulosonic acid (Kdo), glucosamine, and hexose and heptose sugars, a chemical profile unique to Gram-negative LPS. Combined with molecular/structural information collected from Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) MS analysis of putative intact lipid A, these data led us to propose a heterogeneous hexa-acylated lipid A structure (multiple lipid A species). We also confirmed previous reports of *G. obscuriglobus* whole-cell fatty acid (FA) and sterol composition, and detected a novel polyunsaturated FA (PUFA). Our confirmation of LPS, and by implication an OM, in *G. obscuriglobus*, raises the possibility that other Planctomycetes possess an OM. Pursuit of this question, together with studies of the structural connections between Planctomycete LPS and peptidoglycans, will shed more light on what appears to be a Planctomycete variation on the Gram-negative cell plan.
Bacterial species are classified as Gram-positive or -negative, based on cell envelope structure. For 25 years, the envelope of planctomycete bacteria has been considered a unique exception, lacking peptidoglycan and an outer membrane (OM). However, the very recent detection of peptidoglycan in planctomycete species has provided evidence for a more conventional cell wall, and raised questions about other elements of the cell envelope. Here we report direct evidence for lipopolysaccharide in the planctomycete *Gemmata obscuriglobus*, suggesting the presence of an OM, and supporting the proposal that the planctomycete cell envelope is an extension of the canonical Gram-negative plan. This interpretation features a convoluted cytoplasmic membrane and expanded periplasmic space, the function of which provides an intriguing avenue for future investigation.
Introduction

The Planctomycete bacterium *Gemmata obscuriglobus* (1) is of considerable interest to the fields of cell and evolutionary biology due to its uncommon cellular features, and evolutionary placement within the Bacteria (2-5). *G. obscuriglobus* possesses an extensive endomembrane system (6) with superficially eukaryote-like properties, including the presence of membrane coat-like (MC-like) proteins (7), large quantities of sterols (8,9), a proposed tubulovesicular transport system (7), and endocytosis-like behavior (10). *G. obscuriglobus* also exhibits an unusually condensed nucleoid (11) and moderate radiation resistance (12). Two recent tomography studies have enabled 3-D reconstruction of the *G. obscuriglobus* cell (7,13), with conflicting conclusions about the cell plan. On the basis of two-dimensional imagery, *G. obscuriglobus* was initially described as having a nucleoid ‘compartment’ completely enclosed by a double membrane (6,14,15), analogous to the eukaryotic nucleus. However, the first tomography analysis did not support the presence of closed cellular compartments, and indicated the presence of a continuous cytoplasmic volume (7). Additionally, the endomembrane system was proposed to be an invaginated cytoplasmic membrane rather than a unique additional intracellular membrane, suggesting that the *G. obscuriglobus* cell plan should be considered a variant of the ‘classical’ Gram-negative cell plan. In contrast, the second tomography study found closed compartments at certain life cycle stages, and the authors argued that such stage-specific membrane rearrangements would be expected, particularly for nucleoid segregation and transfer to daughter cells (13).
Most recently, the detection of peptidoglycan in the cell wall of Planctomycetes, including *G. obscuriglobus* (16,17), supports the interpretation of the *G. obscuriglobus* cell plan as Gram-negative. The absence of peptidoglycan and presence of a proteinaceous cell wall (18) had long been a distinguishing Planctomycete feature that led to proposal of a unique cell plan for all Planctomycetes (3,5,6,13,19). The proteinaceous barrier was previously suggested to substitute for other classical Gram-negative cell features, such as cell surface protection more typically provided by O-antigen (20), a rigid structural framework in place of peptidoglycan (13), and possibly to serve as an attachment platform for degradative enzymes, as in a related species, *Rhodopirellula baltica* (21). The proteinaceous exterior of *G. obscuriglobus* has not been shown to constitute a surface layer (S-layer), typically composed of only one or two proteins in a lattice configuration, like that found in some other bacteria, including archaea and the Planctomycete *Candidatus Kuenenia stuttgartiensis* (13,22,23). The original cell wall analysis of *G. obscuriglobus* by Stackebrandt and Liesack (18) found the cell wall composition to include many amino acids, and the ability of the *G. obscuriglobus* cell to maintain its integrity was demonstrated by the finding that cell sacculi retained their native cell morphology after 10% SDS treatment (18), thus exhibiting a resistance to SDS that is more like cells that have a peptidoglycan-containing murein sacculus (21). Despite that resistance ability, the cell wall of *G. obscuriglobus* was actually found in that study to not contain muramic acid and diaminopimelic acid, which are considered to be peptidoglycan markers (18). The *G. obscuriglobus* cell also stains as a Gram-negative bacterium (1). However, *G. obscuriglobus* cell wall characteristics have not appeared to exhibit a classical Gram-negative cell composition, exemplified by *Escherichia coli* as a prototype. Most typically such a classical Gram-negative cell envelope arrangement includes an inner membrane (IM), a periplasmic space containing...
peptidoglycan tethered to lipoproteins of an OM, the exterior layer of which holds lipid A linked to oligosaccharides (13, 24).

OM synthesis and retention of integral outer membrane proteins (OMPs) in classical Gram-negative bacteria is typically achieved by two major pathways: that of the very conserved integral OMP insertion complex, β-barrel assembly machinery (BAM) (25) and the lipopolysaccharide (LPS) insertion complex (20), represented primarily by the large subunit, LptD; OstA; Imp and LpxC that catalyzes a major step in lipid A synthesis. Other genes contributing to synthesis of OM features include those of the O-antigen (O-Ag) gene cluster. A particularly prominent component of the outer leaflet of the OM in Gram-negative bacteria is the semi-rigid LPS (26, 27), which is composed of glycolipids that cover a major proportion of the cell surface (24), providing a barrier to protect the cell from the environment (28, 29). The general architecture of LPS includes three distinct regions: lipid A, core-oligosaccharides and O-antigen (28, 30-32). Lipid A is a unique carbohydrate-lipid, typically a (β-1, 6)-D-glucosamine (GlcN) disaccharide linked to a variable number of fatty acids (FAs) and hydroxy-FAs (HOFAs) (30, 33, 34) and is anchored by its lipophilic domain in the OM lipid bilayer (27). A second LPS region, the core-oligosaccharide, typically holds the characteristic component, 2-keto 3-deoxy-D-manno-octulosonate (Kdo) or less typically D-glycero-D-talo-2-octulopyranosonic acid (Ko) (30, 35). Every LPS characterized to date has been shown to contain either Kdo or Ko (31, 34). The inner and outer core oligosaccharides can be composed of up to 15 sugar moieties (31), with the outer core usually consisting of hexoses and hexosamines (30, 31) and the inner core usually holding 1-3 Kdo and 2-3 specific heptoses (30, 35). Finally, the third LPS region, the O-Ag...
repeat, a repetitive glycan structure of varied length, is not present in all organisms that carry an LPS (28,36).

There is extensive component variety within this general LPS structural organization, with each organism displaying specific modifications (24,28,30,31,33,36-38). Moreover, the inherent chemical heterogeneity of a particular LPS, derived from the interconnection of its three very different regions, makes it challenging to isolate and characterize (33,38,39). Beyond this, structural variations can also occur within the LPS of the same organism (39). Regardless of the huge variations possible for LPS structure, identification of lipid A, its characteristic disaccharide and 3-HOFAs, as well as Kdo or Ko, representing the core oligosaccharide, is strong indication for the presence of an LPS in a particular organism.

A previous study (40) that detected LPS in Planctomyces and Pirellula species gave the first indication of possible OM features within the Planctomycetes. Rare glycolipids were found, apparently of LPS origin, that contained a unique HOFA pattern with major proportions of long-chain, normal 3-HOFAs, specifically 3-OH eicosanoic (3-OH 20:0), as well as normal FAs. Results of this study prompted Kerger and associates to propose 3-OH 20:0 as a potential signature FA of Planctomycetales LPS and concluded that their FA composition and LPS demonstrated that these strains had some similarity with classical Gram – negative eubacteria. A subsequent study (41) inventoried whole cell FAs for a variety of organisms, with preliminary findings of eight different 3-HOFAs in G. obscuriglobus specifically, with a FA distribution of 82% normal and 18% unsaturated.
Intriguing hints that components of an OM structure may be present in *G. obscuriglobus* have also been introduced through genomic analyses, including a prediction for a 60 kDa OMP in all Planctomycetes and in no other prokaryotes (42). Although sequencing of the *G. obscuriglobus* genome is incomplete, additional *G. obscuriglobus*-specific genomic OM evidence has been collected. Speth and colleagues utilized the prediction tool Beta-barrel Outer Membrane protein Predictor (BOMP) and found 95 predicted OMPs in *G. obscuriglobus*, with only five of these having a BLAST hit to a known OMP, suggesting a highly unusual OMP composition.

Remarkably, the major OM markers for LPS insertion (LptD; ZP_02735880.1) and OMP assembly (BamA; ZP_0273769.1) were identified in the *G. obscuriglobus* genome (20), despite general thought that *G. obscuriglobus* does not have the capability to produce either of these cell features. *G. obscuriglobus* also possesses the key LPS biosynthetic genes LpxB and LpxC, as well as KdsA, which adds specific moieties to the Kdo structure (25). Although O-antigen biosynthesis genes have not yet been reported for *G. obscuriglobus*, a search of its draft sequence via JGI/IMG does indicate putative O-antigen related candidate genes, including one preliminarily annotated as an O-Ag polymerase (NCBI ZP_02736666) of the ‘Lipid A core - O-Ag ligase and related enzymes’ grouping within the COG3307 category, Cell wall/membrane/envelope biogenesis.

However, despite the expanding list of genomic data suggesting the existence of an OM in *G. obscuriglobus*, no OM gene products have yet been isolated, recovered for proteomic identification (13) or localized to the cell wall of *G. obscuriglobus*. Here we describe an attempt to LPS as an OM signature in *G. obscuriglobus*. We isolated LPS, detected and characterized its key components, including lipid A, its fatty acid constituents and the disaccharide Kdo, as
evidence for the presence of an OM. This was carried out by biochemical and differential staining approaches to enable isolation of *G. obscuriglobus* LPS and lipid A, followed by GC-MS, GC-tandem-MS (MS/MS) to obtain FA and carbohydrate composition (bottom-up), and MALDI-MS analyses for MW and structural information (top-down). This work resulted in the isolation and characterization of the LPS and lipid A unique to this bacterium, and represents the first experimental confirmation that features usually associated with the OM are present in *G. obscuriglobus.*
Materials and Methods

General methods

Bacterial strains and LPS standard. *Gemmata obscuriglobus* DSM5831<sup>T</sup> was obtained from the Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures; Braunschweig, Germany), and *Escherichia coli* #1246 from Dr. Erin Sander (UCLA). Liquid or solid DSMZ 629 Staley’s Maintenance Medium was prepared as described on the DSMZ website (http://www.dsmz.de).

*G. obscuriglobus* cells were grown at 30°C for 6-13 days on solid or in liquid 629 media while shaking at 200 rpm. *E. coli* UCLA #1246 cells were grown overnight at 37°C on solid or in liquid lysogeny broth-Miller (LB-Miller) media while shaking at 300 rpm. Purified lipopolysaccharide (LPS) standard (L2630), derived from *E. coli* strain 0111:B4, was purchased from Sigma-Aldrich, St. Louis, MO.

Criteria for analysis of *G. obscuriglobus* culture purity. Liquid *G. obscuriglobus* cultures prepared for lyophilization in this study were inoculated from liquid starter cultures of smaller volume. These starter cultures represented 1 – 2% of the final scaled up culture volume, and were initially inoculated with colonies from solid agar plates. These solid agar cultures were in turn originally inoculated from our -80°C freezer maintenance stock of *G. obscuriglobus*. To confirm culture purity, the following criteria were used: single colony isolation to analyze colony morphology and growth rate on solid agar plates, phase contrast microscopy for examination of cell morphology in wet mounts of plate colonies and of liquid culture aliquots, and 16S rRNA gene sequencing of DNA derived from lyophilized cell stocks used in this study.
DNA extraction, PCR amplification and sequencing of 16S rRNA genes. To further confirm the purity of the stocks from which lyophilized cells were derived, 16S rRNA gene sequences were recovered and analyzed from DNA extracted from these experimental stocks. PBS-washed, lyophilized *G. obscuriglobus* cells were rehydrated in sterile, nuclease-free dH₂O for extraction of DNA via the Power Soil DNA Extraction Method (MO BIO #12888-50) per manufacturer’s instructions. DNA recovered was eluted in sterile, nuclease-free dH₂O and frozen at -20°C.

PCR amplification of the 16S rRNA gene was completed in a 50 μl reaction mix, utilizing 1.25U Taq DNA Polymerase (NEB #M0273S), 200 μM Deoxynucleotide (dNTP) Solution Mix (NEB #N0447S), 9.4, 10.4 and 10.8 ng of *G. obscuriglobus* DNA templates and 0.2 μM of the 27F and 1492R 16S rRNA primers (Integrated DNA Technologies (IDT). The 27F primer sequence was 5'-AGAGTTTGATCMTGGCTCAG-3' with a Tm 53.2 °C, and the 1492R sequence was 5'-CGGTTACCTTGTTACGACTT-3' with Tm 52.3 °C. Amplification was carried out in a Bio-Rad T-100 Thermal Cycler with reaction conditions as follows: Initial denaturation at 95°C for 2 min, followed by 30 cycles incorporating a 30 sec denaturation (95°C), 1 min at annealing temp (49.8°C) and a 1.5 min extension (72°C). The reaction was completed by a final 5 min extension (72°C), and reaction mixes were maintained for 30 min at 10°C and then frozen at -20°C. 10 μl of each PCR product was combined with 2 μl NEB 6X Loading Dye (NEB# B7021S) and loaded onto a 0.8% Agarose/TAE gel for electrophoresis at 60 V for 1 hr. Recovered PCR reaction mixes were purified using the QIAquick PCR Purification kit (Qiagen #28104) per manufacturer instructions. Purified PCR products were eluted in nuclease-free dH₂O, and concentrations were determined using a NanoDrop spectrophotometer. The purified PCR products were sequenced by Sequetech (Mountain View, CA) using a 519R internal 16S rRNA primer (IDT) with the sequence 5’-GWATTACCGCGGCKGCTG-3’, TM 58.1 °C.
Reagents and supplies. Sodium acetate (RGC-5260), 10X Tris-glycine SDS Buffer (RGF-3390), 1 M Tris-HCl pH 7.5 (RGF-3350), 10% SDS (RGE-3230) and phosphate-buffered saline pH 7.4 (RGF-3210) were purchased from KD Medical, Columbia, MD. Acetic acid (A6283), hydrochloric acid (H1758), chloroform (C2432), Tri-Reagent (T9424), isobutyric acid (I1754) and Biotech grade methanol (49443-16) were obtained from Sigma-Aldrich. Other reagents included: ammonium hydroxide from Fluka (09859); Biotechnology Grade glycerol (0854-14), Proteomic Grade bromphenol blue sodium salt (M116) and magnesium chloride hexahydrate (0288) from Amresco, Solon, OH; ethanol (111ACS200) from PHARMCO-AAPER, Brookfield, CO; SDS (BP166) from Thermo Fisher Scientific, Waltham, MA; sterile deionized water from both Integrated DNA Technologies, Coralville, IA and (Genemate G-3250) ISC Bioexpress, Kaysville, UT; LB agar (L9110) and LB broth (L9135) from Teknova, Hollister, CA. Dialysis tubing (3500 MWCO Spectrapor 3 Membrane #132720) was from Spectrum Medical Industries, Laguna Hills, CA.

Preparation and lyophilization of whole bacterial cells. Aliquots from each liquid culture flask were examined by phase contrast microscopy (Olympus BX41; Olympus, Center Valley, New Jersey) to ensure culture purity. The remaining culture volumes from these *G. obscuriglobus*, or *E. coli*, liquid cultures were transferred to multiple 250 ml sterile centrifuge bottles and centrifuged (13,847 x g for 20 min at 16°C in pre-cooled Beckman JA14 rotor; Beckman J2-21M Induction Drive Centrifuge; Beckman Coulter, Indianapolis, IN) to recover whole cell pellets.
Original liquid culture volumes/flask for *G. obscuriglobus* were as follows: 3 vials of 1.5 ml, 7 of 12 ml, and 1 of 110 ml for a 198 ml total prep; 101 ml, 198 ml, 300 ml and 600 ml for a 1198 ml total prep; 2 of 102 ml, 2 of 150 ml, 153 ml, 3 of 306 ml, and 511 ml for a 2086 ml total prep. *E. coli* liquid culture volumes/flask were: 5 of 506 ml, 512 ml and 1015 ml for a 4057 ml total prep; 1000 ml for a 1000 ml total prep.

Pellets from *G. obscuriglobus*, or *E. coli*, were combined and washed with 100 - 200 ml of PBS by resuspension and further centrifugation, repeated 3 - 4 times. Pellets were resuspended in 100 – 200 ml sterile dH2O, and cells again harvested by centrifugation (13,847 x g for 20 min in Beckman JA14 rotor) two times. The PBS/dH2O-washed cell pellets were resuspended in 4 – 5 ml sterile dH2O, transferred to fresh vials, frozen via liquid nitrogen and lyophilized at -48°C and 2.4 pascal (Pa) for 24 hrs (Freezone 6 Freeze Dry System; Labconco, Kansas City, MO).

Recovered lyophilized *G. obscuriglobus* cell quantities were as follows: 132 mg from the 198 ml prep, 218 mg from the 1198 ml prep and 350 mg from the 2086 ml prep. Recovered lyophilized *E. coli* cell quantities were as follows: 669 mg from the 1000 ml prep and 2470 mg from the 4057 ml prep. The purity of recovered lyophilized *G. obscuriglobus* cells used in this study was confirmed by 16S rRNA gene sequencing of DNA extracted from these lyophilized, experimental cell stocks, as described above.

**Extraction and purification of LPS from whole bacterial cells.** Crude LPS was extracted from washed, lyophilized cells by a modification of the Tri-Reagent method of Yi and Hackett (43), and performed at room temperature using a Beckman Coulter Microfuge 18 centrifuge with
F241.5P rotor. Recovered products were dialyzed extensively vs. dH2O and lyophilized using a
Freezone 6 Freeze Dry System (Labconco). Crude LPS was purified by cold ethanol magnesium
precipitation, based on a method described by Yi and Hackett (43). Details of these procedures
are provided in Supplementary Methods.

Lipid A isolation by two approaches. Lipid A was released from purified LPS by modification
of the mild acid hydrolysis method of Yi and Hackett (43). An alternative method for lipid A
isolation was based on the method of El Hamidi et al (44) and enabled lipid A extraction directly
from whole cells, rather than from purified LPS. Details of both lipid A isolation approaches are
provided in Supplementary Methods. The Sigma LPS standard (L2630) served as an extra
control, in addition to E. coli cells, for lipid A extraction by this direct method.

SDS-PAGE and differential staining of LPS and lipid A extracts. Samples were solubilized
in 2x Laemmli Sample Buffer (62.5 mM Tris-HCl pH 6.7, 2% SDS, 25% glycerol, 0.8% BPB),
boiled for 5 min, centrifuged (13,000 × g for 10 sec at room temperature; Beckman Coulter
Microfuge 18 centrifuge with F241.5P rotor), applied to a 4-20% gradient SDS-PAGE gel
(MiniProtean TGX, BIO-RAD 456-1094; Hercules, CA) and resolved by electrophoresis in 1x
Tris-glycine SDS running buffer at 200 V for 33 min. The molecular weight (MW) markers,
Page Ruler Plus Prestained Protein Ladder (Thermo Scientific 26619), Fermentas Spectra
Multicolor Low Range Protein Ladder (Thermo Scientific H266) and CandyCane Glycoprotein
Molecular Weight Standard (Molecular Probes C21852) were prepared and loaded onto the gel
per manufacturer instructions. Gels were stained using Pro-Q Emerald 300 Glycoprotein Gel
Stain with SYPRO Ruby Protein Stain (Molecular Probes P21855) and silver stain (Pierce Silver
Stain Kit 24612; Thermo Scientific) following manufacturer protocols. Stained gels were visualized by white light or a 300 nm UV transilluminator (BIO-RAD Gel Doc 1000) for fluorescence.

**GC-MS.** Full scan GC-MS analyses were performed with a gas chromatograph (GC, model: Trace) mass spectrometer (MS, model: DSQ-II; both from Thermo, Austin, TX). A 30 m length (0.25 mm internal diameter x 0.25 µm film thickness), fused-silica DB-5 equivalent column (ZB-5 Phenomenex, Torrance, CA) was used for all chromatographic separations. Helium carrier gas was set at a constant flow rate of 1.0 ml/min. Gas chromatography conditions used for the separation of each particular metabolite group are listed in Table S1.

C4:0 to C24:0 straight-chain, even-carbon and 3OH-C14:0 to 3OH-C18:0 straight-chain, standard fatty acid methyl ester (FAME) mixtures were used to calculate the Retention Index (RI) for confirmatory identifications of saturated FAs present in the sample; details of these calculations are provided in Supplementary Methods. **Fig. S1** shows Equivalent Chain Length (ECL), a parameter analogous to RI in the case of FAMEs, as a function of Retention Time (RT) for 3-HOFAs.

The MS was operated in the positive electron ionization (EI+) mode with an electron energy of -70 eV and emission current of 100 µA. The quadrupole mass analyzer was scanned at the rate of 4.5 scans/s in the range of m/z 50 - 550. The ion source was kept at 250°C, and a 4 min solvent delay was used. Chromatograms and mass spectra were collected with the Xcalibur software (ver. 2.0.7, Thermo Fisher Scientific). For MS measurements in the positive ion chemical ionization (PICI) mode, methane was used as the reagent gas. The reagent gas flow (1 ml/min),
emission current (25 µA), and electron energy (100 eV) were all optimized with cis-docosahexaenoic acid methyl ester (DHA-ME) standard.

**Gas Chromatography-Tandem Mass Spectrometry (GC-MS/MS).** Tandem mass spectral analyses were carried out with a gas chromatograph-triple quadrupole mass spectrometer (GC-MS/MS; model: TSQ7000 and Trace GC, Finnigan, Austin, TX). Both product ion scan (MS/MS) and single reaction monitoring (SRM) scans were conducted. The GC was fitted with a DB-5 equivalent column (ZB-5, Phenomenex, Torrance, CA; 30 m x 0.25 mm x 0.25 µm) and using separation conditions listed in Table S1. Full-scan MS analyses followed by MS/MS modes such as product ions, precursor ions, and neutral loss scans were performed using argon gas as the collision gas. Tandem MS (MS/MS and SRM) parameters were optimized with perfluorotributylamine (PFTBA): collision-induced dissociation (CID) pressure, of 1.0 mTorr, CID voltage of 15 V, and data acquisition rate of 0.4 scans/sec (5 data points in 2 s). All other ionization parameters were as stated above.

**Methods for global lipid analysis**

**Reagents and supplies.** HPLC grade chloroform, hexane, and methanol were purchased from Fisher Chemicals (Pittsburg, PA). HPLC-grade water was obtained from Burdick & Jackson (Muskogon, MI). Even-carbon C4:0-C24:0 saturated FAME mixture (49453-U), cis-4, 7, 10, 13, 16, 19 DHA (D2534), acetyl chloride (00990), lanosterol (L5768), N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, M7891), and trimethylchlorosilane (TMCS, 92360) were purchased from Supelco (St. Louis, MO). All solvents and reagents were used without further purification.
Lipid extraction and derivatization. Lyophilized *G. obscuriglobus* cells were subjected to a modified Bligh-Dyer extraction (described in detail in Supplementary Methods, Lipid extraction from whole bacterial cells) followed by a two-step derivatization procedure. Control lyophilized *E. coli* cells and a reagent blank containing only Bligh-Dyer solvent were processed using the same protocol.

A two-step process, methyl esterification of the carboxylate groups, followed by silylation of hydroxyl groups, was used for the complete derivatization of all lipid functional groups prior to GC-MS and MS/MS analyses. A modified method of Schultz and Pugh, (45) was used to carry out microwave-assisted methyl esterification using methanolic hydrochloride. Briefly, dried lipid extract was mixed with 200 µL of methanol and 40 µL acetyl chloride (~ 4 M methanolic hydrochloride), N₂ gas was added to the reaction vial to avoid potential oxidation of lipids, and the sealed vial was irradiated with microwave-radiation with optimized power of 50 W, maximum temperature set at 70°C, and irradiation time of 3 min. Derivatization was performed using a software-controlled analytical microwave oven (CEM-Discovery, Synergy software, Matthews, NC). The reaction mixture was neutralized with 6% (w/v) Na₂CO₃ solution (aq) and extracted with two volumes of 500 µl hexane by liquid-liquid extraction (LLE). Hexane was evaporated in a Speedvac (at room temperature for the organic phase, and at 40°C for the aqueous phase) to make a final volume of 100 µl, and 1 µl of this sample was used for GC-MS analysis. Because methylation with methanolic hydrochloride is ineffective for the derivatization of hydroxyl functional groups, the second derivatization step of silylation, to produce trimethylsilyl (TMS) derivatives, was used to derivatize 3-HO FAMEs and sterols. An aliquot of 50 µL of methyl esterified sample from the previous step was dried under a N₂ gas stream, and...
resuspended in 30 µL of MSTFA containing 1% (v/v) TMCS. The solution was then heated in an oven at 70°C for 20 min, and the derivatization reagent was evaporated in a Speedvac. The sample was re-dissolved in 50 µL hexane, and 1 µl was injected for GC-MS analyses.

Methods for targeted analysis of LPS composition

Reagents and supplies. Lipopolysaccharide (E. coli O111:B4), Kdo ammonium salt, D-glucose, D-galactose, D-mannoheptose, D-glucoheptose, and glucosamine standards were purchased from Sigma Aldrich (St. Louis, MO). The 3-HOFA standards C14:0, C16:0, C17:0, and C18:0 were purchased from Matreya LLC (Pleasant Gap, PA); and C15:0, from Larodan (Malmö, Sweden). Pyridine and acetic anhydride for acetylation were also purchased from Sigma Aldrich. Descriptions of solvents and other reagents are provided above (Methods for global lipid analyses). All solvents, reagents, and standards were used without further purification.

FA compositional analysis of LPS and lipid A compared to whole cell FAs. A microwave-assisted two-step derivatization method (methylation/esterification followed by silylation) was used for FA compositional analysis of LPS and lipid A samples, as described above (Lipid extraction and derivatization). Initial methylation was performed on ~2 mg of LPS and 2 mg of lipid A. The method described by Komagata and Suzuki (46) was used for extraction of total 3-HOFAs from whole cells, as described in Supplementary Methods (Direct extraction and analysis of 3-HOFAs from whole cells). Methylated-silylated 3-HOFAs, under EI+ conditions, yield ions at m/z 175, 133, and a neutral with mass 42 u and these ions/transition were chosen for product ion, precursor ion, and neutral loss MS/MS measurements, respectively. In addition, selected reaction monitoring (SRM) measurements monitoring the transition from the ion at m/z
175 (diagnostic ion for TMS derivatives of 3-HO FAMEs) to \( m/z \) 133 was used as the confirmatory identification of 3-HOFAs.

**Analysis of Kdo and other carbohydrates in LPS.** A two-step hydrolysis and derivatization method, methylation followed by peracetylation, was applied. This was carried out using a modified Rybka and Gamian method (47), described in further detail in Supplementary Methods (Derivatization of LPS for detection of Kdo and other sugars). Kdo ammonium salt and sugar standards were processed in the same way and used for confirmatory identifications of these compounds in *G. obscuriglobus* LPS, when analyzed by GC-MS.

**Methods for determination of lipid A structure**

**Reagents and supplies.** Methanol, the MALDI matrix 5-chloro-2-mercaptobenzothiazole (CMBT), and mass calibration reagents such as angiotensin I, angiotensin II, and neurotensin ACTH clip (1-17) were purchased from Sigma Aldrich (St. Louis, MO); suppliers of other reagents are described above (Methods for global lipid analysis). All solvents, reagents, and matrix were used without further purification.

**Deacylation of lipid A.** Lipid A samples were subjected to two different de-acylation approaches prior to MALDI-MS analysis. In the first procedure, partial de-O-acylation was performed as described by Silipo et al. (48). Briefly, approximately 1 mg of lipid A was mixed with 200 \( \mu \)L of dilute ammonia (1:3 \( \text{NH}_4\text{OH}/ \text{H}_2\text{O} \)) and left at room temperature for 16 hrs (alternatively, 0.25 M aqueous NaOH at 37°C for 15 min gives the equivalent result). This hydrolysis selectively eliminates O-linked acyl chains, such as 3-acyloxyacyl and FA chains. In
the second procedure, partial deacylation was carried out by mixing approximately 1 mg of lipid A with 200 µL of conc. ammonia and incubation at room temperature for 16 hrs. Concentrated NH₄OH completely eliminates O-linked acyl chains and secondary FA residues in the N-linked acyl chains. The primary FA residues in the N-acyl chains are unaffected. In both cases, ammonia was evaporated under a N₂ gas stream.

MALDI-MS instrumentation and lipid A sample preparation. MALDI-MS measurements were performed on both intact lipid A standards, as well as on lipid A samples that had been subjected to de-acylation reactions (above). Intact and partially deacylated lipid A samples were mixed with a 4:4:1 mixture of chloroform/methanol/water to make approximately 1 mg/ml solution. A fresh solution of CMBT MALDI matrix (20 mg/ml) was prepared in the same solvent mixture. Lipid A and matrix solutions were pre-mixed in a 1:1 ratio (v/v), and an aliquot of 1 µl of the sample/matrix solution was deposited onto a stainless steel target. Analyses were carried out using a MALDI-Time-of-Flight-MS (model: Voyager DE-STR, Applied Biosystems), equipped with a 337 nm N₂ laser, in the negative ion mode. An acceleration voltage of 20 kV was used in the delayed extraction (500 ns), reflector mode. The laser intensity was optimized to 10 - 20% above ionization threshold intensity. The low mass gate was set at \( m/z \) 700. The final mass spectra obtained were the average of 50 laser shots/spot from 10 different and random locations (or spots) within a deposited sample (i.e., a total of 500 laser shots/spectrum). A mixture of (20 ppm each) angiotensin II \([\text{C}_{58}\text{H}_{71}\text{N}_{13}\text{O}_{12}]\), monoisotopic (m.i.) exact mass 1045.5345 u, deprotonated m.i. mass 1044.5272 u], angiotensin I \([\text{C}_{52}\text{H}_{89}\text{N}_{17}\text{O}_{14}]\), m.i. mass 1295.6774 u, deprotonated m.i. mass 1294.6701 u) and neurotensin ACTH clip 1-17
(C$_{78}$H$_{121}$N$_{21}$O$_{20}$, m.i. mass 1671.9096 u, deprotonated m.i. mass 1670.9023 u), treated and analyzed in the same way, was used as an external mass calibrant.
Results

Analysis of *G. obscuriglobus* experimental cultures and lyophilized cell stock purity.

In addition to phase contrast microscopy examination of cells from the liquid cultures prepared for lyophilization in this study, plate colonies, from which liquid cultures were inoculated, had previously been streaked to isolate single colonies and examined carefully for plate growth rate and morphology, both of which were consistent with typical *G. obscuriglobus* characteristics. No contaminating colonies of different morphology or growth rate were found. Likewise, when wet mounts of representative cells from these plate colonies were examined using phase contrast microscopy, the cells also exhibited typical *G. obscuriglobus* cell characteristics.

To further confirm the purity of the stocks from which lyophilized cells were derived, 16S rRNA gene sequences were recovered from DNA extracted from these experimental stocks. Following PCR amplification with 16S rRNA universal primers, bands of the appropriate size of ~1.5 kb for the 16S rRNA gene product were detected, when compared to migration of a NEB 1 kb MWM (NEB # N3232S). The ‘No template’ and ‘Extraction Blank’ PCR reaction mixes showed no PCR product. Sequence chromatograms for each of the three independent sequencing reactions showed high signal-to-noise ratio, with clean peaks and no evidence of contaminating signal. The DNA sequences were compared to all partial and complete genome sequences of Bacteria in the JGI IMG genome database via blastn search, and each of the sequence comparisons returned 100% identity to *G. obscuriglobus*. Thus, the purity of these lyophilized experimental cultures was confirmed.
Global lipid analysis

**FAs.** Tentative identifications of all Bligh-Dyer extractable FAs from *G. obscuriglobus* were made by comparison of FAME mass spectra to that of standard FAMEs in the National Institute of Standards and Technology (NIST) 08 Standard Reference Database 1A or the American Oil Chemists’ Society (AOCS) Lipid Library Archives of Mass Spectra (Lipid Library) and by comparison of ECL (Fig. S2c). *G. obscuriglobus* was shown to possess both saturated and unsaturated FAs in the range C14 to C22 (Table 1; Fig. S2a,b), with C18:0 being the most abundant FA, followed by C16:1. Global GC-MS analysis of the Bligh-Dyer extractable fractions from *G. obscuriglobus* showed 69.6 ± 4.7% saturated FAs and 30.4 ± 4.7% unsaturated FAs, in contrast to the dominance of unsaturated FAs in *E. coli* (65.5 ± 5.1% unsaturated, 34.5 ± 5.1% saturated) (data not shown).

Several isomers of the monounsaturated FAs C16:1 and C18:1 were detected; however, no additional measurements were conducted to establish the location or stereochemistry of the double bonds in these positional isomers. In addition, GC-MS analyses of *G. obscuriglobus* determined the presence of methyl-branched *iso* and *anteiso* FAs, with signals at retention times (RT) of 11.14 and 11.20 min (Fig. S3a), through combined analysis of RI data (49), comparison of their mass spectra with the unbranched FA, and the reference mass spectra in published databases (NIST mass spectral database and the AOCS lipid library) (Table 1; Fig. S3a).

**PUFA.** A PUFA was tentatively identified in *G. obscuriglobus* Bligh-Dyer extract FAMEs by the mass spectral fragmentation pattern at RT = 16.70 min in the chromatogram (Fig. 1). Analyses of a *G. obscuriglobus* sample by GC-MS in both EI and PICI modes, with DHA-ME as
the reference compound, suggested that this signal corresponds to the PUFA heptacosatrienoic acid.

3-HOFAs. After silylation, the premethylated Bligh-Dyer extract of *G. obscuriglobus* cells showed multiple 3-HOFAs in the GC-MS extracted ion chromatogram (XIC) of *m/z* 175 (Fig. S4a). Identification of 3-HOFAs in *G. obscuriglobus* were made by comparing to chromatogram RI and mass spectral data obtained for a mixture of silylated 3-HOFAME standards in the range C14 - C18. Assuming equal ionization and detection sensitivity, 3-OH 15:0 and 17:0 were present in highest abundance.

Sterols. As reported previously (8), we detected the presence of two C30 sterols, lanosterol and parkeol, identified through comparison with the TMS derivative of the lanosterol standard (data not shown). Comparison of available mass spectra (NIST mass spectral database and the AOCS lipid library and published articles), for TMS derivatives of several possible lanosterol and parkeol isomers, suggested that the two early-eluting peaks in the *G. obscuriglobus* sample, at retention times 24.11 and 24.17 min, (Fig. S5) may represent isoarborinol isomers.

Detection of LPS and lipid A by differential staining and SDS-PAGE

Evidence for appropriate LPS and lipid A extraction and staining protocols. Because, to the best of our knowledge, LPS and lipid A protocols had not previously been applied to *G. obscuriglobus*, we performed the same lipid A extraction, staining, and electrophoresis methods on a commercial *E. coli* (strain 0111:B4) LPS standard, which thus served as a reference compound. *E. coli* commercial LPS standard, and lipid A preparations derived from it, showed
banding patterns in differentially stained gels (Fig. 2a – c), lane 1, lipid A and lane 2, LPS),
that were consistent with previously published information (50-53) for E. coli strain 0111:B4.
Presumed LPS and lipid A preps from G. obscuriglobus displayed increasing refinement through
the extraction and purification strategy (Fig. 2a, lanes 3-6) with the loss of higher MW bands in
the G. obscuriglobus lipid A samples (Fig. 2a - c, lanes 5, 6), as compared to the corresponding
crude and purified LPS (Fig. 2a - c, lanes 3 and 4 respectively), suggesting successful lipid A
enrichment. The noticeably evident major band at ~5 kDa in the Sypro Ruby stained gel (Fig.
2c, lanes 1, 5, 6) appearing in both E. coli and G. obscuriglobus lipid A preps, is consistent with
enriched lipid A after hydrolysis from LPS. The residual upper (40-90 kDa) MW bands in E.
coli lipid A (Fig. 2a, lane 1) may be relatively more resistant to the acid hydrolysis that liberates
lipid A, as has been reported for longer polysaccharide chains (Sigma L2630 LPS standard
product information). All three stains appropriately detected MW markers, with silver and Sypro
Ruby stains detecting eight CM bands (Fig. 2a, c respectively, lane CM) and six LM bands
(Fig. 2a, c respectively, lane LM), as expected. Sypro Ruby product literature (Molecular
Probes) state that it may negatively stain prestained protein markers, due to the ability of these
markers to quench the fluorescence of this stain. This was noted in our gel staining results as
well, but although stained negatively, the marker bands were still very visible on the Sypro
Ruby-stained gel (Fig. 2c, lane LM). Most importantly, the ProQ glycosylation stain detected
three of the glycosylated proteins, with MWs of 18, 42 and 82 kDa, from the Candy Cane marker
(Fig. 2b, lane CM), while all eight proteins were detected in this marker lane by either silver
stain (Fig. 2a, lane CM), which detects all macromolecules, including lipids, fatty acids,
proteins, nucleic acids, carbohydrates and lipopolysaccharides, or Sypro Ruby stain (Fig. 2c,
Evidence for LPS and lipid A in *G. obscuriglobus*. *G. obscuriglobus* samples yielded banding profiles consistent with the presence of LPS/lipid A in both silver- and differentially stained gels (Fig. 2a – c, lanes 3-6), based on comparison with the *E. coli* controls, as well as general literature on the migration profiles of LPS and lipid A from other bacterial species. Laddering and glycosylation shown in the middle-to-upper (~18 - 82 kDa) MW regions of the *G. obscuriglobus* LPS samples (Fig. 2a - c, lanes 3, 4), and even more distinctly (~20 - 90 kDa for *E. coli* LPS (Fig. 2a - c, lane 2), suggest the presence of LPS O-antigen polysaccharides of various lengths. Although each showed a different pattern, distinct bands for *E. coli* lipid A within the range 5 – 20 kDa (Fig. 2a - c lane 1) and LPS (lane 2), as well as somewhat less distinct bands in the same ranges for *G. obscuriglobus* lipid A (Fig. 2 a – c lanes 5, 6) and LPS (lanes 3, 4), are likely to represent lipid A and inner/outer core LPS components. The numerous bands ranging from ~10 to 90 kDa in *E. coli* LPS (Fig. 2a - c, lane 2), and ~18 to 82 kDa in *G. obscuriglobus* LPS (Fig. 2a - c, lanes 3, 4), in all three stained gels, particularly noting those also detected with the Pro Q glycosylation stain (Fig. 2c), suggest that there are multiple oligosaccharide sugar moieties of differing composition in the LPS of both of these species.

Sypro Ruby (primary amine) staining of *E. coli* (Fig. 2c, lane 1) and *G. obscuriglobus* (Fig. 2c, lanes 5, 6) lipid A preps detected very low MW bands (~5 kDa), that are not visible on silver-stained (Fig. 2a), or glycosylation-stained gels (Fig. 2b), but that are characteristic of lipid A presence.
Beyond these common features, the LPS and lipid A banding profiles of *G. obscuriglobus* and *E. coli*, and thus their presumed biochemical structure, are very different. *G. obscuriglobus* samples show significant glycosylation within the MW range of 18 - 82 kDa (Fig. 2b, lanes 3-6), with particularly strong detection of two major LPS bands at ~25 and 50 kDa that were not observed in *E. coli*. The lower band at 25 kDa is also very distinct in the *G. obscuriglobus* lipid A samples. The Sypro Ruby stain (Fig. 2c, lanes 1-6), which associates with primary amines, revealed an additional difference between the lipid A and LPS samples for both *G. obscuriglobus* and *E. coli*, as a major band of ~5 kDa is detectable, although partially obscured by a gel staining artifact, in only the lipid A of both *E. coli* (Fig. 2c, lane 1) and *G. obscuriglobus* (Fig. 2c, lanes 5 and 6).

**Targeted analysis of LPS composition**

3-HOFA composition of LPS and lipid A. As this method has not been previously applied to *G. obscuriglobus*, we validated our protocol by analyses of isolated LPS and lipid A from controls, i.e., *E. coli* LPS standard (Sigma Aldrich) and *E. coli* UCLA strain #1246, in the same way as for *G. obscuriglobus* LPS. Both controls, when methylated and silylated, showed a single peak in XIC of m/z 175, identified as 3-OH C14:0 (data not shown), the presence of which is in agreement with previous reports (54).

GC-MS analysis of the non-polar fraction of *G. obscuriglobus* LPS after methylation-silylation showed eight 3-HOFAs, in the range C14-C18 (Fig S4b), with 3-OH C14:0 being the most abundant. The other seven FAs included three C15:0 (*iso, anteiso*, and *n*), a C16:0, two C17:0 (*iso or anteiso and n*) and a C18:0. 3-HOFAs were identified by comparison with GC RI.
data obtained for a mixture containing five saturated 3-HOFA standards derivatized in the same way. A similar GC-MS profile was obtained for *G. obscuriglobus* lipid A prepared in the same way (**Fig. S6**). The relative abundances of C14:0, C18:0, C16:0, *iso* & *n*-C15:0, and the remaining OH FAs were in the proportions 4:1:0.5:0.5:0.25, and were indistinguishable for both LPS and lipid A.

Analysis by GC-MS of methylated and silylated 3-HOFAs extracted from *G. obscuriglobus* cells using the Komagata-Suzuki methods (46) showed 13 different 3-HOFAs, in the range C14 - C18, including monounsaturated and methyl-branched compounds. **Fig. 3a** shows these 3-HOFAs, detected in the XIC *m/z* 175 using the single quadrupole instrument, which were identified and confirmed using RI data (i.e., ECL’s). Tandem MS (MS/MS) analysis in SRM mode, using the transition from *m/z* 175 to *m/z* 133, was also performed, confirming the presence of the same signals detected in the XIC of *m/z* 175 measurements (**Fig. 3b, c**).

**Kdo in LPS.** GC-MS analyses of peracetylated, premethylated *G. obscuriglobus* LPS showed three Kdo peaks in XIC of *m/z* 375 (**Fig. 4, Fig. S7**). These peaks were identified based on their combined mass spectral and GC RT data. In contrast to the isomer profile of the Kdo standard, Kdo1 (pyranoside ring) is the dominant isomer in *G. obscuriglobus* LPS, as is also seen in *E. coli* (**Fig. 4, Fig. S7**). Both of these bacterial species have a relative abundance Kdo1 > Kdo2 > Kdo3, based on the signal at *m/z* 375.

**Carbohydrates.** *G. obscuriglobus* LPS, when derivatized and analyzed by GC-MS, was shown to contain both hexose and heptose sugars. The XIC of *m/z* 243, 331 and XIC of *m/z* 315, 403, respectively (**Fig. S8**). These carbohydrates were subsequently identified as glucose and...
mannoheptose, by comparison to a similarly derivatized standard sugar mixture, on the basis of both GC RT and MS fragmentation information (data not shown).

Glucosamine. GC-MS analyses of completely methylated E. coli and G. obscuriglobus LPS, after acetylation, showed peaks tentatively identifiable as glucosamine at RT = 9.65 and 9.75 min, through comparison with a glucosamine standard (Fig. S9). Although glucosamine was identified in both G. obscuriglobus LPS and lipid A (data not shown), the exact location of this chemical moiety (polysaccharide vs lipid A) could not be confirmed.

Determination of lipid A structure

Structural analysis of intact G. obscuriglobus lipid A. MALDI parameters were optimized by analysis of lipid A derived from the E. coli 0111:B4 LPS standard. The mass spectrum of a different E. coli lipid A extract control (UCLA #1246; Fig. 5a) showed a similar fragmentation pattern to that of the lipid A standard (data not shown). However, the lipid A sample isolated from G. obscuriglobus cells showed a completely different MALDI-mass spectrum, with no discernable fragmentation pattern and no apparent loss of 80 u, the characteristic fragmentation pattern for dephosphorylation (Fig. 5b). A series of peaks separated by 14 u, ranging from m/z 2017.16 to 2143.28, with the most intense peak at m/z 2087.22 (monoisotopic mass for the deprotonated molecule, [M-H]'), were observed (Fig. 5c). Given that 3-OH C14:0 was shown to be the most abundant G. obscuriglobus FA by GC-MS analyses, it can be assumed that the most intense peak at m/z 2087.22 corresponds to a dominant lipid A structure having 3-OH C14:0 as the major FA component (and assuming equal MALDI efficiencies for all lipid A moieties). Further investigation revealed another cluster of 14 u-separated peaks in the range m/z 1883.28 -
1953.48 (Fig. 5b). The most intense peak in this cluster, i.e., m/z 1911.13, is 176 u away from the m/z 2087.22. A low-intensity peak at m/z 1684.28 and another cluster of peaks around m/z 1270.44 were also observed.

Use of the Hamidi method (44) of direct lipid A isolation from whole cells provided an alternative isolation method to that of Yi and Hackett (43), which may cause alterations in lipid A structure, such as loss(es) of primary or secondary O-acyl chain and dephosphorylation (55,56). For both controls and G. obscuriglobus, the mass spectra obtained from the Hamidi preps were similar to those from the Yi and Hackett method (data not shown), although of lower signal-to-noise ratio.

**Structural analysis of selectively deacylated lipid A.** To substantiate the G. obscuriglobus lipid A MALDI-mass spectrum fragmentation pattern (Fig. 5b) and to ascertain the location of fatty acid chains, de-O-acylation reactions were carried out, sequentially removing primary O-acyl FA chains, followed by secondary O-acyl-linked FA residues in N-acyl chains, by ammonia hydrolysis. For *E. coli* lipid A, both dilute and conc. ammonia hydrolysates produced similar GC mass spectra. However, *G. obscuriglobus* lipid A did not exhibit any significant evidence of deacylation (data not shown).

**Carbohydrate compositional analyses of LPS and lipid A.** Comprehensive GC-MS analysis of *G. obscuriglobus* LPS, derivatized by methylation-peracetylation, showed the presence of glucosamine and diamino glucose (Fig. 6). Glucosamine was identified by mass spectral and RT comparisons with the glucosamine standard, while diamino glucose (2, 3-diamino, 2, 3-dideoxy glucose; GlcN3N) was tentatively identified on the basis of differences in fragmentation pattern.
and RT shift (data not shown) due to the presence of an extra –NH-Ac group (there was not a
Lipid Library entry available for a diamino diacetylated methyl glycoside derivative). A
hexuronic acid (e.g., GalA, MW 194 u) was also identified in the G. obscuriglobus LPS by GC-
MS analysis (Fig. 6d). Additionally, a G. obscuriglobus lipid A sample, isolated by the Yi and
Hackett method (43), when analyzed in the same way as the LPS, showed all the polar
components described above (Fig. 6). This lipid A sample, when analyzed by GC-MS, showed
two peaks, RT = 26.03 and 26.80 min (data not shown), having similar mass spectra to that of
the glucosamine standard. However, the glucosamine standard, exhibited two peaks consistent
with the presence of α and β anomers, while only one G. obscuriglobus peak matched a RT of
the glucosamine β anomer, and the other G. obscuriglobus peak eluted 6 seconds before the RT
of the α anomer. Thus, GC-MS analyses of both G. obscuriglobus LPS and lipid A produced
peaks identified as glucosamine and another peak tentatively identified as diamino glucose.
Discussion

The principal aim of this study was to experimentally determine the presence of LPS, a hallmark of the OM (26,27), in *G. obscuriglobus*, based on previous predictions from genomic data (20,25,42). Specific components within the *G. obscuriglobus* LPS were elucidated, including lipid A 3-OH fatty acid composition, the presence of Kdo (the disaccharide which links the lipid A to LPS core oligosaccharides (35)), and the presence of glucose and mannoheptose, which may constitute the *G. obscuriglobus* core oligosaccharide or O-Ag components (30,35). Combined data from these analyses provide a tentative lipid A structure for *G. obscuriglobus* (presented below) that is a monophosphorylated hexa-acylated structure with a trisaccharide backbone. Furthermore, we identified a putative PUFA and two sterols that have not previously been reported, and achieved a detailed comparison of FAs present in *G. obscuriglobus* whole cells, LPS, and lipid A. Our isolation and characterization of LPS suggests indirectly that an OM is present.

Global lipid analysis

FAs. Both saturated and unsaturated long-chain fatty acids (LCFAs) were found in *G. obscuriglobus* whole cell extracts. We found that *G. obscuriglobus* contained ~70% saturated vs. ~30% unsaturated FAs, consistent with a previous analysis (41) that reported 82% saturated and 18% unsaturated FAs. As reported in an earlier study (57), C18:0 and C16:1 were found to be the dominant FAs. This profile is also in agreement with non-hydroxy FA composition reported previously by Sittig and Schlesner (41). Long-chain FAs (LCFAs, 16-18 carbons) are necessary for production of lipids required to support the integrity of bacterial membranes (58,59), and...
LCFAs also contribute to low bacterial endotoxicity (38), consistent with the fact that no previous study or literature reference reports endotoxicity in *G. obscuriglobus*. Several isomers of the monosaturated FAs C16:1 and C18:1 were detected, as well as methyl-branched *iso* and *anteiso* FAs, including methyl-branched-chain 3-HOFAs. Branched-chain FAs impact membrane fluidity through effects on the combined FA melting point (60-62).

**PUFA.** In whole cell extracts, we detected a previously unreported very-long-chain FA (VLCFA, >18 carbons) that appears to be a heptacosatrienoic (ω3 C27: 3) acid, a type of omega-3 PUFA (63-65). To the best of our knowledge, a PUFA of this same structure and chain length has been found only in marine sponges (66). A different VLCFA, n-C31:9, has been reported previously for *G. obscuriglobus* (57). VLCFAs are most often found in Alphaproteobacteria and the order Cytophagales (57,67), and can be derived from exogenous sources (68), or synthesized *de novo*; the origin of the *G. obscuriglobus* PUFA remains to be determined. PUFAs exhibit very low melting temperatures and thus contribute to increased membrane fluidity. The *G. obscuriglobus* PUFA may provide an adaptive response to increase membrane fluidity (through low melting temperature), counterbalancing the reduction in fluidity associated with abundant saturated FAs (69). PUFAs also regulate membranes and membrane-bound proteins (68), aid in membrane curvature (70), and facilitate increased membrane permeability, as well as membrane vesicle formation and fusion (71,72). A role for the *G. obscuriglobus* PUFA in membrane curvature, permeability, membrane remodeling, or vesicular transport would be an intriguing avenue for future study, given the presence of MC-like proteins and a proposed tubulovesicular transport system (7).
3-HOFAs. Multiple long-chain 3-HOFAs were identified in *G. obscuriglobus* whole cell extracts, consistent with an earlier study (41) that reported eight different 3-HOFAs, and exceeding the single long-chain normal 3-HOFA reported for other Planctomyces species, *Planctopirus limnophilus*, *Planctomyces* sp. 1317, and *Pirellula staleyi* (40,73). The presence of both branched-chain FAs and 3-HOFAs in a single species is relatively rare (61). The diversity of *G. obscuriglobus* 3-HOFAs may be related to the presence of a PUFA, as discussed above. β-OH FAs may also be components of ornithine-amide lipids (74), and several Planctomyces strains contain ornithine lipids (75). Although we cannot exclude the possibility that the 3-HOFAs of *G. obscuriglobus* derive from other cellular lipids, 3-HOFAs are always components of lipid A (35), as discussed below.

Sterols. We confirmed the presence of two previously reported *G. obscuriglobus* C₃₀ sterols, lanosterol and parkeol (8), but also detected two additional sterols, tentatively identified as isoarborinol isomers. The fact that *G. obscuriglobus* contains both sterols and a PUFA is intriguing, considering reports that a high degree of membrane disorder results from this lipid combination (71,76,77). It is possible that these two lipid classes are differently distributed in the cell's membranes to provide locally specialized functions.

Detection of LPS and lipid A by differential staining and SDS-PAGE

Evidence for LPS and lipid A in *G. obscuriglobus*. Our SDS-PAGE profiles of extracted and purified *G. obscuriglobus* LPS provide the first gel images that are consistent with isolated LPS and lipid A for this species, or any Planctomyces. Increasing refinement of successive extractions was evident, with the loss of higher molecular weight material in the lipid A prep.
Gel profiles of *E. coli* standard LPS and lipid A indicate that the *E. coli* LPS may be more resistant to the hydrolysis required to liberate lipid A, as has been reported for LPS carrying longer chain polysaccharides (Sigma L2630 LPS product information).

Differentially stained SDS-PAGE gel profiles show results consistent with published gel images specific to LPS of the particular *E. coli* strain (0111:B4) (50,51,53,78) represented by the *E. coli* standard, and more generally with data reported in the scientific literature for LPS from various other bacteria (24,28,30,31,37,79-81). Common features for *E. coli* and *G. obscuriglobus* LPS include the presence of multiple oligosaccharide sugar moieties that differ in composition between the two species, inferred from band laddering and glycosylation. This suggests the presence of LPS O-antigen polysaccharides of varied length or other glycosylated components; LPS inner and outer core oligosaccharides are typically composed of up to 15 sugar moieties (31). Some Gram-negative bacteria lack the repeating units referred to as O-Ag, or display a very limited O-Ag chain (28,82). *G. obscuriglobus* may be one of these, based on the smaller number of laddered glycosylated bands laddered in the gel region where O-Ag bands typically migrate. A related bacterium within the *Chlamydiaceae* has been found to carry the most truncated LPS structure reported. The SDS-PAGE gel bands of very low MW, that are visible only in the lipid A preps of both species, are characteristic of lipid A presence (24,28,30,31,37,79). This major component may be most visible in the lipid A gel lanes, because it represents lipid A that is released from LPS by hydrolysis and subsequently enriched.

Bands of ~10-20 kDa in LPS and lipid A from both species may represent lipid A and inner/outer core LPS components, based on literature comparisons (24,28,30,31,37). Differences
between *G. obscuriglobus* and *E. coli* LPS or lipid A include significant glycosylation of *G. obscuriglobus* LPS components in the range of 20 - 85kDa, with two particularly apparent entities (~25 and 50 kDa) not found in the *E. coli* LPS. Because of their laddered appearance, apparent molecular weights, and glycosylation, the LPS of *G. obscuriglobus* could include a very limited O-Ag makeup (discussed above), or alternatively represent part of the core oligosaccharide. One of these glycosylated components (~25 kDa) is also found in the *G. obscuriglobus* lipid A preparation.

**Targeted analysis of LPS composition**

**3-HOFA composition of LPS and lipid A.** GC-MS analysis of *G. obscuriglobus* LPS showed eight 3-HOFAs, in the range C14-C18, with C14 being most abundant. The same GC-MS profile was obtained from analysis of *G. obscuriglobus* lipid A, expected because LPS hydrolysis would release lipid A from its linkage to Kdo, and the lipid A FA content would remain the same as that for the entire LPS. 3-HOFAs are always present in lipid A structures, with the degree of acylation impacting host immune response when exposed to a particular lipid A (24). In contrast to the relatively restricted 3-OH content of the LPS, whole cells were shown to contain 13 different 3-HOFAs (discussed above), suggesting that our purification protocols for LPS and lipid A were effective.

**Kdo in LPS.** GC-MS analyses of *G. obscuriglobus* LPS revealed three Kdo (35,38,79) peaks. Identification of Kdo in *G. obscuriglobus* is a finding of note, because all LPS described to date contains either Kdo or Ko (26,34,83), although Kdo is known to occur in other cell polymers (84). The LPS inner core of a Gram-negative bacterium usually has 1-3 Kdo and 2-3 specific heptoses, and a variety of possible structural configurations (83). Alternative methodologies will
be needed to determine the exact number of Kdo moieties (i.e. Kdo monomer, dimer, trimer etc.). Both *G. obscuriglobus* and *E. coli* (UCLA #1246) had a relative abundance of Kdo1 > Kdo2 > Kdo3, but identifying the specific structural location of each of these isomers in *G. obscuriglobus* requires further study.

**Carbohydrates.** *G. obscuriglobus* LPS, when derivatized and analyzed by GC-MS, was shown to contain both hexose and heptose sugars, consistent with a previous report (18). These sugars, further identified as glucose and mannoheptose, may represent the LPS core oligosaccharide or O-Ag components, although heptose has been known to occur in other non-LPS bacterial glycolipids (85); more detailed structural analysis will be needed to decipher their specific location within the LPS structure.

**Glucosamine.** Although the exact location of glucosamine in the *G. obscuriglobus* LPS, within the core polysaccharide chain or part of lipid A, could not be determined, its presence in both the LPS and lipid A preps supports effective isolation of LPS, because lipid A is composed of a unique carbohydrate-lipid combination that is frequently a β-1,6-D-glucosamine disaccharide linked to a varied number of FAs and OH-FAs.

**Determination of lipid A structure**

Structural analysis of intact *G. obscuriglobus* lipid A suggested that the dominant *G. obscuriglobus* lipid A structure has 3-OH C14:0 (the most abundant FA in whole cells) as the major FA. The presence of multiple repeated peak clusters of a similar fragmentation pattern, and detection of eight different lipid A 3-HOFAs, raises the possibility that *G. obscuriglobus*
contains multiple lipid A species, as previously reported for *Porphyromonas gingivalis* (86). Consistent mass spectra obtained for intact lipid A using the Yi and Hackett and Hamidi methods support our predicted lipid A structure. Structural analysis of deacylated *G. obscuriglobus* lipid A did not show any evidence of sequential removal of O-acyl FA chains by deacylation, which may indicate that it lacks O-acyl chain(s).

**Sugar compositional analyses of LPS and lipid A.** Detection of glucosamine, (tentatively) diamino glucose, and a hexuronic acid in both LPS and lipid A samples from *G. obscuriglobus*, suggest that all three components are contained in the lipid A backbone. Hexuronic acid is found within the glycosaminoglycan family (GAGs) and found in lipid A from other bacterial species e.g., *Rhodospirillum salinarum* 40 (87) and *Rhizobium trifolii* (88). The lipid A backbones of *Rhizobium* species also contain either D-galacturonic acid/D-glucosamine or glucosamine/2,3-diamino glucose (89), similar to our findings in *G. obscuriglobus*.

**Elucidation of a tentative lipid A structure for G. obscuriglobus.** On the basis of combined GC-MS and MALDI-MS results, we propose a tentative structure for the *G. obscuriglobus* lipid A molecule (one representative structure shown in Fig. 7), that is a monophosphorylated hexa-acylated structure with a trisaccharide backbone, featuring a heterodimer of GlcN-GlcN3N with a phosphate at 1 replaced by a hexuronic acid (GalA), i.e., P-GlcN-GlcN3N-GalA. Hexuronic acids are capable of maintaining the negative charge on a lipid A molecule that is more commonly provided by phosphate moieties. Negative charges in close proximity to a membrane are characteristic of known LPS structures and contribute to membrane stability by allowing cross-linking with bivalent cations. For the most intense acyl homolog cluster in the *G.
obscuriglobus MALDI mass spectrum m/z 2087.22 (monoisotopic mass of deprotonated molecule), our proposed structure would correspond to the neutral molecular formula C_{114}H_{214}N_{3}O_{27}P (monoisotopic mass 2088.52 Da). The tentative representative structure (Fig. 7) shows an example FA distribution, with linked FA’s with carbon numbers 16, 14, 16, 14, 18, and 18 (total of 96 carbon atoms, plus 18 carbon atoms in the trisaccharide backbone, for a total of 114 carbon atoms). This tentative structure comprises contributions from three of the most abundant 3-OH FAs, n-14:0, n-C16:0, and n-C18:O, detected in our GC-MS measurements.

The monophosphorylation of G. obscuriglobus lipid A (when one of two typical phosphate group positions is replaced by hexuronic acid) may contribute to lower endotoxicity for this lipid A (90), as this organism has not been reported to exhibit endotoxicity and may be impacted by this lipid A structural replacement. There are several other examples of Gram-negative bacteria that exhibit only one, or a complete lack of, phosphate groups in their lipid A, but a diphosphorylated lipid A is the most highly conserved structure in this bacterial group (34). To our knowledge, Rhizobium etli is the only other organism reported to have a lipid A structure containing the same trisaccharide backbone as that proposed for G. obscuriglobus, although there are 2-3 reported lipid A structures that do carry the heterologous disaccharide backbone of GlcN- GicN3N (28,91). The tentative representative structure we propose is an example of possible FA distribution, 16+14+17+14+18+18 = 97, that incorporates three of the most abundant 3-OH FAs, n-14:0, n-C16:0, and n-C18:O, detected in the GC-MS measurements. However, other structures are possible, and later studies involving further fractionation of the individual components may be necessary.
Our confirmation of a LPS implies the presence of an OM in *G. obscuriglobus*, although verification of the LPS location within this organism by immunogold or fluorescent antibody detection will be necessary, and raises the possibility that other Planctomycetes also have outer membrane components. Pursuit of this question, together with studies of the structural connections between the LPS of *G. obscuriglobus* (and possibly other Planctomycetes) and the newly discovered Planctomycete peptidoglycans (16,17), will shed more light on what is now emerging as the Planctomycete variation on the Gram-negative cell plan (20).

Acknowledgments

This work was supported by an award to N.L.W. from the United States National Science Foundation (MCB-0920667), as well as grants from the National Center for Research Resources (5P20RR016474-12) and the National Institute of General Medical Sciences (8 P20 GM103432-12) from the National Institutes of Health. Acquisition of the GC-MS instrument was made possible through an award to F. B. from the National Science Foundation (NSF CAREER CHE-0844694).

Literature cited


Figure legends

Figure 1. EI mass spectrum of an unknown PUFA in *G. obscuriglobus* with RT 16.70 min showing $m/z$ 108 as diagnostic ion (ω) of ω3 PUFAs and tropylium ion (*) at $m/z$ 91 as base peak. The mass spectrum in inset is of DHA-methyl ester with RT = 13.90 min, showing both ions.

Figure 2. Differential staining of LPS and Lipid A extracts resolved on 4-20% SDS-PAGE. (a) Silver stain; (b) Emerald ProQ glycosylation stain; (c) Sypro Ruby protein stain. Samples include: Sigma Standard *E. coli* strain 0111:B4 lipid A (1) and purified LPS (2); Candy Cane glycosylated protein marker (CM); *G. obscuriglobus* crude LPS (3), purified LPS (4) and lipid A (5,6); Spectra low molecular weight marker (LM).

Figure 3. (a) Identified 3-HOFAs in *G. obscuriglobus* with combined GC and MS data. Analysis performed using the single quadrupole GC-MS: XIC of $m/z$ 175 for *G. obscuriglobus* treated by the Komagata-Suzuki method with identified peaks produced by GC-MS. (b) Analysis performed using the triple-quadrupole GC-MS/MS: Chromatograms comparing 3-HOFA distributions in *G. obscuriglobus* cell by full scan GC-MS/MS and (c) GC-MS/MS in SRM mode. The profile in the chromatogram represents SRM mass spectrum showing peak at $m/z$ 133.

Figure 4. (a) XIC ($m/z$ 375) showing the presence of Kdo in *G. obscuriglobus* LPS analyzed by GC-MS. As found in Gram-negative bacteria, the pyranosidic form (Kdo1) is the predominant Kdo in *Gemmata* LPS. (b) Mass spectrum showing fragmentation pattern of peak at RT 10.16
min. The fragmentation pattern and RT match those of the Kdo standard, confirming the presence of Kdo in *G. obscuriglobus*.

**Figure 5.** Comparative negative ion MALDI-TOF mass spectra of (A) *E.coli* controls and (B) *G. obscuriglobus* lipid A isolated by the Yi and Hackett method. Lack of adequate fragmentations impedes determination of exact molecular structure. However, the presence of a cluster of ions, each separated by 14 u, may indicate the presence of multiple lipid A species in *G. obscuriglobus*. (C) Expanded view of the negative ion mode MALDI-TOF mass spectrum showing the de-protonated molecule region for the lipid A isolated from *G. obscuriglobus* (inset: calculated isotopic pattern for the highest abundant acyl homolog).

**Figure 6.** (a) XIC of m/z 101 showing peaks corresponding to glucosamine and diaminoglucose, when methylated, acetylated *G. obscuriglobus* LPS was analyzed via GC-MS. The corresponding mass spectra for glucosamine (b) and diaminoglucose (c). Glucosamine produces characteristic m/z 330 with the loss of methoxy radical, while diaminoglucose produces m/z 329 by the same loss. (d) EI mass spectrum (GC-MS) of hexuronic acid with the characteristic ion at m/z 317 shown in the profile. The mass spectrum of the hexuronic acid from *G. obscuriglobus* has 80% match with the NIST library hit. All samples were derivatized by methylation-acetylation.

**Figure 7.** A tentative representative molecular structure for *G. obscuriglobus* lipid A, referring to m/z 2087.22. The structure shown is neutral. The secondary FA chain presented in blue or 3-OH C16:0 at C3, when replaced with FA chains=C18:0, yields m/z’s at 2073.22 and 2059.22
respectively. On the other hand, replacement of the 3-OH FA chain drawn in green with FA-C14:0 yields the signals above \( m/z \ 2087.22 \).
Table 1. List of identified FAs in Bligh-Dyer extracts of lyophilized *G. obscuriglobus* cells.

Retention Time (RT); Equivalent Chain Length (ECL), calculated; Identification (ID) from GC Mass Spectrometry (MS); ID from ECL; Final ID (consensus of ID from MS and ECL); Qualifier Ions

<table>
<thead>
<tr>
<th>RT</th>
<th>ECL (Calculated)</th>
<th>ID from MS</th>
<th>ID from ECL</th>
<th>Final ID</th>
<th>Qualifier Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.32</td>
<td>14.05</td>
<td>C14:0</td>
<td>C14:0</td>
<td>C14:0</td>
<td>M⁺ 242, 213, 211, 199, 87, 74*</td>
</tr>
<tr>
<td>9.78</td>
<td>14.72</td>
<td>C15:0</td>
<td>methyl C14:0</td>
<td>iso C15:0</td>
<td>M⁺ 256, 227, 229, 199, 87, 74*</td>
</tr>
<tr>
<td>10.01</td>
<td>15.06</td>
<td>C15:1</td>
<td>C15:1</td>
<td>C15:1</td>
<td>M⁺ 254, 222, 97, 87, 74, 69, 55*</td>
</tr>
<tr>
<td>10.04</td>
<td>15.11</td>
<td>C15:0</td>
<td>C15:0</td>
<td>C15:0</td>
<td>M⁺ 256, 227, 229, 199, 87, 74*</td>
</tr>
<tr>
<td>10.69</td>
<td>16.06</td>
<td>C16:1</td>
<td>C16:1</td>
<td>C16:1</td>
<td>M⁺ 268, 236, 97, 87, 74, 69, 55*</td>
</tr>
<tr>
<td>10.73</td>
<td>16.12</td>
<td>C16:0</td>
<td>C16:0</td>
<td>C16:0</td>
<td>M⁺ 270, 241, 239, 199, 87, 74*</td>
</tr>
<tr>
<td>11.14</td>
<td>16.73</td>
<td>C17:0</td>
<td>methyl C16:0</td>
<td>iso C17:0</td>
<td>M⁺ 284, 255, 253, 199, 87, 74*</td>
</tr>
<tr>
<td>11.21</td>
<td>16.83</td>
<td>C17:0</td>
<td>methyl C16:0</td>
<td><em>anteiso</em> C17:0</td>
<td>M⁺ 284, 255, 253, 199, 87, 74*</td>
</tr>
<tr>
<td>11.29</td>
<td>16.95</td>
<td>C17:1</td>
<td>C17:1</td>
<td>C17:1</td>
<td>M⁺ 282, 250, 97, 87, 74, 69, 55*</td>
</tr>
<tr>
<td>11.39</td>
<td>17.10</td>
<td>C17:0</td>
<td>C17:0</td>
<td>C17:0</td>
<td>M⁺ 284, 255, 253, 199, 87, 74*</td>
</tr>
<tr>
<td>11.90</td>
<td>17.85</td>
<td>C18:1</td>
<td>C18:1</td>
<td>C18:1</td>
<td>M⁺ 296, 264, 97, 87, 74, 69, 55*</td>
</tr>
<tr>
<td>11.97</td>
<td>17.95</td>
<td>C18:1</td>
<td>C18:1</td>
<td>C18:1</td>
<td>M⁺ 296, 264, 97, 87, 74, 69, 55*</td>
</tr>
<tr>
<td>Time</td>
<td>Peak 1</td>
<td>Peak 2</td>
<td>Peak 3</td>
<td>M⁺ Peaks</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>12.02</td>
<td>18.02</td>
<td>C18:0</td>
<td>C18:0</td>
<td>298, 269, 267, 199, 87, 74*</td>
<td></td>
</tr>
<tr>
<td>12.39</td>
<td>18.57</td>
<td>C19:0</td>
<td>methyl C18:0</td>
<td>312, 283, 281, 199, 87, 74*</td>
<td></td>
</tr>
<tr>
<td>12.45</td>
<td>18.66</td>
<td>C19:0</td>
<td>methyl C18:0</td>
<td>312, 283, 281, 199, 87, 74*</td>
<td></td>
</tr>
<tr>
<td>12.61</td>
<td>18.89</td>
<td>C19:0</td>
<td>C19:0</td>
<td>methyl C18:0</td>
<td>312, 283, 281, 199, 87, 74*</td>
</tr>
<tr>
<td>13.08</td>
<td>19.59</td>
<td>C20:1</td>
<td>C20:1</td>
<td>methyl C18:0</td>
<td>324, 292, 97, 87, 74, 69, 55*</td>
</tr>
<tr>
<td>13.14</td>
<td>19.67</td>
<td>C20:1</td>
<td>C20:1</td>
<td>methyl C18:0</td>
<td>324, 292, 97, 87, 74, 69, 55*</td>
</tr>
<tr>
<td>13.18</td>
<td>20.19</td>
<td>C20:0</td>
<td>C20:0</td>
<td>methyl C18:0</td>
<td>326, 297, 295, 199, 87, 74*</td>
</tr>
<tr>
<td>13.73</td>
<td>21.02</td>
<td>C21:0</td>
<td>C21:0</td>
<td>methyl C18:0</td>
<td>340, 311, 309, 199, 87, 74*</td>
</tr>
<tr>
<td>14.15</td>
<td>21.65</td>
<td>C22:1</td>
<td>C22:1</td>
<td>methyl C18:0</td>
<td>352, 320, 97, 87, 74, 69, 55*</td>
</tr>
<tr>
<td>14.26</td>
<td>21.82</td>
<td>C22:0</td>
<td>C22:0</td>
<td>methyl C18:0</td>
<td>354, 325, 323, 199, 87, 74*</td>
</tr>
</tbody>
</table>

Here, M⁺ is the molecular ion peak and m/z value marked with an asterisk (*) indicates the base peak in the mass spectrum.
Unknown PUFA-methyl ester in G. obscuriglobus
a. XIC m/z 175 showing 3-HOFAs in *G. obscuriglobus*

b. Full scan in GC-MS/MS: XIC m/z 175

c. SRM m/z 175 → m/z 133
a. *G. obscuriglobus* LPS: Kdo analysis, XIC m/z 375

![Graph showing Kdo1, Kdo2, and Kdo3 peaks with relative abundance and time (min).]

b. *Loss of acetic acid (60 u)*
   *Loss of CH₂CO (42 u)*

\[m/z \text{ 213 > 217}\]

![Graph showing m/z values with Kdo1 peak at 375.18.]

**M+ -59**