An Outer Membrane Enzyme That Generates the 2-Amino-2-deoxy-gluconate Moiety of Rhizobium leguminosarum Lipid A

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The structures of Rhizobium leguminosarum and Rhizobium etli lipid A are distinct from those found in other Gram-negative bacteria. Whereas the more typical Escherichia coli lipid A is a hexa-acylated disaccharide of glucosamine that is phosphorylated at positions 1 and 4', R. etli and R. leguminosarum lipid A consists of a mixture of structurally related species (designated A–E) that lack phosphate. A conserved distal unit, comprised of a diacylated glucosamine moiety with galacturonic acid residue at position 4' and a secondary 27-OH-C28 acyl chain as part of a 2' acyloxyacyl moiety, is present in all five components. The proximal end is heterogeneous, differing in the number and lengths of acyl chains and in the identity of the sugar itself. A proximal glucosamine unit is present in B and C, but an unusual 2-amino-2-deoxy-gluconate moiety is found in D-1 and E. We now demonstrate that membranes of R. leguminosarum and R. etli can convert B to D-1 in a reaction that requires added detergent and is inhibited by EDTA. Membranes of Sinorhizobium meliloti and E. coli lack this activity. Mass spectrometry demonstrates that B is oxidized in vitro to a substance that is 16 atomic mass units larger, consistent with the formation of D-1. The oxidation of the lipid A proximal unit is also demonstrated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry in the positive and negative modes using the model substrate, 1-dephospho-lipid IV A. With this material, an additional intermediate (or product) is detected that is tentatively identified as a lactone derivative of 1-dephospho-lipid IV A. The enzyme, presumed to be an oxidase, is located exclusively in the outer membrane of R. leguminosarum as judged by sucrose gradient analysis. To our knowledge, an oxidase associated with the outer membranes of Gram-negative bacteria has not been reported previously.

The Rhizobiaeae are agriculturally important Gram-negative bacteria that are able to establish a symbiotic relationship with the root cells of certain plants (1). The symbiotic bacteria provide the plant with \( \text{NH}_4^+ \) by fixing \( \text{N}_2 \), whereas the plant provides the bacteria with reduced carbon sources (2). A detailed understanding of the many factors contributing to the complex interplay between the plant host and the microbes is beginning to emerge (3). A few of the key components needed for effective symbiosis and nitrogen fixation include flavonoids (4), nod factors (5, 6), receptor kinases (7), various exopolysaccharides (8, 9), cyclic \( \beta \)-glucans (10, 11), K-antigens (12), and lipopolysaccharides (LPSs) (13, 14).

LPSs are macromolecular glycolipids present on the outer surfaces of the outer membranes of Gram-negative bacteria (15–18). The structure of LPS may be subdivided into the lipid A region, which embeds the LPS in the outer membrane, the nonrepeating core oligosaccharide, and the distal O-antigen polysaccharide. The effects of changing LPS structure on symbiosis and nitrogen fixation are not fully characterized (14). Mutants of Rhizobium leguminosarum and Rhizobium etli that lack O-antigen are defective in the infection process, producing poorly differentiated, nonfunctional nodules (13, 19–21). Whether or not mutations in lipid A biosynthesis are deleterious to symbiosis remains to be determined.

Recent studies of the LPS of R. leguminosarum and R. etli, which are distinct bacterial species based on their ribosomal RNA sequences (22), have revealed that both bacteria possess unusual lipid A molecules (23–25) and core oligosaccharides (14, 20, 26, 27). Although the classical lipid A structure, typified by that of Escherichia coli, consists of a hexa-acylated disaccharide of glucosamine that is phosphorylated at positions 1 and 4' (15–18), R. etli and R. leguminosarum lipid A is recovered as a mixture of structurally related components (A to E) that are not phosphorylated (Fig. 1) (24, 25, 28). A conserved distal unit, comprised of a diacylated glucosamine residue with a secondary 27-OH-C28 acyl chain as part of an acyloxyacyl moiety at position 2' and a galacturonic acid residue at position 4', is present in all of the components (Fig. 1) (24, 25, 28). The microheterogeneity of R. etli lipid A arises mainly in the proximal unit, which may contain acyl chains of different lengths at position 2 or may be decacylated at position 3 (Fig. 1). Although glucosamine constitutes the proximal units of components B and C, its oxidized form, 2-amino-2-deoxy-gluconate (2-amino-2-deoxygluconate), is present in D-1 and E (Fig. 1) (24, 25, 28). Components C and E are 3-O-deacylated derivatives of the more abundant species B and D-1, respectively (24, 25, 28). Component A (not shown in Fig. 1) appears to be an elimination product generated from D-1 during the mild acid hydrolysis procedure used to release lipid A from the LPS core (24, 25). D-2 is an isomer formed from D-1 in a nonenzymatic reaction.

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The abbreviations used are: LPS, lipopolysaccharide; 2-amino-2-deoxy-gluconate, 2-amino-2-deoxy-gluconate; MES, 2-(N-morpholino)-ethanesulfonic acid; MOPS, 3-(N-morpholino)-propanesulfonic acid; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; PQQ, pyrroloquinoline quinone.
Lipid A Oxidation in \textit{R. leguminosarum}.

R. \textit{etli} lipid A, which is thought to be the same as that of \textit{R. leguminosarum}, is a mixture of several related species (23–25). In contrast to \textit{E. coli} lipid A, all \textit{R. etli} lipid A species lack phosphate groups (24, 25). Instead, each one contains a galacturonic acid moiety at position 4°, and is attributed to acyl chain migration on the proximal unit (24, 25).

To date, the occurrence of the 2-aminogluconate moiety is limited to the lipid A of \textit{R. etli} and \textit{R. leguminosarum} (23, 24). A plausible pathway for 2-aminogluconate formation could involve the oxidation of the proximal glucosamine unit of component B to generate D-1. In conjunction with our recent re-evaluation of the structures of \textit{R. etli} and \textit{R. leguminosarum} lipid A (25), we discovered that crude extracts of \textit{R. etli} and \textit{R. leguminosarum} can convert $^{14}$C-labeled component B to a lipid that migrates with D-1 during thin layer chromatography. We now report the detailed characterization of this novel oxidative reaction using both the natural product B and the relatively simple model substrate, 1-dephospho-lipid IV$_A$. Remarkably, the enzyme is an outer membrane protein and is presumed to be an oxidase. These findings are consistent with the idea that 2-aminogluconate formation is a late step in \textit{R. leguminosarum} lipid A biosynthesis. As demonstrated in the accompanying manuscript, a novel gene, designated \textit{lpqQ}, encodes the putative oxidase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glass-backed 0.25-mm Silica Gel 60 thin layer chromatography plates were from Merck. Chloroform, ammonium acetate, and sodium acetate were obtained from EM Science. Pyridine, methanol, and formic acid were from Mallinckrodt. [U-$^{14}$C]acetate was purchased from Amersham Biosciences.

**Bacterial Growth Conditions and Membrane Preparations—**\textit{R. leguminosarum} 3855 was grown at 30°C in TY broth (5 g of tryptone and 3 g of yeast extract/liter) supplemented with 10 mM CaCl$_2$, \textit{R. etli} CE3 and \textit{Sinorhizobium meliloti} 1021 were grown in TY broth supplemented with 10 mM CaCl$_2$, 20 μg/ml nalidixic acid, and 200 μg/ml streptomycin sulfate. \textit{E. coli} strains were grown at 37°C in LB broth (29) with one of the following antibiotics, depending on the resistance markers of the plasmid that the strain harbors: ampicillin (100 μg/ml), tetracycline (15 μg/ml), and kanamycin (25 μg/ml). Table I describes the various bacterial strains used.

**Rhizobium cultures** (0.5 to 1 liter) were grown to late log phase (OD$_{550}$ ~ 1.0) and then harvested by centrifugation at 6,000 × g for 15 min at 4°C. All subsequent steps were carried out at 4°C. The cell pellets were washed with 50 mM HEPES, pH 7.5, with a volume that was 1/10th of that of the original culture volume. The cells were collected by centrifugation and resuspended in a minimal volume (usually 5 ml) of 50 mM HEPES, pH 7.5, and stored at ~80°C if they were not immediately lysed. The frozen cells were thawed, resuspended in 50 mM HEPES, pH 7.5, at a protein concentration of ~3–10 mg/ml, and broken by three passages through an ice-cold French pressure cell (SLM Instruments, Urbana, IL) at 18,000 p.s.i. Large cell debris and unbroken cells were removed by centrifugation at 12,100 × g for 10 min. The membranes were recovered from the cytosol by ultracentrifugation at 149,000 × g for 1 h. The pellet containing the membranes was washed by homogenization in the same volume of 50 mM HEPES, pH 7.5. After a second ultracentrifugation, the washed membranes were homogenized in 50 mM HEPES, pH 7.5, at a protein concentration of ~5–15 mg/ml. The samples were stored in aliquots at ~80°C. The protein concentrations were determined by the bicinchoninic acid assay with bovine serum albumin as the standard (30).

**Subcellular Localization of the Lipid A Oxidase—**Subcellular localization of the lipid A oxidase in \textit{R. leguminosarum} 3855 membranes was determined using a protocol similar to that described by Trent et al. (31). Briefly, a 1-liter culture of \textit{R. leguminosarum} 3855 was grown with shaking (225 rpm) at 30°C for 16 h (OD$_{550}$ ~ 1.0). The culture was centrifuged at 6,000 × g for 10 min at 4°C. The cell pellet was resuspended in 7.8 ml of 50 mM HEPES, pH 7.5, containing 0.5 mM EDTA, and the cells were lysed by passage through a French pressure cell at 10,000 p.s.i. Crude cell-free extract was prepared by removal of unbroken cells and large debris by centrifugation at 12,100 × g for 10 min at 4°C, and the supernatant was recovered. One-half of the crude extract was stored at ~80°C, whereas the remaining half was used to prepare washed membranes, as described above. The washed membranes were homogenized with a 25-gauge 1/2 syringe needle in a total volume of 2.5 ml of 50 mM HEPES, pH 7.5, containing 0.5 mM EDTA. After being layered on top of a seven-step isopycnic sucrose gradient as described by Guy-Caffey et al. (32), the membranes were centrifuged in a swinging bucket rotor at 155,000 × g for 18 h at 4°C. The fractions (0.5 ml) were collected, and their protein content was determined by the bicinchoninic acid assay, as described above. The following undiluted fraction volumes were used for the various assays: 3 μl for the lipid A oxidase reaction, which was terminated after 30 min; 50 μl for the NADH oxidase assay (33, 34); and 20 μl for the phospholipase A assay (35). The turbidity (OD$_{600}$) of each fraction was measured to confirm the presence of membrane fragments. The activity for each fraction was calculated as a percentage of the total activity throughout the entire gradient.
Preparation of Nonradioactive and 14C-Labeled R. etli Lipid A Component B—Nonradioactive B was prepared from R. etli CE3 as described previously (24, 25). To make 14C-labeled component B, a 50-mg culture of R. etli CE3 was grown to an absorbance at 600 nm of 1 in the presence of 500 μCi of [U-14C]-acetate (50 μCi/mmol) (24, 25). A combination of DEAE-cellulose column chromatography and preparative thin layer chromatography (24) was used to purify 14CIB.

Preparation of 1-Dephospho-lipid IVα—Lipid IVα was isolated as described by Raetz et al. (36) and further purified by reversed phase high-pressure liquid chromatography (37). To cleave the phosphate at the anomeric position (38, 39), 3.2 mg of lipid IVA in a 16 × 125-mm glass tube with a Teflon-lined cap was resuspended by sonication in 3.8 ml of an acidic single-phase Bligh-Dyer solution, consisting of CHCl3, MeOH, H2O (2:2:1, v/v). The solution was then centrifuged briefly and then spotted onto a 20-cm silica gel plate. Preparative TLC was carried out as described above for the 1-dephospho-lipid IVA, which is referenced against internal tetramethoxypropane and its cross-peaks are also detected. Following NMR spectroscopy, the final compound was diluted 4 mg of a fresh lower phase of water into 12 ml of CHCl3, MeOH, H2O (2:3:1, v/v) (24, 36, 41). The solutions were then redissolved in 12 ml of CHCl3, MeOH, H2O (2:3:1, v/v) and spotted in a line 20 mm from the edge of a 20-cm silica TLC plate. The plate was allowed to dry and developed in CHCl3, pyridine, 88% formic acid, H2O (50:50:16:5, v/v) until the solvent front was 5 cm from the top of the plate. The solvents were removed by drying the plate with a cold air stream for 30 min. The band of interest, its cross-peaks are also detected. Following NMR spectroscopy, the final compound was dried down under N2 and resuspended in 150 μl of 50 mM MOPS, pH 7.0, to make a 1 mg aqueous dispersion. This was stored at −80 °C and subjected to brief sonic irradiation prior to use in assays.

Preparation of [4-32P]1-dephospho-lipid IVα—The starting material [4-32P]lipid IVα was synthesized enzymatically as previously described (43) and then was converted into [4-32P]1-dephospho-lipid IVα by hydrolysis in 0.1 M HCl. Typically, a sample containing 80–100 μCi of [4-32P]lipid IVα was re-suspended in 150 μl of 0.1 M HCl. Following sonic irradiation for 2 min in a bath apparatus, the mixture was placed in a 100°C heat block for 15 min. After cooling, the hydrolyzed material was centrifuged briefly and then spotted onto a 20 × 20-cm silica gel plate. Preparative TLC was carried out as described above for the nonradioabeled lipid IVα. After drying, the product was detected by a brief autoradiography and recovered by extraction from the silica chips (see above). The final substrate was typically dispersed in 50 mM HEPES, pH 7.5, that such the working stock solution contained 50,000 cpm/μl.

Assay Conditions for Measuring the Conversion of 14CIB to 14C-DIC—The standard reaction mixture (10 μl) contained 10 μM 14CIB (∼500 cpm/reaction), 0.5–1 mg/ml membrane protein, 0.1% Triton X-100, 1 mM MgCl2, and 50 mM MOPS buffer, pH 6.5, unless otherwise indicated. The reactions were incubated under ambient conditions at 30°C and terminated at the indicated times by spotting 4-μl samples onto a 20 × 20-cm silica gel TLC plate. The spots were dried for 30 min with a cold air stream, and the plate was then developed in the solvent CHCl3, MeOH, H2O (2:3:1, v/v). The remaining substrate and product(s) were detected with a Molecular Dynamics Storm PhosphorImager equipped with ImageQuant software. Enzyme specific activity (usually expressed as nmoi/min/mg) was calculated based on the percentage of conversion of substrate to product(s).

Assay Conditions for Detecting the Oxidation of 1-Dephospho-lipid IVα—The standard reaction mixture (10 μl) contained 5–10 μM [4-32P]1-dephospho-lipid IVα (∼5000 cpm/tube), 0.5–1 mg/ml membrane protein, 1 mM MgCl2, and 50 mM MOPS, pH 7.0, to partially suppress 4′ phosphatase activity. After incubation at 30°C for the indicated times, the reactions were stopped by spotting 4-μl samples onto a 20 × 20-cm silica gel TLC plate. The plate was dried with the solvent CHCl3, pyridine, 88% formic acid, H2O (50:50:16:5, v/v) and imaged as described above.

Isolation of the Product Generated from Component B in Vitro by R. leguminosarum Membranes—A 7.3-ml reaction mixture containing 50 μM R. etli component B (24, 25), 50 mM MES, pH 6.5, 1 mM MgCl2, 0.1% Triton-X-100, and 0.5 mg/ml R. leguminosarum 3855 membranes was incubated overnight (17.5 h) at 30°C. TLC analysis of the reaction at

| Strain | Relevant properties | Source |
|--------|---------------------|--------|
| R. etli strain CE3 | biовар phaseolii, derivative of CFN42, Nal', Str' | Refs. 83 and 84 |
| R. etli strain CE309 | biовар phaseolii, Str', Kan' (gal-, galUA) | Refs. 83 and 84 |
| R. leguminosarum 3855 | biовар vicieae, lipid A similar to R. etli | Ref. 86 |
| R. leguminosarum 8401 | biовар vicieae, lipid A similar to R. etli, Rif' | Ref. 49 |
| S. melloti 1021 | Nal', Str' | Ref. 87 |
| E. coli | recA, Str' (from BRL) | Ref. 88 |

**Table I.** Bacterial strains used in this study.
the initial and final time points indicated complete conversion of B to a compound migrating with a component D-1 standard. Half of the reaction mixture (contained in a sterile 16 × 125-mm glass tube) was transferred to an identical glass tube. Each half was then converted into a two-phase Bligh-Dyer mixture by the addition of CHCl₃ (4 ml) and MeOH (4 ml) to each tube. After mixing, the phases were separated by centrifugation for 10 min at room temperature in a clinical centrifuge. The lower phase was recovered and transferred to a clean glass tube. After two extractions of the upper phases with pre-equilibrated lower phases, all of the resulting lower phases were pooled, dried under N₂, and stored at −20 °C.

A 1.5-ml DEAE-cellulose column (Whatman DE-52) (24) was used to purify the lipids in the reaction mixture. The dried lipid product was dissolved in 1.6 ml of CHCl₃, MeOH, H₂O (2:3:1, v/v). After equilibration of the DEAE column as the acetate form in CHCl₃, MeOH, H₂O (2:3:1, v/v), the sample was loaded and the flow through was collected as one fraction. The column was then washed with 1.5 ml of CHCl₃, MeOH, H₂O (2:3:1, v/v), which was collected as one fraction. Next, the column was washed successively with the following solvent mixtures: 8 ml of CHCl₃, MeOH, 60 mM aqueous NH₄Ac (2:3:1, v/v), 8 ml of CHCl₃, MeOH, 120 mM aqueous NH₄Ac (2:3:1, v/v), 6 ml of CHCl₃, MeOH, 240 mM aqueous NH₄Ac (2:3:1, v/v), and finally 6 ml of CHCl₃, MeOH, 500 mM aqueous NH₄Ac (2:3:1, v/v). 1-ml fractions were collected throughout the elution. Silica gel TLC analysis of the fractions was done with the solvent system CHCl₃, MeOH, H₂O (2:3:1, v/v), which was collected as one fraction. Next, the tube was washed successively with the following solvent mixtures: 8 ml of CHCl₃, MeOH, 30 mM aqueous NH₄Ac (2:3:1, v/v), 6 ml of CHCl₃, MeOH, 500 mM aqueous NH₄Ac (2:3:1, v/v), and finally 6 ml of CHCl₃, MeOH, 500 mM aqueous NH₄Ac (2:3:1, v/v). 1-ml fractions were collected throughout the elution. The in vitro product emerged in the late 60 mM NH₄Ac wash, in accordance with the behavior of R. etli lipid A component D-1 (24). Lipids in fractions from the flow-through, 30 mM NH₄Ac, and 60 mM NH₄Ac washes were pooled separately and recovered by two-phase Bligh-Dyer partitioning, as discussed above for the preparation of 1-dephospho-lipid IV₅. The lower phases for each pool were dried down with a stream of N₂ and stored at −20 °C.

Isolation of the Products Generated in Vitro from 1-Dephospho-lipid IV₅ by R. leguminosarum Membranes—In a sterile 16 × 125-mm glass tube equipped with Teflon-lined cap, 100 μl 1-dephospho-lipid IV₅ was incubated with 0.36 mg/ml R. leguminosarum 3855 membranes in a buffer containing 50 mM MOPS, pH 7.0, 0.1% Triton X-100, and 1 mM MgCl₂. The final reaction volume was 7 ml. A parallel reaction was done in a 1.5-ml microcentrifuge tube containing 96 μl of the above reaction mixture and 4 μl of [14C]B (50,000 cpm/μl) was carried out to monitor product formation with a PhosphorImager. After 14 h at 30 °C, half of the nonradioactive reaction mixture was transferred to a second identical glass tube. CHCl₃ (4 ml), MeOH (4 ml), and 0.1 M HCl (360 μl) were added to each of the two tubes, which were mixed and centrifuged for 10 min in a clinical centrifuge. After two more extractions of the upper phase with pre-equilibrated lower phase, all of the lower phases were pooled in a clean glass tube. Two drops of pyridine were added before the pooled lower phases were dried with N₂ and stored at −20 °C.

The sample was redissolved in 5 ml of CHCl₃, MeOH, H₂O (2:3:1, v/v) and loaded onto a 1.0-ml DEAE-cellulose column (Whatman DE-52) that was previously equilibrated as the acetate form in CHCl₃, MeOH, H₂O (2:3:1, v/v) (24). The column was washed with 1 ml of CHCl₃, MeOH, H₂O (2:3:1, v/v). Elution was done stepwise with the following solvent mixtures: 6 ml of CHCl₃, MeOH, 30 mM aqueous NH₄Ac (2:3:1, v/v), 6 ml of CHCl₃, MeOH, 60 mM aqueous NH₄Ac (2:3:1, v/v), 5 ml of CHCl₃, MeOH, 85 mM aqueous NH₄Ac (2:3:1, v/v), 6 ml of CHCl₃, MeOH, 120 mM aqueous NH₄Ac (2:3:1, v/v), 5 ml of CHCl₃, MeOH, 240 mM aqueous NH₄Ac (2:3:1, v/v), and 5 ml of CHCl₃, MeOH, 500 mM aqueous NH₄Ac (2:3:1, v/v). 0.5-ml fractions were collected from the 30 mM to the 120 mM NH₄Ac washes, and 1-ml fractions were collected for the subsequent steps. Ten μl of each fraction was analyzed using silica gel TLC plates developed in CHCl₃, pyridine, 88% formic acid, H₂O (60:55:10:5:2, v/v). Fractions from each elution step were pooled separately and recovered by two-phase Bligh-Dyer partitioning as described above for 1-dephospho-lipid IV₅. The desired lower phases were dried down, and the lipids were transferred to an identical glass tube. CHCl₃ (4 ml), MeOH (4 ml), 0.1 M HCl (360 μl) were added to each of the two tubes, which were mixed and centrifuged for 10 min in a clinical centrifuge. After two more extractions of the upper phase with pre-equilibrated lower phase, all of the lower phases were pooled in a clean glass tube. Two drops of pyridine were added before the pooled lower phases were dried with N₂ and stored at −20 °C.

RESULTS

Conversion of [14C]B to [14C]D-1 by R. leguminosarum membranes. The substrate [14C]B, prepared from R. etli cells labeled with [14C]-acetate (24, 25), was converted to a substance that migrates like [14C]D-1 in the presence of R. leguminosarum 3855 membranes and 0.1% Triton X-100 when analyzed on silica gel TLC plates with the solvent system CHCl₃, MeOH, H₂O, pyridine (40:25:4:2, v/v). All of the incubations were at 30 °C for 1 h at a protein concentration of 0.5 mg/ml in a reaction mixture containing 50 mM MES, pH 6.5, 0.1% Triton X-100, and 10 μM substrate. Recombining the cytosol and washed membranes did not stimulate the reaction (not shown). S.F., solvent front.

As shown in Fig. 2, the membranes of R. leguminosarum 3855 efficiently convert [14C]B to a substance migrating like [14C]D-1 in the presence of Triton X-100. The cytosolic fraction is completely inactive (Fig. 2), and it does not further stimulate the activity present in membranes (not shown). Other nonionic detergents such as Nonidet P-40 can substitute for Triton X-100, but ionic detergents, like SDS or LDAO, are inhibitory (not shown). Product formation is nearly linear with time for about 30 min at 0.25 mg/ml membrane protein (Fig. 3). The enzyme is inhibited by addition of 5 mM EDTA to the assay mixture (Fig. 4A, lane 2a). However, nearly complete reactivation of the EDTA-inhibited enzyme is observed upon addition of excess (10 mM) MgCl₂ and incubation for another 20 min (Fig. 4B, lane 7). Mg²⁺, Ni²⁺, and Mn²⁺ are comparable with Mg²⁺ in reactivating the EDTA-treated enzyme (Fig. 4B). Ca²⁺, Zn²⁺, Cu²⁺, and Fe²⁺ are minimally effective, but Mo²⁺ is inactive (Fig. 4B). The chelators dipiprydyl and o-phenanthroline did not inhibit the reaction when added at 10 mM.

The membranes of other strains of R. leguminosarum or R. etli catalyze the conversion of [14C]B to [14C]D-1 at rates that are comparable with those seen with membranes of R. leguminosarum 3855 (Fig. 5). The membranes of E. coli and S. melliotii 1021, which make lipid A species that are fully phosphorylated and do not contain 2-amino-muconate, are unable to metabolize [14C]B to [14C]D-1 (Fig. 5).
of B to D-1 is shown in the lower panel, as shown in the upper panel. D-1 generated from authentic D-1 for DEAE-cellulose (24), from which it elutes because of the partial substitution with certain divalent cations added in excess (10 mM) to a portion of the EDTA inhibited reaction mixture from lane 2a of A and the incubation was allowed to proceed for another 20 min (B), the activity was restored in some cases, as indicated. Product formation was analyzed with a PhosphorImager as for Fig. 2.

MALDI/TOF Mass Spectrometry of the D-1-like Substance Synthesized in Vitro—The TLC method (Figs. 2–5) used to monitor the conversion of [14C]B to [14C]D-1 by R. leguminosarum membranes strongly suggests that the product accumulating with time is authentic D-1 (Fig. 1), given its migration with a D-1 standard isolated from R. etli (24, 25). In addition, the D-1-like material synthesized in vitro has the same affinity as authentic D-1 for DEAE-cellulose (24), from which it elutes with CHCl₃, MeOH, 120 mM aqueous NH₄Ac (2:3:1, v/v) (data not shown). In contrast, the substrate [14C]B elutes with CHCl₃, MeOH, 30 mM aqueous NH₄Ac (2:3:1, v/v) because it is less negatively charged (24).

A scaled-up, nonradioactive enzymatic reaction mixture was used to prepare D-1 for mass spectrometry. Following purification on a DEAE-cellulose column, MALDI/TOF mass spectra of the remaining substrate B (Fig. 6, lower panel) and of the D-1-like material (Fig. 6, upper panel) were recorded in the positive reflectron mode. As noted in Fig. 1, both the substrate and the product are mixtures of related lipid species differing in acyl chain length by two methylene groups (i.e. 28 atomic mass units) on the proximal unit (24, 25). The observed peaks at m/z 1980.1 and 2008.1 (Fig. 6, lower panel) may be interpreted as the monoisotopic [M + Na]⁺ ions for the two predominant acyl chain lengths present in B (Fig. 1) (24). The putative D-1 generated in vitro by R. leguminosarum membrane gives strong peaks at m/z 1996.5 and 2024.5 (Fig. 6, upper panel), consistent with the incorporation of a single oxygen atom by oxidation of the proximal glucosamine unit to the 2-amino glucosamine residue (24). As with B, both signals may be interpreted as the monoisotopic [M + Na]⁺ ions arising from the two predominant acyl chain lengths present in D-1 (Fig. 1). The distributions of the additional peaks at progressively higher m/z in each of the clusters shown in Fig. 6 are consistent with the molecular weights of B and D-1 (Fig. 1) and are in agreement with previous studies of the natural products isolated from R. etli CE3 using conventional MALDI/TOF mass spectrometry (24). The additional microheterogeneity of B and D-1 because of the partial substitution with β-hydroxybutyrate (Fig. 1) (24) is not seen in the region of the spectrum shown in Fig. 6.

Use of [4'-32P]1-Dephospho-lipid IVₐ as an Alternative Substrate for Demonstrating Oxidation of the Proximal Glucosamine Unit—The E. coli lipid A precursor [4'-32P]lipid IVₐ, which is a tetra-acylated disaccharide of glucosamine that is phosphorylated at positions 1 and 4 (36, 44), is not metabolized appreciably by R. leguminosarum membranes under the oxidase assay conditions (Fig. 7A, lower arrow). A 1-phosphatase is present in R. leguminosarum that converts R. leguminosarum lipid A precursors to their 1-dephosphorylated derivatives (45), but this activity is barely detectible with [4'-32P]lipid IVₐ as the substrate under the conditions employed. However, when [4'-32P]1-dephospho-lipid IVₐ (Fig. 8 and supplementary figure) is prepared by chemical hydrolysis of lipid IVₐ with 0.1 M HCl at 100 °C and used as a substrate under the oxidase assay conditions, it is metabolized to several new products (Fig. 6).
7B). These compounds migrate slightly faster than the substrate in this solvent system (Fig. 7B). Almost all of the 1-dephospho-lipid IV A is consumed after overnight incubation at 30 °C, as shown by mass spectrometry (see below). The same lipid A oxidizing enzyme that converts B to D-1 is responsible for the metabolism of 1-dephospho-lipid IV A by R. leguminosarum membranes (89).

Negative Ion MALDI/TOF Mass Spectrometry of the Products Made by R. leguminosarum Membranes from 1-Dephospholipid IV A—The conversion of 1-dephospho-lipid IV A to its putative oxidation products (Figs. 7 and 8) was scaled up to permit mass spectrometry, as described under “Experimental Procedures.” Fig. 9 shows the MALDI/TOF spectra in the negative mode of the 1-dephospho-lipid IV A substrate, its putative lactone derivative, and the proposed 2-aminogluconate product (Fig. 8). The latter two compounds elute from DEAE-cellulose at about CHCl3, MeOH, 60 mM aqueous NH4Ac (2:3:1, v/v) and CHCl3, MeOH, 120 mM aqueous NH4Ac (2:3:1, v/v), respectively.

The substrate 1-dephospho-lipid IV A (Fig. 9A) shows the expected peak at m/z 1324.8, which is interpreted as [M – H]−. The proposed 2-aminogluconate product (Fig. 9C) displays an intense peak at m/z 1341.2, consistent with [M – H]− of a compound that has gained an oxygen atom (Fig. 8). The peak at m/z 928.9 (Fig. 9C) could not be assigned and might be an impurity carried over from the R. leguminosarum membranes used as the enzyme source.

The negative mode MALDI/TOF mass spectrum of the second (less abundant) product derived from 1-dephospho-lipid IV A, which emerges from DEAE-cellulose with the 60 mM NH4Ac wash, is shown in Fig. 9B. An intense signal is observed at m/z 1078.9. The putative lactone derivative of 1-dephospho-lipid IV A (Fig. 8) has a molecular weight of 1323.7 and should give rise to a peak at m/z 1322.7 in the negative mode. However, such lactones might undergo elimination of the fatty acid moiety at position 3 (24, 25). In the case of the putative lactone derived from 1-dephospho-lipid IV A, the elimination of hydroxymyristic acid would generate an αβ-unsaturated lactone (Fig. 8) with a molecular weight of 1079.3. Consequently, the major peak seen at m/z 1078.9 in Fig. 9B could be interpreted as [M – H]− derived from the lactone elimination product (Fig. 8). We do not believe that the putative elimination product forms prior to mass spectrometry, because it would migrate more slowly during TLC than 1-dephospho-lipid IV A, which is not observed (Fig. 7B).

A small amount of residual substrate is evident in the sample shown in Fig. 9B, consistent with its charge (Fig. 8), as judged by the peak at m/z 1325.2. As in Fig. 9C, the peak
at m/z 928.9 (Fig. 9B) is not assigned and may represent an impurity.

Positive Ion MALDI/TOF Mass Spectrometry of Products Made by R. leguminosarum Membranes from 1-Dephospho-lipid IVA—The positive ion MALDI/TOF mass spectra (Fig. 10) of the same three substances are consistent with the interpretation of their negative mode spectra (Fig. 9). Most significantly, the positive mode spectra reveal an intense B1/H11001 ion at m/z 695.3 in the substrate (Fig. 10A), the proposed 2-aminogluconate (Fig. 10C), and the putative lactone elimination product (Fig. 10B). The presence of a common B1/H11001 ion clearly demonstrates that the distal units of these lipids are identical and supports the view that the proximal sugar unit of 1-dephospho-lipid IVA is converted to the 2-aminogluconate derivative (Fig. 8) during the course of the reaction shown in Fig. 7. However, these findings do not clearly distinguish the aldehyde oxidation pathway (Fig. 8, reaction 1) from the alternative of a lactone intermediate (Fig. 8, reactions 2 and 3), because the lactone might also be formed from the 2-aminogluconate product of reaction 1 by a nonenzymatic process (Fig. 8, reaction 4).

Outer Membrane Localization of the Lipid A Oxidase of R. leguminosarum—In previous studies, we have described methods for separating inner and outer membranes of various *Rhzobium* strains using isopycnic sucrose gradient centrifugation (46). As in *E. coli*, the heavier (outer) membrane fraction is characterized by its phospholipase A activity, whereas the lighter (inner) membranes are detected using NADH oxidase. Remarkably, the lipid A oxidase activity, which was measured by following the conversion of [14C]B to [14C]D-1, is recovered almost entirely with the outer membrane fragments of *R. leguminosarum* 3855 (Fig. 11). The same profile was obtained when 1-dephospho-lipid IV was used as the substrate (not shown). Among the various *R. etli* or *R. leguminosarum* lipid A enzymes studied to date (45–51), only the oxidase is found in the outer membrane. The outer membrane localization of the oxidase therefore demonstrates that the formation of 2-aminogluconate represents a late modification in the maturation of *R. leguminosarum* lipid A.

**DISCUSSION**

*R. leguminosarum* and *R. etli* are the only organisms known to synthesize 2-aminogluconate, an oxidized derivative of D-glucosamine (23–25, 52). This material substitutes for the proximal glucosamine unit that is usually present in lipid A (Fig. 1) (23–25, 52). In re-evaluating the microheterogeneity and structures of *R. leguminosarum* and *R. etli* lipid A, Que et al. (24, 25,
28) discovered that about one-third of the lipid A isolated from these organisms does in fact contain the conventional glucosamine disaccharide backbone found in most other Gram-negative bacteria (Fig. 1, structure B). In earlier studies by Bhat et al. (23), B had been overlooked, because the lipid A released from lipopolysaccharide by acid hydrolysis was not further purified. Given that B lacks the anomeric phosphate residue (24, 25), it might be the immediate precursor of component D-1 (Fig. 1). We have now developed a quantitative assay for following the conversion of B to D-1 using membranes of *R. leguminosarum* or *R. etli* (Figs. 2–5) and have demonstrated the presence of an additional oxygen atom in the D-1 product generated *in vitro* from B. The peak at m/z 928.9 could not be assigned and may reflect contaminating *R. leguminosarum* membrane lipids.

A remarkable feature of the oxidase is its outer membrane localization. This finding suggests that the formation of 2-aminogluconate occurs as a late modification of the lipid A molecule. All of the early conserved reactions of lipid A biosynthesis in *R. leguminosarum* and *R. etli*, as well as the 4' and 1 phosphatases that are unique to these organisms, appear to be associated with the inner membrane (45–51). Very few outer membrane enzymes have ever been described. All of the known outer membrane enzymes are lipases (35, 53), acyltransferases (54, 55), or proteases (56). The outer membrane localization of the enzyme that generates D-1 from B suggests that it is a novel kind of oxidase. Oxygen is indeed required for the reaction, as demonstrated in the accompanying manuscript. However, it is not yet possible to show stoichiometric formation of H$_2$O$_2$ and D-1 from O$_2$ and B when using whole membrane preparations as the enzyme source (see below).

The ability of the oxidase to utilize 1-dephospho-lipid IV$_A$ (Figs. 7 and 8) as a model substrate demonstrates that it does not require the 2' acyloxyacyl or the 4' galacturonic acid residues characteristic of *R. leguminosarum* or *R. etli* lipid A (Fig. 12 M. J. Karbarz and C. R. H. Raetz, manuscript in preparation.)

**Fig. 9.** Negative-mode MALDI/TOF mass spectrometry of the oxidation products of 1-dephospho-lipid IV$_A$. The lipids extracted from an overnight reaction mixture containing *R. leguminosarum* 3855 membranes and 1-dephospho-lipid IV$_A$ were fractionated on a DEAE-cellulose column. A, the spectrum of the substrate 1-dephospho-lipid IV$_A$ prior to incubation with membranes. B, the lipids from the CHCl$_3$, MeOH, 60 mM aqueous NH$_4$Ac (2:3:1, v/v) wash, consisting of residual substrate and the putative lactone. C, the lipids from the CHCl$_3$, MeOH, 120 mM aqueous NH$_4$Ac (2:3:1, v/v) wash. The latter consist primarily of the proposed 2-aminogluconate derivative, as judged by the peak at m/z 1341.2. The peak at m/z 928.9 could not be assigned and may reflect contaminating *R. leguminosarum* membrane lipids.

**Fig. 10.** Positive-mode MALDI/TOF mass spectrometry of the oxidation products of 1-dephospho-lipid IV$_A$. The lipids extracted from an overnight reaction mixture containing *R. leguminosarum* 3855 membranes and 1-dephospho-lipid IV$_A$ were fractionated on a DEAE-cellulose column as in Fig. 9. A, the substrate 1-dephospho-lipid IV$_A$. B, the lipids from the CHCl$_3$, MeOH, 60 mM aqueous NH$_4$Ac (2:3:1, v/v) wash. C, the lipids from the CHCl$_3$, MeOH, 120 mM aqueous NH$_4$Ac (2:3:1, v/v) wash. The peaks at m/z 931.1, 854.0, and 813.4 could not be assigned and may reflect contaminating *R. leguminosarum* membrane lipids. The B$_1$ ions of the substrate 1-dephospho-lipid IV$_A$, the lactone elimination product, and the aminogluconate are all observed at m/z 695.3, demonstrating that the distal unit is unchanged and that only the proximal unit of the product is modified under these conditions.
Although the membrane-bound glucose dehydrogenases oxidize various monosaccharides, they cannot process disaccharides. In contrast, an atypical soluble glucose dehydrogenase found only in Acinetobacter calcoaceticus (63, 64) can oxidize both mono- and disaccharides. Interestingly, this periplasmic enzyme is rather different from the membrane-bound glucose dehydrogenases, not only with regard to its sugar substrate specificity but also in its preference for electron acceptors. It does not utilize ubiquinone (61) but instead slowly reduces a soluble cytochrome b that does not appear to interact with the electron transport chain (65). Furthermore, the periplasmic dehydrogenase shares very little amino acid sequence similarity with the membrane-bound enzymes (66), and it lacks the conserved 11-residue tryptophan docking motif found in most other PQQ-containing dehydrogenases (67).

Another enzyme that oxidizes the C1 of a hexose moiety is cellulose dehydrogenase, which is found in the lignin-degrading white rot fungi Phanerochaete chrysosporium (68–72) and Humicola insolens (73). Cellulose dehydrogenase is an extracellular enzyme that oxidizes various di- and oligosaccharides and can utilize either Fe3+ or O2, or various organic molecules as electron acceptors (74, 75). As shown in the accompanying manuscript, the lipid A oxidase of R. leguminosarum does not share significant sequence homology with any of the above enzymes.

Another alternative for 2-amino-6-glucuronic acid formation might involve ring opening of the proximal lipid A unit, followed by oxidation the aldehyde (Fig. 8, reaction 1). This possibility is reminiscent of the mechanism proposed for D-xylose isomerase (76) (also known as D-glucose isomerase), which interconverts a broad spectrum of aldoses and ketoses (77). X-ray data suggest the presence of an extended open chain sugar substrate in the enzyme active site (78, 79). Mg2+ is believed to be the sole cofactor needed by the enzyme in vivo (80), but other divalent cations can be substituted in vitro.

Given the outer membrane localization of the lipid A oxidase, we consider it unlikely that its enzymatic mechanism involves pyridine or flavin nucleotides. This idea is supported by the observation that there is no stimulatory effect on the rate of conversion of B to D-1 by addition of exogenous NAD, NADP, FAD, FMN, ubiquinone, or cytochrome c (data not shown). Furthermore, the reaction is not inhibited by cyanide. A mechanism involving PQQ deserves consideration in view of the inner membrane or periplasmic localization of the PQQ-dependent glucose dehydrogenases discussed above, but again no stimulation of B to D-1 conversion was seen with added PQQ (data not shown). At present, the only clue to the mechanism of the oxidation is the inhibition by added EDTA and the reaction with excess Mg2+ and some other divalent cations, especially Co2+, Ni2+, and Mn2+ (Fig. 4). Although a direct involvement of Mg2+ in substrate binding, catalysis, or maintenance of tertiary structure is certainly a possibility, we cannot exclude the alternative that the enzyme requires a redox-active heavy metal, which is removed by EDTA and then transferred back in the presence of excess Mg2+ or other divalent cations (Fig. 4).

A mechanism in which electrons are transferred from B to molecular oxygen in the outer membrane without the involvement of the inner membrane electron transport chain seems attractive based upon the available data. This hypothesis predicts that H2O2 would be formed as a by-product. Attempts to demonstrate stoichiometric formation of H2O2 with the Amplex Red detection kit (Molecular Probes) during the conversion of 50 μM B to D-1 have not been successful so far (data not shown).

However, the membrane preparations used as the enzyme source rapidly consume 50 μM H2O2 added exogenously (data
Lipid A Oxidation in R. leguminosarum

not shown). Purification of the oxidase to homogeneity, as well as required to structural and mechanistic studies, will therefore be
ecessary to identify the intermediates and by-products of the
conversion of B to D-1.

Although the occurrence of 2-aminogluconate is limited to a
few Gram-negative bacteria involved in nitrogen fixation (23–
25), an emerging general theme in lipid A biogenesis is that
sugar A modification reactions may occur within the outer
membrane (31, 54). For instance, the lipid A acyltransferase PagP
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