An unusual feature of the lipid A from the plant endosymbionts *Rhizobium etli* and *Rhizobium leguminosarum* is the presence of a proximal sugar unit consisting of a 2-amino-2-deoxy-gluconate moiety in place of glucosamine. An outer membrane oxidase that generates the 2-amino-2-deoxy-gluconate unit from a glucosamine-containing precursor is present in membranes of *R. leguminosarum* and *R. etli* but not in *S. meliloti* or *Escherichia coli*. We now report the identification of a hybrid cosmid that directs the overexpression of this activity by screening 1800 lysates of individual colonies of a *R. leguminosarum* 3841 genomic DNA library in the host strain *R. etli* CE3. Two cosmids (p1S11D and p1U12G) were identified in this manner and transferred into *S. meliloti*, in which they also directed the expression of oxidase activity in the absence of any chromosomal background. Subcloning and sequencing of the oxidase gene on a 6.5-kb fragment derived from the ∼20-kb insert in p1S11D revealed that the enzyme is encoded by a gene (*lpxQ*) that specifies a protein of 224 amino acid residues with a putative signal sequence cleavage site at position 28. Heterologous expression of *lpxQ* using the *T7lac* promoter system in *E. coli* resulted in the production of catalytically active oxidase that was localized in the outer membrane. A new outer membrane protein of the size expected for LpxQ was present in this construct and was subjected to microsequencing to confirm its identity and the site of signal peptide cleavage. LpxQ expressed in *E. coli* generates the same products as seen in *R. leguminosarum* membranes. LpxQ is dependent on O₂ for activity, as demonstrated by inhibition of the reaction under strictly anaerobic conditions. An ortholog of LpxQ is present in the genome of *Agrobacterium tumefaciens*, as shown by heterologous expression of oxidase activity in *E. coli*.

As demonstrated in the accompanying article (1), the outer membranes of *Rhizobium leguminosarum* and *Rhizobium etli* contain an unusual oxidase that converts the proximal glucosamine unit of 1-dephosphorylated lipid A (or related molecules) to a novel 2-aminoglucuronate moiety. The membranes of *Sinorhizobium meliloti* and *Escherichia coli* do not normally contain such an oxidase activity. Although the function of this unusual covalent modification of lipid A is unknown (2–4), the existence of an oxidative enzyme in the outer membrane of a Gram-negative bacterium is without precedent. The few outer membrane enzymes described to date are all phospholipases (5, 6), acyltransferases (7, 8), or proteases (9). Other characterized outer membrane proteins function either as porins or specialized transporters (10–12). The presence of the oxidase in outer membranes of certain strains of *Rhizobium* suggests that lipid A oxidation (1), when it occurs, is a late event in lipopolysaccharide assembly.

Given the considerable progress that has recently been made with the structural biology of outer membrane proteins by x-ray crystallography (12) and NMR spectroscopy (8, 13) and the great interest in lipid A modifications in the context of microbial pathogenesis (14–16), we now report the expression cloning of a novel *R. leguminosarum* gene, designated *lpxQ*, that encodes the oxidase. Hydropathy analysis predicts that LpxQ is a typical outer membrane protein of 224 amino acid residues with a leader sequence that is cleaved during export. The *R. leguminosarum* oxidase is properly localized to the outer membrane when expressed in *E. coli* behind the *T7lac* promoter. The only other bacterial genomes that contain clear-cut orthologs of LpxQ are those of two *Agrobacterium tumefaciens* strains, the sequences of which were recently reported (17, 18). Although 2-aminoglucuronate has not been described as a component of lipid A in *A. tumefaciens*, we show that *A. tumefaciens* LpxQ is in fact fully active as a lipid A oxidase when overexpressed in *E. coli*. The availability of the lpxQ genes of *R. leguminosarum*, *R. etli*, and *A. tumefaciens* should enable purification to homogeneity and mechanistic studies of the oxidase and should also facilitate genetic studies of 2-aminoglucuronate function during plant infection and symbiosis.

**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-¹⁴C]acetate was purchased from Amersham Biosciences, and [¹³C]-labeled component B was prepared as described in the accompanying article (1).

**Bacterial Growth Conditions**—Briefly, *R. leguminosarum* 3855 was grown at 50 °C in TY broth (5 g of tryptone and 3 g of yeast extract/liter) supplemented with 10 mM CaCl₂, 20 μg/ml naldixic acid, and 200 μg/ml streptomycin sulfate. *E. coli* strains were generally grown at 37 °C in LB broth (23) with one of the following antibiotics, depending on the

---

*Nanette L. S. Que-Gewirth*, Mark J. Karbarz‡§, Suzanne R. Kalb¶, Robert J. Cotter¶, and Christian R. H. Raetz‡

From the ‡Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710; and the ¶Middle Atlantic Mass Spectrometry Laboratory, Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2185

Received for publication, January 13, 2003, and in revised form, January 15, 2003

Published, JBC Papers in Press, January 15, 2003, DOI 10.1074/jbc.M300379200

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
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 plasmids and bacterial strains used in this study. Cell-free extracts and washed membranes were prepared as described in the accompanying article (1).

Quantitative Assay for Measuring the Conversion of [14C]B to [14C]-D-1—As described fully in the accompanying article (1), the standard reaction mixture (10 µl) contained (unless otherwise indicated) 10 µM [14C]B (500 cpm/tube), 0.5–1.0 mg/ml membrane protein, 0.1% Triton X-100, 1 mM MgCl₂, and 50 mM MES buffer, pH 6.5. The reactions were incubated under aerobic conditions at 30 °C and terminated at the indicated times by spotting 4- to 5-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, 1:1:0.5. The remaining substrate and product(s) were detected with a Molecular Dynamics Storm PhosphorImager equipped with ImageQuant software. Enzyme specific activity (usually expressed as nmol/min/mg) was calculated based on the percentage of conversion of substrate to product(s).

Anaerobic Assay Conditions—For demonstrating oxygen dependence, an assay system consisting of 25 µM [14C]B, 50 mM MES buffer, pH 6.5, 0.1% Triton X-100, 1 mM MgCl₂, and 0.1 mg/ml BLR(DE3)/pLysS/pQ235 membranes was set up in an anaerobic chamber. The reactions were started by the addition of membranes as the enzyme source after equilibration of all tubes in the absence of oxygen for ~30 min. To remove traces of oxygen, glucose oxidase (20 µg/ml), catalase (2 µg/ml), and glucose (0.1%) were included in some cases, as indicated. After 30, 60, or 90 min, portions of the reaction mixtures were spotted onto a silica gel TLC plate, which was developed as described above. A parallel set of reaction mixtures was assayed simultaneously under anaerobic conditions.

Expression. Cloning of the R. leguminosarum Lipid A Oxidase Gene—A genomic DNA library of the R. leguminosarum strain 3841 was obtained from Dr. L. Dony of the John Innes Institute (Norwich, UK). This library was constructed by the ligation of ~20–25-kb frag-

1 The abbreviations used are: MES, 2-(N-morpholino)ethanesulfonic acid; 2-aminogluconate, 2-amino-2-deoxy-gluconate; CAPS, 3-(cyclohexylamino)propanesulfonic acid; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; Kdo, 3-deoxy-d-manno-2-octulosonic acid.

Table I. Bacterial strains and plasmids used in this study

| Name             | Description                                      | Source       |
|------------------|--------------------------------------------------|--------------|
| pRK404a         | Shuttle vector, Tet’                            | Ref. 32      |
| pLAFLT-1        | Shuttle vector, Tet’                            | Ref. 25      |
| pS11D           | ~20-kb cosmid in pLAFLT-1                       | this work    |
| pS11D           | ~20-kb cosmid in pLAFLT-1                       | this work    |
| pQN208          | ~3.9-kb EcoRI fragment in pRK404a               | from pS11D   |
| pQN200          | ~4.7-kb HindIII fragment in pRK404a             | from pS11D   |
| pQN210          | ~6.5-kb HindIII fragment in pRK404a             | from pS11D   |
| pQN211          | ~4.2-kb HindIII fragment in pRK404a             | from pS11D   |
| pQN212          | ~3.5-kb HindIII fragment in pRK404a             | from pS11D   |
| pQN214          | ~1.3-kb HindIII fragment in pRK404a             | from pS11D   |
| pQN215          | ~0.5-kb EcoRI fragment in pRK404a               | from pS11D   |
| pQN217          | ~6.5-kb HindIII fragment in pRK404a             | from pS11D   |
| pQN231          | IpxQ in pRK404a                                 | this work    |
| pQN233          | IpxQ in pET21a(-)                               | this work    |
| pQN234          | IpxQ in pET28c(+) with N-terminal His tag       | this work    |
| pQN235          | IpxQ in pET21b(+) with C-terminal His tag       | this work    |
| pQN240          | A. tumefaciens IpxQ in pET21b+                  | this work    |
| pET21a+ and pET21b+ | Tlac expression vector, Amp’              | Novagen      |
| Tlac expression vector, Kan’ | Novagen                  | Novagen      |
| Rhizobium strains |                                                 |              |
| R. etli CE3      | Derivative of CFN42, Nal’, Str’                 | Refs. 19 and 20 |
| R. leguminosarum 3855 | Lipid A structure similar to R. etli CE3   | Ref. 52      |
| S. meliloti 1021 | Contains phosphorylated lipid A, Nal’, Str’     | Ref. 21      |
| R. leguminosarum 24AR | LPS mutant with phosphorylated lipid A          | Ref. 53      |
| E. coli strains  | reca, Str’                                      | BRL/88       |
| Novablue (DE3)  | K-12 strain, DE3 lysogen                        | Novagen      |
| BL21 (DE3/pLysS)| B strain, DE3 lysogen, contains pLysS           | Novagen      |
| BLR (DE3/pLysS) | B strain, DE3 lysogen, contains pLysS           | Novagen      |
| XLI-Blue        | K-12 strain                                     | Stratagene   |

The library was transferred from E. coli 803 into R. etli CE3 by tri-parental mating (27, 28) with E. coli MT616 as the helper (28). Three thousand individual R. etli CE3 colonies harboring random fragments of the library were picked into microtiter dishes containing (per well) 150 µl of TY medium with 20 µg/ml nalidixic acid, 200 µg/ml streptomycin, 12 µg/ml tetracycline, and 10 mM CaCl₂ and then were grown at 30 °C with shaking at 25 rpm to stationary phase. The cultures were stored at ~80 °C as master stocks in the same dishes after adjusting the medium to 20% glycerol by addition of an appropriate volume of 60% glycerol. To prepare fresh lysates for screening purposes, 1800 colonies were taken out of storage and regrown in 96-well microtiter plates. Each well was inoculated with a 5-µl portion of the master glycerol stock culture into 150 µl of TY broth supplemented with 10 mM CaCl₂, 20 µg/ml nalidixic acid, 200 µg/ml streptomycin sulfate, and 12 µg/ml tetracycline. The microtiter plates were then shaken at 225 rpm in a 30 °C incubator for ~24 h or until the OD₅₅₀ reached ~0.6, as measured with a microtiter plate reader. (Back-up glycerol stocks of these master plates were made by transferring 50 µl from each well into a new microtiter plate, in which each well contained 25 µl of 60% glycerol. The new stocks were stored at ~80 °C.)

The remaining 100 µl in each well of the microtiter plate was centrifuged at 3,660 × g for 5 min at 4 °C. The cell pellets were washed with 50 µl of 50 mM HEFES, pH 7.5, and centrifuged as above. After decanting the supernatant, the cell pellets were resuspended in 10 µl of lysis buffer, which consists of 100 mg/ml lysozyme in 50 mM HEFES, pH 7.5. The plates were sealed, and the contents were vigorously mixed for a minute. Lysis was allowed to proceed at room temperature for 30 min. The lysates were frozen by placing the plate into a ~80 °C freezer for several hours, after which they were thawed. Each 96-well microtiter plate contained eight rows (rows A–H) of 12 wells (wells 1–12). To facilitate the screening, each row was further grouped into four pools of three lysates. Row A, for example, was grouped into pools A(1–3), A(4–6), A(7–9), and A(10–12). The pooled lysates were assayed for their ability to convert [14C]B to [14C]-D-1. A portion of each pool (9 µl) was mixed with 2 µl of a concentrated reaction buffer (consisting of 5 mM MgCl₂, 250 mM MES, pH 6.5, and 0.5% Triton X-100) and 0.5 µl of 0.05 µM [14C]B (500 cpm/reaction tube). After 30 and 60 min of incubation at 30 °C, 5-µl portions of each reaction mixture were spotted onto a 20 ×
The following combinations of primers were used for PCRs and subsequent ligations. The PCR product of 5'—TGTGAQ/1 and 3'—Qx/Q3 was digested with HindIII and ligated into the shuttle vector pRK404a to yield pQN231. The lpxQ gene amplified with primers 5'—TGTGAQ/2 and 3'—Qx/Q1 was digested with NdeI and HindIII and then ligated into pET21a+, yielding pQN233. The DNA generated with 5'—TGTGAQ/2 and 3'—Qx/Q2 was digested with NdeI and HindIII and then ligated into pET21a+, yielding pQN235.

A typical PCR mixture contained 100 ng of template DNA (pQN210), 1× Pfu polymerase buffer (Stratagene), 10% MeSO, 1% glycerol, 200 μM of each of the dNTPs, 125 ng of each primer, and 2.5 units of Pfu polymerase (Stratagene) in a total volume of 100 μl. The reaction was placed in a DNA thermal cycler and subjected to 5 min of denaturation (94 °C), followed by five cycles consisting of denaturation (95 °C), annealing (1 min, 55 °C), and extension (2 min, 72 °C). Full amplification was then achieved with 25 cycles of the following: denaturation (1 min, 95 °C), annealing (1 min, 68 °C), and extension (2 min, 72 °C). After the 30th overall cycle, an additional 6-min extension at 72 °C was performed. The reaction was terminated by cooling the tubes to 4 °C. In each case, the product was analyzed on a 0.8% agarose gel, excised and purified using the Gene Clean II gel DNA purification kit (Bio 101), digested with the appropriate restriction enzymes as indicated above, and ligated into a vector that had been similarly digested.

The ligation mixtures that resulted in the construction of pQN233 and pQN235 were first transformed into competent E. coli XL1-Blue cells. These colonies were screened for the desired insert by restriction enzyme digestion. The insert and flanking regions of pQN233 were confirmed by DNA sequencing. The final recombinant plasmids were then transformed, as indicated below, into competent E. coli cells of strain BLR(DE3)/pLysS or Novablue(DE3) (Novagen) to evaluate the overexpression of LpxQ upon induction of mid-log phase cells at 37 °C with 1 mM IPTG.

The ligation mixture that yielded pQN231 was first transformed into competent cells of E. coli BL21. The four candidate plasmids containing the proper insert were then moved into S. meliloti 1021 by the tri-parental mating procedure. Of these four hybrid plasmids, only two were found to direct the expression of LpxQ activity and were designated pQN231 and pQN235. These plasmids were screened for the desired insert by restriction enzyme digestion. The insert and flanking regions of pQN233 were confirmed by DNA sequencing. The final recombinant plasmids were then transformed, as described in the accompanying article (1).

**PCR Amplification and Cloning of an A. tumefaciens lpxQ Ortholog—**

The A. tumefaciens lpxQ gene was amplified by PCR following ligation into the pET21a+ vector. The N-terminal primer for the PCRs had the following sequence: 5'—CGCCCGATTTCGCGCGCCATCTTACTATTGTTGGC3'. This primer was designed with a G/C clamp, an NdeI restriction site (in italics) that overlaps with the initiation codon ATG and the first 26 base pairs of the A. tumefaciens lpxQ gene coding sequence (in bold type). The C-terminal primer was designed with a G/C clamp, an HindIII restriction site (in italics) overlapping to a region that is 52 base pairs downstream of the lpxQ TAA termination codon. The sequence of the C-terminal primer was: 5'—CGCCCGATCTGGAAACTCTGGGAGCTGGTGATGG3'.

A. tumefaciens C58 genomic DNA purchased from the American Type Culture Collection (Manassas, VA) was used as the template. The PCR mixture (100 μl) contained 250 ng of template DNA, 1× Pfu polymerase buffer (Stratagene), 10% MeSO, 1% glycerol, 200 μM of each of the dNTPs, 125 ng of each primer, and 2.5 units of Pfu polymerase (Stratagene). The reaction was placed in a DNA thermal cycler and subjected to a 5-min denaturation at 94 °C, followed by five cycles consisting of denaturation (1 min, 95 °C), annealing (1 min, 55 °C), and extension (2 min, 72 °C). Full amplification was achieved with 25 cycles of the following: denaturation (1 min, 95 °C), annealing (1 min, 65 °C), and extension (2 min, 72 °C). After the 30th overall cycle, an additional 6-min extension at 72 °C was performed. The PCR was terminated by cooling the tubes to 4 °C.

The PCR product was analyzed on a 0.9% agarose gel and was gel purified with the Gene Clean DNA system (see above). The purified lpxQ gene, and the vector were both digested with NdeI and BamHI and then ligated at 16 °C overnight. The ligation mixture was transformed into competent E. coli XL-Blue cells (Stratagene). Several ampicillin-resistant colonies were picked, and the purified plasmids were sequenced for the desired insert by digestion with NdeI and BamHI. The construct containing the A. tumefaciens lpxQ gene was then ligated with the second fragment and was transformed into BLR(DE3)/pLysS (Novagen) for T7lac-directed overexpression.

**Preparation of E. coli Membranes Containing A. tumefaciens LpxQ for Assay—**

For this purpose, a single colony of BLR(DE3)/pLysS/
The membranes were resuspended in 0.15 ml of 50 mM HEPES, pH 7.5, mM CAPS, pH 11, containing 10% methanol, at 15 V for 30 min, using bilox-P polyvinylidene fluoride membrane (Millipore) equilibrated in 10

fragments were collected by centrifugation at 149,000

The membranes were washed without dilution for the various enzyme assays: 5

The active cosmids p1U12G and p1S11D were transferred

Expression Cloning of the Lipid A Oxidase of \textit{R. leguminosarum}—The gene encoding the lipid A oxidase was found by assaying ~600 pools of three individual lysates of an \textit{R. leguminosarum} genomic DNA library harbored in \textit{R. etli} CE3 for their ability to convert \textsuperscript{14}C\textsuperscript{B} to \textsuperscript{14}C\textsuperscript{D}-1 (Fig. 1A). A basal level of oxidase activity was present in all of the pools because of the chromosomal copy of the oxidase gene present in the CE3 host. As shown in Fig. 1B, this background activity was quantified at about 11% conversion of \textsuperscript{14}C\textsuperscript{B} to \textsuperscript{14}C\textsuperscript{D}-1 in 60 min under the assay conditions employed. Occasional samples, such as the one indicated by the arrow, derived from the pool of wells 10–12 from row D of plate 1S (Fig. 1B), catalyzed about 2.5-fold more rapid conversion of \textsuperscript{14}C\textsuperscript{B} to \textsuperscript{14}C\textsuperscript{D}-1 than did the others. The lysates making up these active pools were analyzed individually from their native promoters, whereas p1U12G and p1S11D contained the same insert. Three positive cosmids (p1S11D, p1E11D, and p1U12G) were identified in this manner. Based on restriction enzyme digests, p1S11D and p1E11D contained the same insert.

The active cosmids p1U12G and p1S11D were transferred via tri-parental mating from an \textit{E. coli} HB101 stock culture into \textit{S. meliloti} HB101. The latter does not contain endogenous oxidase activity and lacks the 2-aminogluconate unit in its lipid A. \textit{S. meliloti} expresses \textit{R. leguminosarum} genes very effectively from their native promoters, whereas \textit{E. coli} does not. As

![Fig. 1. Expression cloning of the \textit{R. leguminosarum} lipid A oxidase in \textit{R. etli}. A, a genomic \textit{R. leguminosarum} DNA library in cosmids pLAFR-1 (24, 32, 44) was transferred into \textit{R. etli} CE3. Cells from a 5-ml culture of Novablue(DE3)/pQN240 was used to inoculate 5 ml of LB broth containing 100

- \textbf{Results}

- \textbf{Expression Cloning of the Lipid A Oxidase of \textit{R. leguminosarum}}—The gene encoding the lipid A oxidase was found by assaying ~600 pools of three individual lysates of an \textit{R. leguminosarum} genomic DNA library harbored in \textit{R. etli} CE3 for their ability to convert \textsuperscript{14}C\textsuperscript{B} to \textsuperscript{14}C\textsuperscript{D}-1 (Fig. 1A). A basal level of oxidase activity was present in all of the pools because of the chromosomal copy of the oxidase gene present in the CE3 host. As shown in Fig. 1B, this background activity was quantified at about 11% conversion of \textsuperscript{14}C\textsuperscript{B} to \textsuperscript{14}C\textsuperscript{D}-1 in 60 min under the assay conditions employed. Occasional samples, such as the one indicated by the arrow, derived from the pool of wells 10–12 from row D of plate 1S (Fig. 1B), catalyzed about 2.5-fold more rapid conversion of \textsuperscript{14}C\textsuperscript{B} to \textsuperscript{14}C\textsuperscript{D}-1 than did the others. The lysates making up these active pools were analyzed individually (data not shown). In this manner, three positive cosmids (p1U12G, p1S11D, and p1E11D) capable of directing the overexpression of oxidase activity in CE3 were identified. Because p1E11D and p1S11D contained exactly the same inserts (data not shown), only p1S11D and p1U12G were further characterized.

The active cosmids p1U12G and p1S11D were transferred via tri-parental mating from an \textit{E. coli} HB101 stock culture into \textit{S. meliloti} 1021. The latter does not contain endogenous oxidase activity and lacks the 2-aminogluconate unit in its lipid A. \textit{S. meliloti} expresses \textit{R. leguminosarum} genes very effectively from their native promoters, whereas \textit{E. coli} does not. As

- \textbf{Synthesis of Lipid A Containing 2-Aminogluconate}
shown in Fig. 2, no background oxidase activity is present in cell extracts or membranes of the control strain \(S.\) meliloti\(\) \(\text{pLAFR-1} (\text{lanes 2 and 5})\). In contrast, robust conversion of \([14C]B\) to \([14C]D-1\) is observed in both cell extracts (\text{lanes 3 and 4}) and membranes (\text{lanes 6 and 7}) of \(S.\) meliloti\(\) \(\text{p1S11D}\) and \(S.\) meliloti\(\) \(\text{p1U12G}\). These results provide compelling evidence for the presence of the lipid A oxidase gene on the inserts in each of the above cosmids.

Subcloning of \(\text{p1S11D}\) Localizes the Oxidase Gene to a 6.5-kb \(\text{HindIII}\) Fragment—To determine the exact location of the oxidase gene, \(\text{DNA inserts in both p1S11D and p1U12G were digested with}\) \(\text{EcoRI}\) and \(\text{HindIII}\) (Fig. 3), as well as with \(\text{PstI}\) (not shown). The resulting DNA fragments were ligated into \(\text{pRK404a}\) and transformed into \(\text{E. coli}\) \(\text{HB101}\). The subclones obtained in this manner were transferred into \(\text{S. meliloti}\) \(1021\) by tri-parental mating (Fig. 3 and Table I). Upon assaying membranes of the various constructs, only \(\text{pQN210}, \text{which contains a 6.5-kb HindIII fragment (Fig. 3)}\) directed the overexpression of oxidase activity (Fig. 4). None of the fragments recovered from the \(\text{EcoRI}\) or \(\text{PstI}\) digestions of \(\text{p1S11D}\) were active.

Although the DNA inserts in \(\text{p1U12G}\) and \(\text{p1S11D}\) are not identical, their restriction enzyme digestion patterns suggested that they share a common ~9-kb segment of DNA (data not shown). In fact, the insert in \(\text{p1U12G}\) contains a ~6.5-kb \(\text{HindIII}\) fragment (Fig. 3) that appears to be identical to the one from \(\text{p1S11D}\), as judged by the fact that it also can direct the expression of oxidase activity (data not shown). Thus, \(\text{p1U12G}\) and \(\text{p1S11D}\) appear to share overlapping DNA segments containing the oxidase structural gene.
Identification of orfE (lpxQ) as the Structural Gene for the Oxidase—Based upon DNA sequencing, at least nine complete or partial open reading frames were tentatively identified on the 6.5-kb HindIII fragment present in pQN210 (Fig. 5). Sequence similarity searches with the BLASTx program indicated that orfA and orfM (Fig. 5) encode glycolate oxidase subunits. However, when orfA was cloned into pRK404a and then transferred into S. meliloti, no lipid A oxidase activity was observed in cell extracts (data not shown).

Both OrfC and OrfD (Fig. 5) share significant sequence similarity with a set of hypothetical membrane proteins of unknown function found in various members of the Rhizobiaceae and other bacteria, including R. leguminosarum, Mesorhizobium loti, and S. meliloti. Expression cloning of orfC and orfD (Fig. 5) failed to induce oxidase activity in cell extracts (data not shown). A similar hybrid plasmid harboring orfE, which encodes a protein with strong similarity to DNA-3-methyladenine glycosylase I, was likewise inactive in S. meliloti.

Although OrfE does not show strong similarity to any functionally assigned protein in the NCBI database, it does display weak similarity to an outer surface protein of R. leguminosarum and to other bacteria, including Pseudomonas. As discussed further below, a significant OrfE ortholog of unknown function is present in the plant pathogen A. tumefaciens (Fig. 6). Interestingly, extracts of S. meliloti/pQN231, which contains R. leguminosarum orfE behind a lac promoter on pRK404a, display robust oxidase activity (Fig. 7). Additional constructs containing orfE in various T7lac promoter-driven pET vectors (designated pQN233 through pQN235 in Table I) direct high levels of oxidase expression in E. coli cell extracts and membranes (Fig. 7), providing unequivocal evidence that orfE is the oxidase structural gene. Given its unique function in lipid A modification, we suggest that orfE be renamed lpxQ in accordance with the nomenclature used for other genes encoding lipid A biosynthetic enzymes (16, 38, 39).

Expression and Function of the lpxQ Ortholog of A. tumefaciens in E. coli—As shown in Fig. 6, the genomes of both sequenced strains of A. tumefaciens encode a protein that is ~59% identical and ~77% similar to R. leguminosarum LpxQ (17, 18). Expression of the putative A. tumefaciens lpxQ gene behind the T7lac promoter in two strains of E. coli resulted in the appearance of significant lipid A oxidase activity in cell extracts, as judged by the conversion of [14C]B to [14C]D-1 (Fig. 8). This finding suggests that A. tumefaciens may be able to synthesize 2-amino-3-glycerophosphoethanolamine in vitro by Recombinant LpxQ. The predicted amino acid sequence of the R. leguminosarum lipid A oxidase LpxQ (accession number AY228164) is compared with an ortholog of unknown function from A. tumefaciens. The predicted amino acid sequence of the R. leguminosarum lipid A oxidase LpxQ (accession number AY228164) is compared with an ortholog of unknown function from A. tumefaciens (17, 18).
product in the positive mode (Fig. 9B) reveals ions at m/z 1996.2 and 2024.6, which are interpreted as [M+Na]^+ of D-1 species differing in acyl chain length. Fig. 9A shows the positive mode spectrum of an authentic sample of D-1 isolated from R. etli. The resulting hybrid plasmid is designated pQN240. Membranes of E. coli BL21(DE3)/pLysS/pQN240 (lanes 2 and 3) or BLR(DE3)/pLysS/pQN240 (lanes 5 and 6), grown and induced as described in the legend to Fig. 7, were assayed under standard conditions for 15 min. The no enzyme control is shown in lane 1. Membranes derived from cells containing the vector were assayed in parallel (lanes 4 and 7). A positive control (i.e. membranes of the R. leguminosarum lpxQ overexpressing strain E. coli BL21(DE3)/pLysS/pQN233) is shown in lane 8.

Disappearance of LpxQ Activity under Anaerobic Conditions in Vitro—As discussed in the accompanying article (1), a plausible mechanism for the oxidation B to D-1 involves transferase PagP (7). The further addition of glucose oxygen atom into the proximal unit of B, this spectrum also reveals that about half of the D-1 was further modified with a palmitate residue.

LpxQ Localizes to the Outer Membrane when Expressed in E. coli—Isoelectric sucrose density gradient centrifugation of membranes prepared from induced cells of Novablue(DE3)/pQN233 was used to evaluate the subcellular localization of the recombinant LpxQ oxidase. The inner and outer membranes of the induced construct were well separated, as shown in Fig. 11A, by assay of the marker enzymes NADH oxidase and phospholipase A, respectively. Most of the lipid A oxidase is associated with the outer membrane in this construct (Fig. 11B), as in R. leguminosarum 3855 (1).

A protein corresponding in size to that expected for LpxQ (~23 kDa) is present in the outer membranes of Novablue(DE3)/pQN233 (Fig. 12), as judged by SDS-PAGE, but not in the outer membranes of the vector control Novablue(DE3)/pET21a+. The program SignalP (40) does in fact predict that LpxQ is an outer membrane protein with an N-terminal signal peptide that may be cleaved between Ala27 and Glu28. Signal sequences of this kind are present in virtually all outer membrane proteins of Gram-negative bacteria, because they are essential for proper translocation (41). The N-terminal sequence of the first 10 amino acids of the putative mature LpxQ protein band present in the outer membranes of Novablue(DE3)/pQN233 was determined as EDLQFSIYG. This result corresponds precisely to the predicted cleavage site and establishes conclusively that LpxQ is a genuine outer membrane protein.
All Gram-negative bacteria synthesize the lipid A component of lipopolysaccharide by a means of a constitutive seven-step pathway that starts with UDP-GlcNAc and proceeds via the tetra-acylated precursor, Kdo2-lipid IV A, as indicated schematically in Fig. 13 (16, 42). At least one additional secondary acyl chain is usually added after Kdo incorporation, most commonly at the 2/position (16, 42). In the case of R. leguminosarum, this additional acyl chain is 28 carbon atoms long and is hydroxylated at position 27 (Fig. 1) (1, 43, 44). A special acyl carrier protein is required for 27-OH-C28 synthesis and transfer to lipid A (Fig. 13) (43, 44). In E. coli, a secondary laurate chain is added at the 2/position, and myristate is added at 3/ (1, 45–48). All of the reactions leading to Kdo2-lipid IV A are cytosolic or associated with the inner membrane, as is the incorporation of 27-OH-C28 (Fig. 13).

**DISCUSSION**

All Gram-negative bacteria synthesize the lipid A component of lipopolysaccharide by a means of a constitutive seven-step pathway that starts with UDP-GlcNAc and proceeds via the tetra-acylated precursor, Kdo2-lipid IV A, as indicated schematically in Fig. 13 (16, 42). At least one additional secondary acyl chain is usually added after Kdo incorporation, most commonly at the 2/position (16, 42). In the case of R. leguminosarum, this additional acyl chain is 28 carbon atoms long and is hydroxylated at position 27 (Fig. 1) (1, 43, 44). A special acyl carrier protein is required for 27-OH-C28 synthesis and transfer to lipid A (Fig. 13) (43, 44). In E. coli, a secondary laurate chain is added at the 2/position, and myristate is added at 3/ (1, 45–48). All of the reactions leading to Kdo2-lipid IV A are cytosolic or associated with the inner membrane, as is the incorporation of 27-OH-C28 (Fig. 13).
The reactions that incorporate the lipid A disaccharide in the outer membrane suggest that the GalUA transfer to prenyl-phosphate-GalUA, has not actually been isolated from cells or characterized. Although the hypothetical glycolipid, undecaprenyl-phosphate-GalUA, has not actually been isolated from cells or characterized, it is predicted to have a periplasmic active site. The gene encoding the lipid A 1-phosphate transferase, which generates the substrate for LpxQ (Fig. 13), to-
been cloned in our laboratory, and its properties will be described elsewhere. It is an inner membrane protein with an active site that is predicted to face the periplasm (Fig. 13).

Our results demonstrate that formation of the 2-amino- gluconate unit in component D-1 occurs late in the \textit{R. leguminosarum} lipid A pathway. The lipid A of \textit{R. leguminosarum} pQ9210, which overexpresses lpxQ, is highly enriched in component D-1 with very little remaining B (data not shown). In contrast, the ratio between B to D-1 in wild type \textit{R. leguminosarum} lipid A is usually about 1:2 (3, 4). The extra copies of the oxidase present in \textit{R. leguminosarum} pQ9210 appear to deplete the available component B.

The availability of the lpxQ gene should facilitate the purification of the oxidase to homogeneity. With pure enzyme it should be possible to explore the proposed role of oxygen as the electron accepting cosubstrate in the conversion of B to D-1 and to confirm the stoichiometric formation of hydrogen peroxide. The identification of possible organic cofactors, the characterization of the catalytic mechanism, and the evaluation of the significance of the EDTA inhibition should be greatly simplified with pure protein. Finally, the structural biology of LpxQ should also be of great interest in relation to other outer enyzmes of know structure, like PagP (8), OmpT (9), and PldA (6).

The tertiary structure of LpxQ might reveal how the characteristic inside out \textit{b}-barrel folds of outer membrane proteins have evolved to generate novel catalytic sites.

Acknowledgments—We thank Kimberly Johnson and Margo Ruebenes of the Rajagopal laboratory at Duke University for assistance with the anaerobic chamber.

REFERENCES

1. Que-Gewirth, N. L. S., Lin, S., Cotter, R. J., and Raetz, C. R. H. (2003) J. Biol. Chem. 278, 12109–12119
2. Bhat, U. R., Forsberg, I. S., and Carlson, R. W. (1994) J. Biol. Chem. 269, 14492–14410
3. Que, N. L. S., Lin, S., Cotter, R. J., and Raetz, C. R. H. (2000) J. Biol. Chem. 275, 28017–28027
4. Que, N. L. S., Ribeiro, A. A., and Raetz, C. R. H. (2000) J. Biol. Chem. 275, 28017–28027
5. Nishijima, M., Nakaike, S., Tamori, Y., and Nojima, S. (1977) Eur. J. Biochem. 73, 115–124
6. Snijder, H. J., Dharraneta-Belanda, I., Blauw, M., Kalk, K. H., Verheij, H. M., Egmond, M. R., Dekker, N., and Dijkstra, B. W. (1999) Nature 401, 711–717
7. Bishop, R. E., Gibbons, H. S., Guina, T., Trent, M. S., Miller, S. L., and Raetz, C. R. H. (2000) EMBO J. 19, 5071–5080
8. Hwang, P.-M., Choy, W. Y., Lo, E. I., Chen, L., Forman-Kay, J. D., Raetz, C. R. H., Fong, G. G., Bishop, R. E., and Kay, L. E. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 13566–13569
9. Vandeputte-Rutten, L., Kramer, R. A., Kroon, J., Dekker, N., Egmond, M. R., and Gros, A. (2001) EMBO J. 20, 5033–5035
10. Ferguson, A. D., Hofmann, E., Coulton, J. W., Diederichs, K., and Welte, W. (1998) Science 282, 2215–2220
11. Koronska, V., Sharff, A., Koronakis, E., Luisi, B., and Hughes, C. (2000) Nature 405, 914–918
12. Schulz, G. E. (2002) Biochim. Biophys. Acts 1565, 308–317
13. Fernandez, C., Hilty, C., Bonjour, S., Adesivshi, K., Pervushin, K., and Wuthrich, K. (2001) FEBS Lett. 504, 173–178
14. Grisman, E. A. (2001) J. Bacteriol. 183, 1835–1842
15. Ohl, E. M., and Miller, S. I. (2001) Annu. Rev. Med. 52, 259–274
16. Raetz, C. R. H., and Westphal, C. (2002) Annu. Rev. Biochem. 71, 635–700
17. Wood, D. W., Setubal, J. C., Kall, R., Monens, D. E., Kitajima, J. P., Okura, V. K., Zhou, Y., Chen, L., Wood, G. E., Almeida, N. P. F., Jr., Won, L., Chen, Y., Paulsen, I. T., Eisen, J. A., Karp, D. P., Bevye, D. R., Chapman, R. F., Deverall, J., Deatherage, J., Deatherage, G., Gillett, W., Grant, C., Kutyavin, T., Levy, R., Li, M. J., McClelland, E., Palmeri, A., Raymond, C., Rous, G., Sgoultschoukamchak, C., Wu, Z., Romero, P., Gordon, D., Zhang, S., You, H., Tao, Y., Bidde, P., Jung, M., Krespan, W., Perry, M., Gordon-Ramsom, B., Liao, L., Kim, S., Hendrick, C., Zhao, Z. Y., Dolan, M., Chumley, F., Tinge, S. V., Torn, J. F., Gordon, P. M., Olson, M. V., and Nester, E. W. (2001) Science 291, 2017–2023
18. Goodner, B., Hinkle, G., Gattung, S., Miller, N., Blanchard, M., Qurollo, B., Goldman, B. S., Cao, Y., Akszenii, M., Halling, C., Mullin, L., Howard, K., Gordon, J., Vauzun, M., Itouchi, T., Kysa, A., Liu, F., Wallman, C., Allinger, M., Doughty, D., Scott, C., Lappas, C., Marks, B., Flanagan, C., Crowell, C., Sourdun, J., Lomo, C., Bear, C., Strub, G., Ciolo, C., and Slater, S. (2001) Science 294, 2325–2328