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The Dioxygenase-Encoding *olsD* Gene from *Burkholderia cenocepacia* Causes the Hydroxylation of the Amide-Linked Fatty Acyl Moiety of Ornithine-Containing Membrane Lipids

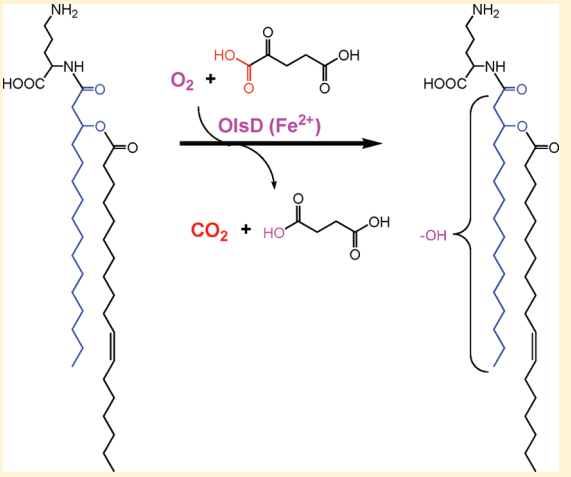
Napoleón González-Silva,[†] Isabel M. López-Lara,[†] Rodrigo Reyes-Lamothe,[†] Adrian M. Taylor,[‡] David Sumpton,[‡] Jane Thomas-Oates,[‡] and Otto Geiger[*,†]

[†]Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Apdo. Postal 565-A, Cuernavaca, Morelos CP62251, Mexico

[‡]Department of Chemistry, University of York, Heslington, York YO10 5DD, United Kingdom

Supporting Information

ABSTRACT: *Burkholderia cenocepacia* is an important opportunistic pathogen, and one of the most striking features of the *Burkholderia* genus is the collection of polar lipids present in its membrane, including phosphatidylethanolamine (PE) and ornithine-containing lipids (OLs), as well as the 2-hydroxylated derivatives of PE and OLs (2-OH-PE and 2-OH-OLs, respectively), which differ from the standard versions by virtue of the presence of a hydroxyl group at C2 (2-OH) of an esterified fatty acyl residue. Similarly, a lipid A-esterified myristoyl group from *Salmonella typhimurium* can have a 2-hydroxy modification that is due to the LpxO enzyme. We thus postulated that 2-hydroxylation of 2-OH-OLs might be catalyzed by a novel dioxygenase homologue of LpxO. In *B. cenocepacia*, we have now identified two open reading frames (BCAM1214 and BCAM2401) homologous to LpxO from *S. typhimurium*. The introduction of *bcam2401* (designated *olsD*) into *Sinorhizobium meliloti* leads to the formation of one new lipid and in *B. cenocepacia* of two new lipids. Surprisingly, the lipid modifications on OLs due to OlsD occur on the amide-linked fatty acyl chain. This is the first report of a hydroxyl modification of OLs on the amide-linked fatty acyl moiety. Formation of hydroxylated OLs occurs only when the biosynthesis pathway for nonmodified standard OLs is intact. The hydroxyl modification of OLs on the amide-linked fatty acyl moiety occurs only under acid stress conditions. An assay has been developed for the OlsD dioxygenase, and an initial characterization of the enzyme is presented.



Burkholderia cenocepacia J2315 is a highly virulent member of the *Burkholderia cepacia* complex (BCC), a subgroup of important opportunistic pathogens of the *Burkholderia* genus that infects individuals with cystic fibrosis and chronic granulomatous disease or the immunocompromised, and causes high mortality rates.[1] Although a number of factors that contribute to BCC virulence are known, often BCC infections are not efficiently eliminated by treatment with common antibiotics.[1] Among the most striking features of the *Burkholderia* genus are the polar lipids present in their membranes. *B. cenocepacia* J2315, like other members of its genus, possesses phosphatidylglycerol (PG), cardiolipin (CL), phosphatidylethanolamine (PE), and ornithine-containing lipids (OLs), as well as the 2-hydroxylated derivatives of PE and OLs (2-OH-PE and 2-OH-OLs, respectively), which differ from the standard versions by the presence a hydroxyl group at C2 (2-OH) of a fatty acyl residue.[2,3] The standard OLs possess an ornithine residue, the α-amino group of which is amide-bound to a 3-hydroxy fatty acyl group. This amide-linked moiety is esterified through its hydroxyl to another nonhydroxylated fatty acyl residue.[4,5] The biosynthesis pathway for such nonhydroxylated OLs has been resolved and consists of two steps. In the first step, the *N*-acyltransferase OlsB catalyzes the transfer of a 3-hydroxy fatty acyl group from 3-hydroxy fatty acyl−acyl carrier protein to the α-amino group of ornithine, forming lyso-ornithine lipid.[6] In the second step, the *O*-acyltransferase OlsA catalyzes the transfer of a fatty acyl group from fatty acyl−acyl carrier protein to the hydroxy group of lyso-ornithine lipid, forming OL.[7]

In 2-OH-PE from *B. cenocepacia* J2315, the 2-hydroxy fatty acyl residue is exclusively linked to the *sn*-2 position of this glycerolipid, whereas in 2-OH-OLs, the 2-hydroxy fatty acyl chain is the ester-linked residue.[2,3] The 2-hydroxy fatty acyl residues are not formed

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Table 1. Bacterial Strains and Plasmids Used in This Study

strain or plasmid	relevant characteristics	ref or source
S. meliloti 1021	SU47 *str-21*	12
S. meliloti AAK1	*olsB::kan*	6
S. meliloti CS111	*pssA::gm*	13
B. cenocepacia J2315	wild type	LMG Bacteria Collection
B. cenocepacia AQ3	*olsD::cat*	this work
B. cenocepacia NG1	*olsB::cat*	this work
E. coli		
DH5α	*recA*1, Φ80 *lacZΔM15*	14
S17-1	modified RP4 plasmid integrated into the genome	15
pBluescriptSK+	cloning vector, Cb^R	Stratagene
pUC18	cloning vector, Cb^R	16
pRK404	broad host range vector, Tc^R	17
pBBR1MCS	broad host range vector, Cm^R	18
pBBR1MCS-5	broad host range vector, Gm^R	19
pLysS	production of lysozyme for repression of T7 polymerase, Cm^R	20
pET9a	expression vector, Kn^R	20
pET16b	expression vector, Cb^R	20
pET17b	expression vector, Ap^R	21
pK18*mobsacB*	suicide vector, Kn^R	22
pJG16	*Bam*HI-restricted pET9a in pRK404	6
pJG20	$olsB_{Sm}$ in pET9a	6
pILAS03	$olsB_{Sm}$ in pBBR1MCS-5	this work
pCAT	pUC18 containing chloramphenicol resistance gene	this work
pSphx01	*bcam1214* in pET9a	this work
pSphx02	*olsD* in pET9a	this work
pSphx03	*Bgl*II-restricted pSphx01 in pRK404	this work
pSphx04	*Bgl*II-restricted pSphx02 in pRK404	this work
pRRL01	1.1 kb downstream of *olsD* in pBluescriptSK+	this work
pSphx06	flanking regions of *olsD* interrupted by a chloramphenicol resistance gene in pBluescriptSK+	this work
pSphx08	flanking regions of *olsD* interrupted by a chloramphenicol resistance gene in pK18*mobsacB*	this work
pNG10	1.1 kb upstream of $olsB_{Bc}$ in pBluescriptSK+	this work
pNG11	1.2 kb downstream of $olsB_{Bc}$ in pBluescriptSK+	this work
pNG14	flanking regions of $olsB_{Bc}$ in pBluescriptSK+	this work
pNG15	flanking regions of $olsB_{Bc}$ interrupted by a chloramphenicol resistance gene in pBluescriptSK+	this work
pNG16	flanking regions of $olsB_{Bc}$ interrupted by a chloramphenicol resistance gene in pK18*mobsacB*	this work
pNG23	$olsB_{Bc}$ in pET17b	11
pNG24	*Bgl*II-restricted pNG23 in pRK404	this work
pNG28	*Bam*HI-restricted pET17b in pRK404	this work
pNG40	*olsD* in pET16b	this work

during standard fatty acid biosynthesis, and specific enzymatic activities are required for the introduction of a hydroxyl group onto C2 of a fatty acyl residue.

In *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), a gene (*lpxO*) has been identified as being responsible for the introduction of a 2-hydroxy group onto the lipid A-esterified myristoyl group.[8] The hydroxylation reaction is catalyzed by the $Fe^{2+}/O_2/$α-ketoglutarate-dependent LpxO-encoded dioxygenase.[9] It has been suggested that such extra hydroxyl groups might increase the extent of hydrogen bonding between adjacent lipid A molecules, enhancing the outer membrane's ability to resist penetration by certain compounds under some growth conditions.[9] A homologue of LpxO, OlsC from *Rhizobium tropici*, is responsible for the formation of two of the four classes of OLs encountered in this organism.[10] Recently, it has been shown that the expression of OlsC modifies OLs by hydroxylation at the 2 position of the esterified fatty acyl residue.[11]

As the functional roles associated with the 2-OH modifications of PE and OLs are not known in *B. cenocepacia*, we embarked on a

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genomic approach in an attempt to identify potential genes that might be responsible for the introduction of the 2-OH modifications. The *B. cenocepacia* J2315 genome contains two open reading frames (ORFs) [BCAM1214 and BCAM2401 (OlsD)] homologous to LpxO from *S. typhimurium*, which were cloned and introduced into *Sinorhizobium meliloti* 1021 and *B. cenocepacia* J2315. The introduction of *olsD* into *S. meliloti* leads to the formation of one new lipid and the introduction into *B. cenocepacia* to the formation of two new lipids. Mass spectrometric data suggest that OlsD can modify preexisting OLs of *S. meliloti* and *B. cenocepacia* by introducing a hydroxyl group. Surprisingly, the OlsD-derived modification does not occur on the ester-linked fatty acyl chain but on the amide-linked fatty acyl chain. The *olsD* gene is part of a biosynthetic pathway for OLs not previously described for any species.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions. All bacterial strains and plasmids used and their relevant characteristics are listed in Table 1.[11−22] Construction of burkholderial mutants is described in the Supporting Information. *S. meliloti* strains were grown either on complex tryptone/yeast extract (TY) medium that contained 4.5 mM $CaCl_2$[23] or on minimal medium[24] with succinate (8.3 mM) replacing mannitol as the carbon source at 29 °C on a gyratory shaker. Growth in minimal medium that contained low concentrations (0.02 mM) of inorganic phosphate (P_i) was performed as previously described.[7] *B. cenocepacia* strains were grown at 29 °C and *Escherichia coli* strains at 37 °C in Luria-Bertani (LB) medium[25] on a gyratory shaker. To study the effect of acidic stress, *B. cenocepacia* was grown in complex LB medium that contained additionally a 50 mM Homopipes [homopiperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]/NaOH buffer (pH 4) instead of the unbuffered, nearly neutral LB medium. Antibiotics were added to the medium at the following concentrations when required: 70 μg/mL gentamicin, 40 μg/mL piperacillin, and 4 μg/mL tetracycline in the case of *S. meliloti*; 100 μg/mL carbenicillin, 20 μg/mL tetracycline, 10 μg/mL gentamicin, 50 μg/mL kanamycin, and 20 μg/mL chloramphenicol in the case of *E. coli*; and 500 μg/mL tetracycline (for solid medium) or 300 μg/mL tetracycline (for liquid medium) and 300 μg/mL chloramphenicol for *B. cenocepacia* J2315.

The pRK404-, pBBR1MCS-5-, or suicide-plasmid derivatives were mobilized into *S. meliloti* or *B. cenocepacia* strains by diparental mating using *E. coli* S17-1.[15]

DNA Manipulations. Recombinant DNA techniques were performed according to standard protocols.[26] In all polymerase chain reactions, the XL-PCR kit from Applied Biosystems was used. To obtain plasmid pILAS03, the 987 bp *Bgl*II−*Bam*HI fragment containing *olsB* of *S. meliloti* 1021 ($olsB_{Sm}$) and the regulating region was obtained from pJG20[6] and ligated into pBBR1MCS-5[19] that had been linearized with *Bam*HI. Cloning of burkholderial genes is described in the Supporting Information.

In Vivo Labeling of Bacterial Strains with [^{14}C]Acetate, [^{14}C]Ornithine, or $^{32}P_i$. The lipid compositions of *S. meliloti* 1021 and *B. cenocepacia* J2315 derivatives were analyzed following labeling with [^{14}C]acetate, [^{14}C]ornithine, or $^{32}P_i$. Cultures (1 mL) in minimal or complex medium were inoculated from precultures grown in the same medium. After addition of 2 μCi of [1-^{14}C]acetate (60 mCi/mmol), 1 μCi of DL-[1-^{14}C]ornithine (56 mCi/mmol), or 1.3 μCi of $^{32}P_i$ to each culture, the cultures were incubated for 24 h (on low P_i-containing minimal medium) or 4 h (on TY medium) in the case of *S. meliloti* and for 12 h in the case of *B. cenocepacia*. Cells were harvested by centrifugation and resuspended in 100 μL of water. The lipids were extracted using the method of Bligh and Dyer.[27] The chloroform phase was used for lipid analysis on TLC plates after two-dimensional[5] separation. The individual lipids were quantified as described previously[28] or by using a Phosphor-Imager (Storm 820, Molecular Dynamics). A chloroform/methanol/water mixture (130:50:8, v/v/v) was used as the solvent system for development in one dimension. Ninhydrin staining of lipids was performed as described previously.[7]

Preparation of the Radiolabeled OL Substrate for OlsD Enzyme Assays. PE-deficient strain *S. meliloti* CS111 × pNG24 expressing OlsB from *B. cenocepacia* was cultivated in 100 mL of complex medium in the presence of 100 μCi of [1-^{14}C]acetate (60 mCi/mmol) and harvested at an OD_{620} of 1. Cells were extracted using the method of Bligh and Dyer,[27] and the chloroform phase was separated by one-dimensional TLC using a chloroform/methanol/acetic acid mixture (130:50:20, v/v/v). Radiolabeled OL was localized by autoradiography and extracted from the OL-containing silica gel fraction, and the OL stock was stored in a chloroform/methanol mixture (1:1, v/v).

Preparation of Cell-Free Extracts for Analysis of the OlsD Dioxygenase. Cultures (1 L) of exponentially growing *E. coli* BL21(DE3) × pLysS, harboring additionally pET16b or pNG40, were induced with 1 mM isopropyl β-D-thiogalactoside at a density of 5×10^8 cells/mL, and cells were incubated for an additional 3 h at 37 °C. After cells had been harvested by low-speed centrifugation at 4 °C, each cell pellet was washed once with 50 mM HEPES/KOH buffer (pH 7.5), and cells were resuspended in 3 mL of the same buffer. Cell suspensions were passed three times through a French pressure cell at 20000 lb/in.^{2} Unbroken cells and cell debris were removed by centrifugation at 4000*g* for 10 min, yielding cell-free extracts as supernatants. For some assays, cell-free extracts were centrifuged at 150000*g* for 1 h to separate soluble proteins and membranes with their associated proteins. Protein concentrations were determined by the bicinchoninic acid assay.[29]

In Vitro Assay for the OlsD Dioxygenase. An in vitro assay for OlsD, using OL as the putative acceptor substrate, was developed on the basis of the assay previously reported for the Fe^{2+}/O_2/α-ketoglutarate-dependent LpxO hydroxylase.[9] The reaction conditions, unless otherwise described, included 50 mM HEPES/KOH buffer (pH 8.0), 1 mM α-ketoglutarate, 2 mM ascorbate, 10 μM $Fe(NH_4)_2(SO_4)_2$, 0.1% Triton X-100, 4 mM dithiothreitol (DTT), and 169 μM OL (25,960 cpm/reaction or 3072 cpm/nmol). Assays were conducted at 30 °C in a final volume of 50 μL. For each assay, first the respective amount of Triton X-100 in water was mixed with radiolabeled OL in a chloroform/methanol mixture (1:1, v/v) and brought to dryness in an Eppendorf concentrator. Then, buffer and the remaining components were added; reactions were initiated via addition of *E. coli* crude extracts (final concentration usually of 1 mg/mL), and they were stopped via addition of 125 μL of methanol and 62.5 μL of chloroform. After lipids had been extracted using the method of Bligh and Dyer,[27] the chloroform phase was separated by one-dimensional TLC with a chloroform/methanol/acetic acid mixture (130:50:20, v/v/v). In this chromatographic system, the unmodified OL exhibited an R_f value of 0.37 whereas the OlsD-modified, hydroxylated OL exhibited an R_f value of 0.29.

Preparation of Lipids for Mass Spectrometric Analysis. For spectrometric analysis of lipids from *S. meliloti* strains carrying

pRK404 derivatives, after two passages in Sherwood minimal medium with a low phosphate concentration, 500 mL cultures were grown in the same medium until they reached the late exponential phase (OD_{620} = 0.570). Cells were harvested by centrifugation, and the lipids were extracted using the method of Bligh and Dyer.[27] The chloroform phase was analyzed by mass spectrometry. Cultures of 1 L of *B. cenocepacia* were grown to an OD_{620} of 1. Cells were harvested by centrifugation, and the lipids were extracted using the method of Bligh and Dyer.[27] The chloroform phase was either directly analyzed by mass spectrometry or further fractionated as follows. Concentrated lipid preparations of *B. cenocepacia* strains were separated on high-performance TLC silica 60 plates by two-dimensional TLC.[5] After the samples had been stained with iodine, the individual iodine-stained spots were scraped off, and lipids were repeatedly extracted from the silica gel as previously described[28] and analyzed by mass spectrometry.

Mass Spectrometric Analysis. The *S. meliloti* lipid extracts were analyzed as follows. ESI mass spectra were recorded on an Applied Biosystems (Warrington, U.K.) QSTAR pulsar i hybrid quadrupole time-of-flight tandem mass spectrometer. The ion source contained a microelectrospray arm mounted in an Applied Biosystems ion source housing. A 100 μL Hamilton 1710N syringe with a 1.46 mm inside diameter was fitted to a fused silica capillary, which was used to deliver the sample, dissolved in methanol (Fischer Scientific, Loughborough, U.K.), to the ion source at a rate of 1 μL/min controlled by the integral syringe driver. The ion source gas reading was set to 4 and the curtain gas to 20, and the capillary was held at 5500 V. The instrument was operated in the positive mode with declustering and focusing potentials of 65 and 265 V, respectively. For Pulsar operation, the ion release delay was set to 6 and the ion release width to 5. In MS mode, the collision gas was set to read 3 and increased to 6 during tandem MS. The "collision energy offset" for tandem MS was varied between 25 and 70 V. Nitrogen was used for both the collision gas and the curtain gas. The data were recorded and analyzed using Applied Biosystems/MDS SCIEX Analyst QS.

The *B. cenocepacia* lipid extracts were analyzed as follows. ES CID tandem mass spectra were recorded on an Applied Biosystems QSTAR pulsar i hybrid quadrupole time-of-flight tandem mass spectrometer with a nanoflow electrospray ion source. The supplied sample solutions were diluted in methanol (1:10, v/v) and were delivered to the ion source using a fused silica continuous flow sample introduction system and a syringe pump that delivered the sample solution at a rate of 0.2 μL/min. When the instrument was operated in positive mode, the ion spray voltage was set to 4800 V, declustering potential 1 to 50 V, declustering potential 2 to 10 V, and the focusing potential to 220 V. In negative mode, the ion spray voltage was set to −4800 V, declustering potential 1 to −50 V, declustering potential 2 to −10 V, and the focusing potential to −220 V. Ionization was assisted via the application of curtain gas (nitrogen) set at 25, and spectra were recorded over the m/z 100−1000 range. Tandem mass spectra were recorded over the m/z 50−800 range depending on the chosen precursor ion; nitrogen was used as the collision gas, and the spectra were recorded using "collision offsets" of 10−45 "V". Both MS and MS/MS data were recorded and processed using Analyst.

RESULTS

OlsD of *B. cenocepacia*, a Homologue of LpxO of *S. typhimurium*, Can Modify OL of *S. meliloti*. We have searched

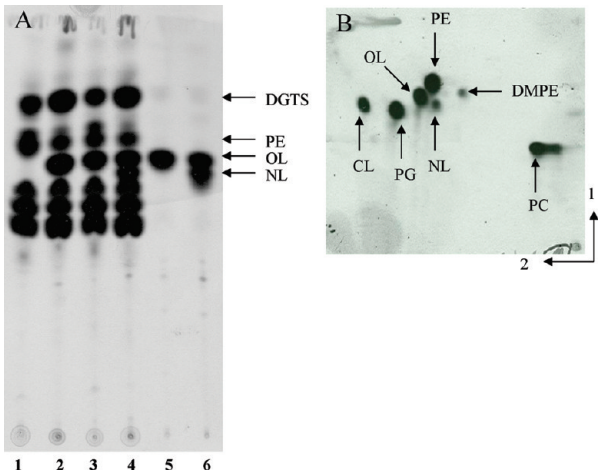

Figure 1. Expression of BCAM2401 (OlsD) in *S. meliloti* causes the formation of a new ornithine-containing lipid (NL). (A) Lipid analysis of *S. meliloti* strains carrying different plasmids after growth on Sherwood minimal medium containing a low phosphate concentration and grown either in the presence of [^{14}C]acetate (lanes 1−4) or in the presence of [^{14}C]ornithine (lanes 5 and 6): lane 1, AKK1 × pJG16, a strain deficient in OL production;[6] lanes 2 and 5, *S. meliloti* 1021 × pJG16; lane 3, *S. meliloti* 1021 × pSphx03, expressing *bcam1214*; lanes 4 and 6, *S. meliloti* 1021 × pSphx04, expressing *bcam2401*. (B) Separation of [^{14}C]acetate-labeled lipids by two-dimensional TLC from *S. meliloti* harboring pILAS03 and pSphx04 (expressing $olsB_{Sm}$ and *bcam2401*) after growth on complex TY medium. The lipids PC, dimethylphosphatidylethanolamine (DMPE), PE, PG, CL, diacylglyceryl *N,N,N*-trimethylhomoserine (DGTS), OL, and new lipid (NL) are indicated.

for homologues of LpxO from *S. typhimurium* (GenBank entry AAF87784) using the BLAST server of the genome sequencing project of *B. cenocepacia* J2315 at http://www.sanger.ac.uk/cgi-bin/blast/submitblast/b_cenocepacia. Searching with the LpxO protein (302 amino acids) in the *B. cenocepacia* genome, we found two homologues; BCAM1214 (299 amino acids) that is 57% identical and 72% similar to *S. typhimurium* LpxO (E value = 1.7 × 10^{-94}) and BCAM2401 (OlsD, 249 amino acids) that is 28% identical and 46% similar in an overlap of 180 amino acids to *S. typhimurium* LpxO (E = 1.7 × 10^{-18}). The ORFs for these two proteins are located on chromosome 2, and in the recently published genome of *B. cenocepacia* J2315,[30] putative β-hydroxylase functions were suggested for both proteins. To determine whether any of these proteins is able to modify OL and/or PE, the ORFs encoding each of the proteins were amplified from genomic DNA of *B. cenocepacia*, and each was cloned in a broad host-range vector (see Experimental Procedures) and designated pSphx03 for that carrying *bcam1214* and pSphx04 for that carrying *olsD*. These vectors, as well as empty vector pJG16,[6] were individually transferred into *S. meliloti* 1021 that produces only nonhydroxylated forms of OL and PE.[5] The three different strains were grown in Sherwood minimal medium at phosphate-limiting concentrations (20 μM P_i) in the presence of [^{14}C]acetate, and lipids were extracted and analyzed using one-dimensional thin-layer chromatography (TLC). Introduction of plasmid pSphx03 into *S. meliloti* gives the same lipid profile found in *S. meliloti* carrying empty vector pJG16 (Figure 1A, lanes 2 and 3). However, the introduction of pSphx04 leads to the formation of a new lipid (Figure 1A, lane 4). The new lipid (NL) reacts positively when stained with ninhydrin (data not shown), indicating that it is an amine-containing compound, but NL does

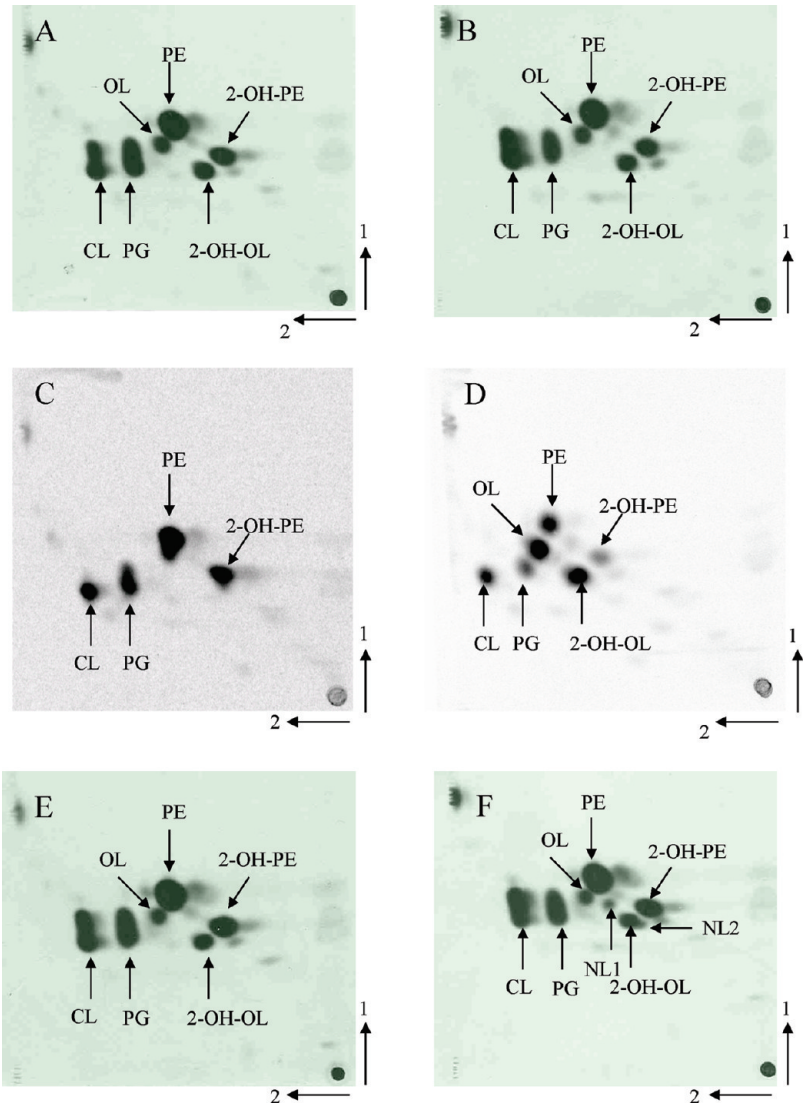

Figure 2. Membrane lipid profiles of *B. cenocepacia* strains defective with respect to OL biosynthesis genes (*olsB* or *olsD*) or overexpressing them. Separation of [^{14}C]acetate-labeled lipids by two-dimensional TLC from wild-type *B. cenocepacia* J2315 (A), wild-type *B. cenocepacia* J2315 containing empty vector pJG16 (B), the *olsB*-deficient knockout mutant *B. cenocepacia* NG1 that lacks OLs (C), the *olsB*-deficient knockout mutant with *olsB*-expressing plasmid pNG24 (D), the *olsD*-deficient knockout mutant *B. cenocepacia* AQ3 (E), and wild-type *B. cenocepacia* J2315 with *olsD*-expressing plasmid pSphx04 (F), after growth on LB medium. The lipids PE, 2-OH-PE, OL, 2-OH-OL, PG, and CL and the two new lipids (NL1 and NL2) are indicated.

not incorporate ^{32}P-containing inorganic phosphate (P_i), suggesting that it might lack phosphorus in its structure. Lipid extracts obtained from *S. meliloti* 1021 × pJG16 grown in the presence of [^{14}C]ornithine show the specific incorporation of radioactivity into OL,[6] and only the compound corresponding to OL can be detected[6] (Figure 1A, lane 5). In contrast, lipid extracts obtained from *S. meliloti* 1021 × pSphx04 contain a second lipidic compound into which [^{14}C]ornithine is specifically incorporated (Figure 1A, lane 6). Taken together, these data suggest that the new compound, due to *olsD*, was a modified form of the OL described previously.[5]

Previously, we have shown that in *S. meliloti* the biosynthesis of OL is regulated at the level of *olsB* gene expression because the introduction of *olsB* in a stable plasmid into *S. meliloti* leads to constitutive high-level production of OL, even in media with high concentrations of phosphate.[6] Now, we have constructed a new broad host-range plasmid, pILAS03, that carries *olsB* from *S. meliloti* ($olsB_{Sm}$) and is compatible with pSphx04 (see Experimental Procedures). As in the case of pJG21,[6] introduction of pILAS03 into *S. meliloti* provokes the formation of high levels of OL even when the *S. meliloti* is grown on TY medium (data not shown). When *S. meliloti* carries both plasmids (pSphx04 and pILAS03), the new lipid compound (NL) dependent on *olsD* is formed even in cultures grown on TY medium (Figure 1B).

OlsB Is Required for the Formation of Unmodified OL and 2-OH-OL in *B. cenocepacia*. *B. cenocepacia* J2315 grown on LB medium forms four ninhydrin-positive spots (data not shown and Figure 2A) that were interpreted as PE, OL, and the respective 2-hydroxylated derivatives (2-OH-PE and 2-OH-OL), as described previously for members of the *Burkholderia* genus.[2,3] Introduction of an empty broad host-range vector (pJG16) does not change the lipid profile of wild-type *B. cenocepacia* (Figure 2B). To determine if formation of standard OLs was essential for the biosynthesis of modified OLs, a mutant

Table 2. Membrane Lipid Composition of Wild-Type *B. cenocepacia* J2315, *olsD*- or *olsB*-Deficient Mutants (AQ3 or NG1), the *olsD*-Deficient Mutant Complemented with the *olsD* Gene (AQ3 × pSphx04) or Containing an Empty Broad Host-Range Vector (AQ3 × pJG16), and the *olsB*-Deficient Mutant Complemented with the *olsB* Gene (NG1 × pNG24) or Containing an Empty Broad Host-Range Vector (NG1 × pNG28) after Growth on Complex LB Medium[a]

	composition (% of total ^{14}C)						
lipid	wild type	AQ3	AQ3 × pSphx04	AQ3 × pJG16	NG1	NG1 × pNG24	NG1 × pNG28
PG	12.2 ± 0.3	14.1 ± 1.9	15.0 ± 0.6	11.8 ± 0.9	11.2 ± 0.6	10.4 ± 0.8	14.2 ± 0.8
CL	9.9 ± 1.1	11.9 ± 1.9	10.2 ± 1.1	11.4 ± 1.2	12.6 ± 1.9	11.3 ± 1.6	11.1 ± 1.3
PE	64.9 ± 2.1	56.0 ± 1.1	61.2 ± 1.8	59.5 ± 0.5	60.3 ± 5.3	11.7 ± 0.9	61.5 ± 2.8
2-OH-PE	6.5 ± 1.2	11.4 ± 0.3	7.6 ± 1.2	9.2 ± 0.7	16.0 ± 6.6	1.8 ± 0.2	13.3 ± 2.0
OL	2.1 ± 0.1	1.8 ± 0.4	1.8 ± 0.4	2.3 ± 0.9	nd[b]	47.2 ± 4.6	nd[b]
2-OH-OL	4.4 ± 0.7	4.8 ± 0.7	1.9 ± 0.3	5.9 ± 0.4	nd[b]	17.8 ± 2.7	nd[b]
NL1	nd[b]	nd[b]	1.3 ± 0.1	nd[b]	nd[b]	nd[b]	nd[b]
NL2	nd[b]	nd[b]	1.1 ± 0.3	nd[b]	nd[b]	nd[b]	nd[b]

[a] The values shown are means ± the standard deviation derived from three independent experiments. [b] Not detected.

of *B. cenocepacia* in the *olsB* gene was created and named NG1. We have searched for the homologue of the OlsB product from *S. meliloti* ($OlsB_{Sm}$) (GenBank entry NP_384499) using the BLAST server of the genome sequencing project of *B. cenocepacia* J2315 at http://www.sanger.ac.uk/cgi-bin/blast/submitblast/b_cenocepacia. Searching with the $OlsB_{Sm}$ protein (296 amino acids) in the *B. cenocepacia* genome, we found a homologue, BCAL1281 ($OlsB_{Bc}$, 268 amino acids), that is 33% identical and 50% similar to $OlsB_{Sm}$ ($E = 6.7 \times 10^{-31}$). The *olsB*-deficient knockout mutant *B. cenocepacia* NG1 does not synthesize any form of OL (Figure 2C). Quantitative analysis of individual burkholderial lipids shows that when OLs are absent, the level of 2-OH-PE increases ~2.5-fold to comprise 16% of total membrane lipids (Table 2). When we complemented the mutant NG1 *in trans* with plasmid pNG24 that contains the $olsB_{Bc}$ gene, the levels of OL and 2-OH-OL increase dramatically, comprising 47 and 18% of total membrane lipids, respectively (Figure 2A,D and Table 2). In this OL-overproducing strain, levels of PE and 2-OH-PE are drastically reduced to 12 and 2% of the total level of membrane lipids, respectively (Table 2). It seems that the cells try to keep the amount of zwitterionic lipid in balance. This effect was not observed in the mutant containing empty vector pNG28 (Table 2).

Mutation of *olsD* Does Not Lead to Any Change in the Membrane Lipid Profile of *B. cenocepacia* under Standard Growth Conditions; However, Its Expression Does. To study the function of *olsD* in *B. cenocepacia*, a knockout mutant in *olsD* was created and was named AQ3. Surprisingly, analysis via two-dimensional TLC (2D-TLC) of [^{14}C]acetate-labeled lipids from the wild type and the AQ3 mutant after growth on LB medium does not show any detectable difference between them (Figure 2A,E and Table 2), and notably, *olsD*-deficient mutant AQ3 still forms the previously described 2-hydroxylated derivatives of PE and OL. Also, the *B. cenocepacia* strain that contained the *bcam1214* gene showed a lipid profile similar to that of the wild type (data not shown). However, the constitutive expression of *olsD* in the AQ3 (Table 2) and *B. cenocepacia* J2315 (Figure 2F) backgrounds led to the formation of two new compounds in each strain, named NL1 and NL2, that could not be detected in a wild-type strain harboring an empty plasmid (Figure 2B and Table 2). The lipids NL1 and NL2, like NL, stained positive with ninhydrin. Therefore, under our standard experimental conditions, the

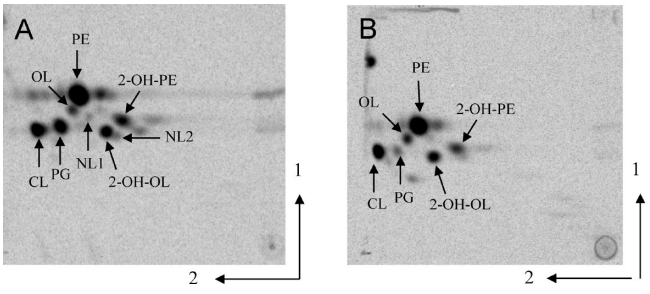

Figure 3. OlsD-dependent modification of OLs in *B. cenocepacia* occurs under acidic growth conditions. Separation of [^{14}C]acetate-labeled lipids by 2D-TLC from wild-type *B. cenocepacia* J2315 (A) or *olsD*-deficient knockout mutant *B. cenocepacia* AQ3 (B) after growth on acidic (pH 4) Homopipes/Na-buffered LB medium. The lipids PE, 2-OH-PE, OL, 2-OH-OL, PG, and CL and the two new lipids (NL1 and NL2) are indicated.

products dependent on *olsD* were not detected in wild-type *B. cenocepacia*. Spot NL1 produced in *B. cenocepacia* (Figure 2F) shows the same mobility in 2D-TLC as spot NL observed in *S. meliloti* after expression of *olsD* and *olsB* (Figure 1B). Quantitative analysis of the individual burkholderial lipids (Table 2) indicates that even if OlsD is expressed in *B. cenocepacia* × pSphx04, NL1 and NL2 are only minor lipids, each of them comprising slightly more than 1% of the total membrane lipids. Remarkably, the strain forming NL1 and NL2 contains reduced amounts of 2-OH-OL as if the organism tries to maintain a constant level of OL hydroxylation for a given physiological condition. When we expressed the *olsD* gene in OL-deficient mutant NG1, a membrane lipid profile similar to that of the mutant that contains empty vector pJG16 was observed (data not shown). Our results show that the formation of standard OLs is required for the formation of OLs hydroxylated on the external, ester-linked fatty acyl residue (2-OH-OL) as well as for the formation of OlsD-derived OLs in which the internal, amide-linked fatty acyl residue bears an extra hydroxyl group (*N*-acyl-OH-OL) as we show later.

Modification of OLs by OlsD Occurs under Acidic Conditions. Distinct environmental stresses can provoke hydroxylations of standard membrane lipids.[3,8,11,31] An increase in the growth temperature of *B. cenocepacia* J2315 over the range of 29−42 °C led to increased proportions of 2-OH-OL and 2-OH-PE

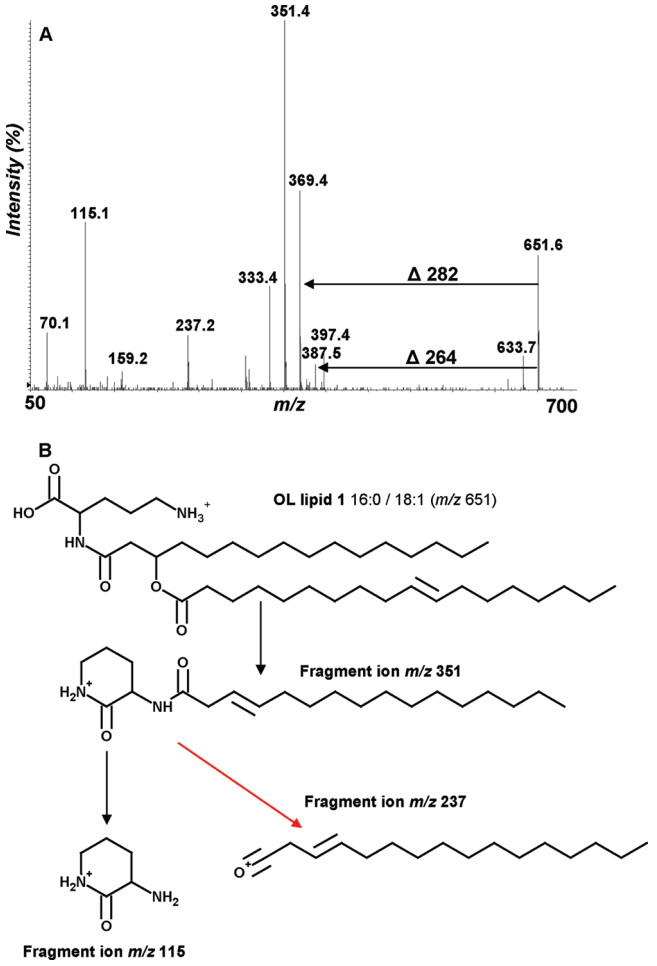

Figure 4. Positive ion mode product ion mass spectrum of OL lipid 1 (m/z 651) (A) and proposed fragmentation scheme for OL (B). The step shown with the red arrow shows the loss of the newly formed unsaturated internal fatty acyl residue from ornithine as an acylium cation, producing the proposed diagnostic fragment ion that has one more degree of unsaturation than the original internal fatty acid residue. The cyclic structures and protonation sites shown are consistent with similar OL positive ion structures presented in the literature.[32,33] Peak intensities are normalized to the most intense fragment ion.

(data not shown), similar to those reported previously for *B. cepacia* NCTC 10661.[3] When *B. cenocepacia* J2315 was grown in complex medium at pH 4 instead of in unbuffered LB medium at neutral pH, two new minor compounds were formed that migrated like NL1 and NL2 on 2D-TLC (Figure 3A). In contrast, neither NL1 nor NL2 was formed when OlsD-deficient mutant AQ3 was grown in complex medium at pH 4 (Figure 3B). Therefore, in *B. cenocepacia* J2315, the formation of NL1 and NL2 under acidic conditions depends on an intact *olsD* gene, with NL1 and NL2 each amounting to ~1% of the total membrane lipids (data not shown).

OlsD in *B. cenocepacia* Produces OH-PE and OH-OL; PG and CL Are Not Hydroxylated. Individual lipids formed by *B. cenocepacia* J2315 × pSphx04 were separated, extracted, and concentrated from preparative 2D-TLC plates (see Experimental Procedures), and fractions corresponding to lipids PE, 2-OH-PE, PG, CL, OL, and NL1, the mixture of 2-OH-OL and NL2, and the complete lipid extracts of some burkholderial or sinorhizobial strains were examined using mass spectrometry. Although the acyl chain substitutions of *B. cenocepacia* PG and CL were found to be similar to those of PE, clearly 2-OH-PE was substituted with hydroxylated fatty acyl chains at the *sn*-2 position (see Figure S1 of the Supporting Information).

New MS Fragmentation of OLs Aids in Structural Assignment. The positive ESI mass spectrum of lipid spot OL (Figure S2A of the Supporting Information) contains ions for at least five distinct species ($M + H^+$ at m/z 651, 665, 667, 625, and 615). The tandem mass spectrum of OL with an ion at m/z 651 (Figure 4A) contains fragment ions in the low-m/z region consistent with an OL lipid (m/z 115 = ornithine b_1 ion, m/z 133 = ornithine protonated molecule, m/z 70 = ornithine immonium ion − NH_3). The fragment ions at m/z 369 and 387 correspond to the elimination and direct cleavage, respectively, of a C18:1 acyl chain, with m/z 351 corresponding to the elimination product minus H_2O (Figure 4B). These data are characteristic of an *N*-acyl-OL in which a C16:0 hydroxy fatty acid is amide-bound to ornithine, which is ester-linked to a C18:1 fatty acid.

Upon interpretation of a large number of different OL tandem mass spectra, an additional, characteristic, fragmentation of the amide-bound hydroxy fatty acid residue was consistently observed (regardless of the acyl chain structures) that has to the best of our knowledge not been described before. This ion is proposed to be formed following elimination of the esterified fatty acid side chain; this results in a new double bond within the amide-bound chain (Figure 4B). Upon further fragmentation (indicated in Figure 4B by the red arrow), this now unsaturated chain is lost from the ornithine as an acylium cation, producing a fragment ion that now has a double bond marking the position of the hydroxyl group of the original residue (Figure 4B). For example, in the fragmentation spectrum of OL with an ion at m/z 651 (Figure 4A) (assigned a C16:0 hydroxyacyl amide-bound moiety on the basis of the other fragment ions observed), an ion at m/z 237 that is formed from the cleavage of the original amide-bound C16:0 hydroxy fatty acid as a C16:1 acylium cation is observed. This new fragmentation pathway has proved to be invaluable for supporting the assignment of OL structures from their product ion spectra, especially because many of the OL product ion spectra are more complicated than that of the ion at m/z 651, due to the presence of a mixture of isobaric species. In this way, the results of the tandem MS analyses of the OL lipids with $M + H^+$ ions at m/z 651, 665, 667, 625, and 615 suggest that in all five lipids, a C16:0 hydroxy fatty acyl is the amide-linked chain, while the ester-linked chains are C18:1 (for m/z 651), C19:1 (for m/z 665), C19:0 (m/z 667) (Figure 5A), C16:0 (m/z 625), and C13:0 (m/z 615), which seems to be an OL−methanol adduct (data not shown). It is worth noting that this fragmentation can also be observed in spectra of other workers,[33] although in that publication its origin was not recognized because only one OL was described in detail, and the fatty acids on that structure made it impossible to distinguish; our study in which so many different structures were analyzed made it possible to identify this useful diagnostic ion.

OlsD Is Responsible for Hydroxylating the Fatty Acyl Residue That Is Amide-Bound to Ornithine in *B. cenocepacia*. During the 2D-TLC separation of the total lipid extract, new lipid 1 (NL1) migrated in a manner analogous to that of the altered OL (NL) observed in the study of *S. meliloti* carrying the *olsD* gene. NL1 isolated via TLC was analyzed using positive mode ESI MS (Figure S2B of the Supporting Information) and product ion experiments; ions at m/z 631, 667, 681, and 683 were identified as OLs, each of which is 16 Da higher in mass than the OLs identified from the OL spot extract (Figure S2A of the

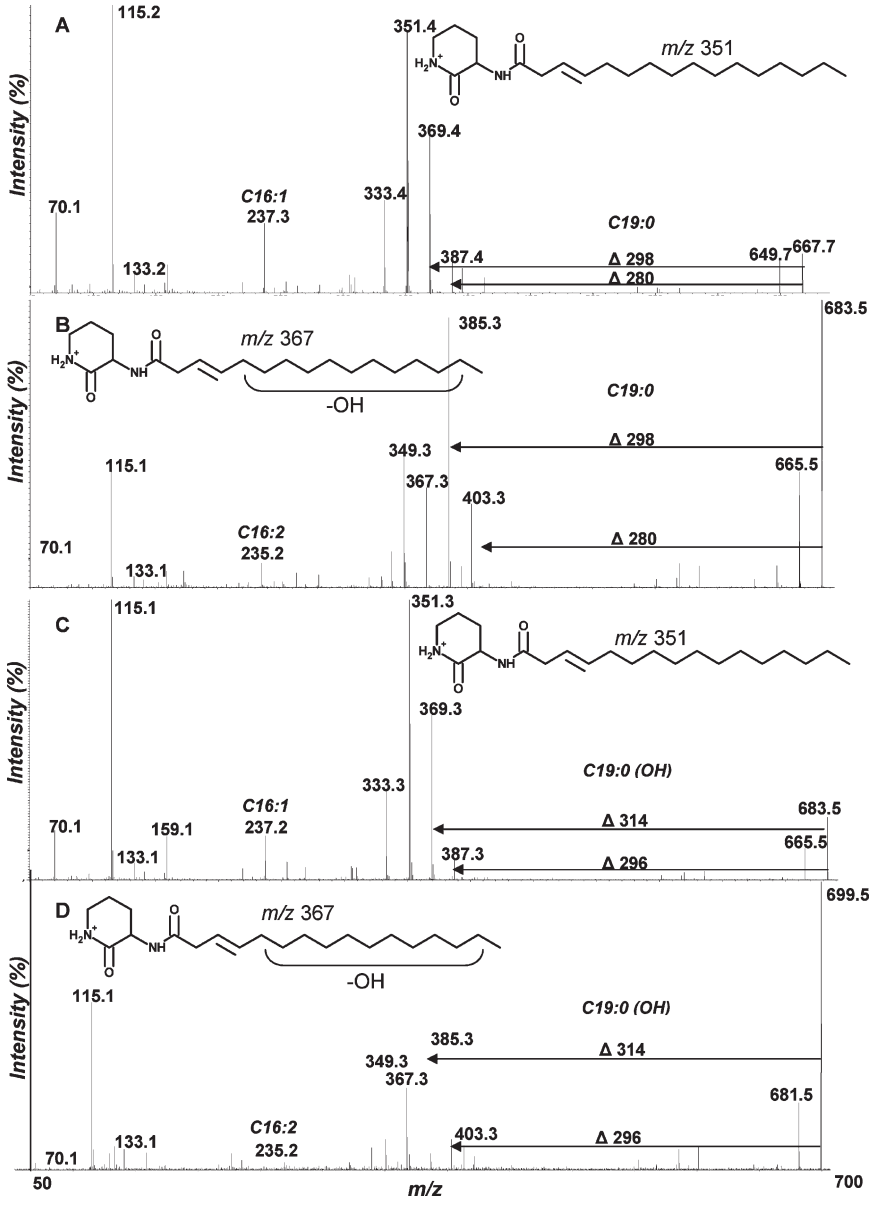

Figure 5. Positive mode product ion mass spectra of the ions at *m*/*z* 667 from OL spot extract (A), *m*/*z* 683 from NL1 spot extract (B), *m*/*z* 683 from 2-OH/NL2 spot extract (C), and *m*/*z* 699 from 2-OH-OL/NL2 spot extract (D). Peak intensities are normalized to the most intense fragment ion.

Supporting Information), consistent with hydroxylated versions of the OLs characterized previously. The tandem mass spectrum of *m*/*z* 683 (Figure 5B) contains fragment ions in the low-*m*/*z* region (*m*/*z* 70, 115, and 133) for an OL lipid and ions at *m*/*z* 385 and 403 for the elimination and direct cleavage of the ester-bound C19:0 acyl chain. The ion at *m*/*z* 235 corresponds to the mass of a C16:2 acylium cation derived from the amide-bound residue by elimination of both its OH substituents. These results identify an *N*-acyl-OL in which a C16:0$(OH)_2$ dihydroxy fatty acid is amide-bound to ornithine, which in turn is ester-linked via one of its hydroxyl groups to a C19:0 fatty acid. This result is consistent with an OL lipid with an $M + H^+$ ion at *m*/*z* 683 being the hydroxylated version of the previously characterized OL lipid with an $M + H^+$ ion at *m*/*z* 667, with the additional hydroxylation on the amide-linked hydroxy fatty acid residue (compare the product ion spectra in panels A and B of Figure 5).

Similar analysis of the product ion spectrum of the ion at *m*/*z* 681 (not shown) showed that it corresponds to a hydroxylated version of OL lipid 2 (*m*/*z* 665), again with the hydroxylation on the internal fatty acid residue. The product ion spectrum of the ion at *m*/*z* 667 (OL lipid 11) is consistent with a mixture of two isobaric OLs: OL [16:0$(OH)_2$/18:1] corresponding to the hydroxylated version of OL lipid 1 (*m*/*z* 651) and an OL that is not additionally hydroxylated [16:0(OH)/19:0].

The signal in the spectrum of the NL1 spot extract at *m*/*z* 631 (OL lipid 8) corresponds to the hydroxylated version of OL lipid 7 (*m*/*z* 615). OL lipids 7 and 8 correspond to methanol adducts, presumably formed in the electrospray interface of protonated species with ions at *m*/*z* 599 and 583. Fragmentation is consistent with the hydroxylation being present on the amide-bound hydroxy fatty acid (data not shown).

OlsD Also Hydroxylates the OL Amide-Bound Fatty Acyl Residue in *S. meliloti*. MS analysis of lipids from *S. meliloti*

1021 × pJG16 yielded an intense $[M + H]^+$ ion at m/z 693, together with a less intense ion at m/z 679. The product ion spectrum of the ion at m/z 693 yielded an intense ion at m/z 397, and a much less intense ion at m/z 415 for elimination and direct cleavage of a C19:1 fatty acid, and ornithine-derived fragment ions were observed at m/z 70, 155, and 133. Product ion analysis of the ion at m/z 679 also yielded an ion at m/z 155 for ornithine (data not shown). These results are identical to those reported previously.[5] The generation of ions by the facile loss of only a C19:1 fatty acid is consistent with an *N*-acyl-OL in which a C18 hydroxy fatty acid is amide-bound to ornithine, and to which a C19:1 fatty acid is esterified to the hydroxyl group of the *N*-C18 fatty acid.[5] Analogous ions were observed in the CID mass spectrum obtained from the ion at m/z 679, consistent with it bearing an ester-linked C18:1 fatty acid on an *N*-C18 hydroxyornithine.

Comparison of the mass spectrometric data of *S. meliloti* × pSphx03 lipids with those of the lipid extract from the control strain (*S. meliloti* × pGJ16) shows the presence of ions similar to those mentioned above. This suggests that the *B. cenocepacia* J2315 *bcam1214* gene that was more similar to the LpxO dioxygenase from *S. typhimurium* does not cause the formation of any additional extractable lipid by the method used.[27] In contrast, when we analyzed the mass spectra of lipids extracted from *S. meliloti* × pSphx04, we detected the presence of an additional ion at m/z 709 (Figure S3A of the Supporting Information), which was not detected in *S. meliloti* strains harboring plasmid pJG16 or pSphx03 (data not shown). The ion at m/z 709 (m/z 693 + 16) is consistent with a hydroxylated version of the ion at m/z 693. Product ion analysis of the ion at m/z 709 yielded ions at m/z 413 and 431 for elimination and direct cleavage of a C19:1 fatty acid (Figure S3B of the Supporting Information). Significantly, this analysis suggests that it is again the amide-bound C18 fatty acyl chain that is additionally hydroxylated in OLs of *S. meliloti* and not the ester-linked C19:1 residue. Therefore, the site of OlsD-derived hydroxylation of *S. meliloti* OL is analogous to that in OLs extracted from *B. cenocepacia* carrying the *olsD* gene.

OlsD in *B. cenocepacia* Also Causes Hydroxylation of the Amide-Linked Fatty Acyl Chain in the Presence of a Hydroxylated Esterified Fatty Acid. It proved to be impossible to extract the 2-OH-OL and NL2 spots independently. The ESI mass spectrum of the extract of the combined 2-OH-OL and NL2 spots (Figure S2C of the Supporting Information) appears to be very similar to that of the NL1 extract, with signals observed at m/z 631, 667, 681, and 683. Product ion analysis of the ion at m/z 683 (OL lipid 17) (Figure 5C) was consistent with an OL in which a C16:0 hydroxy fatty acid is amide-bound to ornithine (confirmed by the presence of the C16:1 acylium ion), which in turn is ester-linked via its hydroxyl group to a C19:0 (OH)-fatty acid, confirming that OL 17 (m/z 683) is the hydroxylated version of the previously characterized OL lipid 3 (m/z 667). However, unlike lipid 11 (Figure 5B), which is additionally hydroxylated on the amide-bound hydroxy fatty acid, the extra hydroxyl group in the *B. cenocepacia* lipid is now positioned on the external ester-linked fatty acyl residue. Product ion analysis of the ion at m/z 681 (OL lipid 16) shows that it corresponds to hydroxylated OL 2 (m/z 665), again with the hydroxylation group on the ester-bound residue. Similarly, the ion at m/z 641 corresponds to hydroxylated OL 5 (m/z 625), again with the hydroxyl group on the external residue, although the hydroxylated amide-bound fatty acid version of OL 5 was not detected in the NL1 extract, probably because of the very small amount of this lipid (apparent from the low abundance of this signal in the spectra of the different extracts). The mass spectrometric data of OL lipid 15 (m/z 667) are consistent with it being hydroxylated OL lipid 1 (m/z 651) with the hydroxyl group on the ester-linked fatty acid residue. However, because an 18:1(OH) and a 19:0 fatty acyl residue are isobaric, it is impossible to distinguish, solely on the basis of these MS data, between these two possible structures for OL 15. However, OL 15 migrates on TLC with OLs with hydroxylated ester-linked fatty acids, and no unhydroxylated species were detected in the 2-OH-OL/NL2 extract.

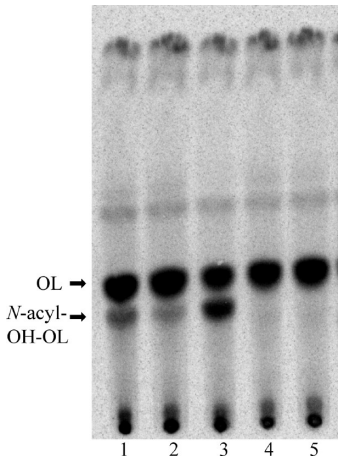

Figure 6. Membrane-associated OlsD dioxygenase converts OL to OL that is hydroxylated in the amidified acyl residue. Cell-free extracts of *E. coli* BL21(DE3) pLysS pNG40 expressing OlsD (lane 1), BL21(DE3) pLysS containing the empty pET16b vector (lane 4), the soluble protein fraction from *E. coli* BL21(DE3) pLysS pNG40 (lane 2), the membrane fraction from *E. coli* BL21(DE3) pLysS pNG40 (lane 3), and buffer only (lane 5) were incubated with 8.45 nmol of $[^{14}C]$OL (3072 cpm/nmol) and other soluble cofactors for 2 h as described in Experimental Procedures. OlsD enzyme assays usually contained a final protein concentration of 1 mg/mL. After extraction, radiolabeled lipids were analyzed by one-dimensional TLC using a chloroform/methanol/acetic acid mixture (130:50:20, v/v/v) as a solvent system and detected and quantified with a phosphorimager. Unmodified OL and OL that is hydroxylated at the amidified acyl residue (*N*-acyl-OH-OL) are indicated.

Intriguingly, ions at m/z 697 (OL lipid 18) and m/z 699 (OL lipid 19) in the spectrum of the 2-OH-OL/NL2 extract from *B. cenocepacia* are 16 m/z units higher than those for OLs 16 (m/z 681) and 17 (m/z 683), respectively (Figure S2C of the Supporting Information), and 32 m/z units higher than OLs 2 (m/z 665) and 3 (m/z 667), respectively, in the OL extract. OLs 16 and 17 have been shown to be hydroxylated on their ester-linked fatty acids. The observation of "+16" OLs 16 and 17 suggests the existence of hydroxylated versions of OLs 16 and 17 (i.e., doubly hydroxylated forms of OLs 2 and 3, respectively). Product ion analysis of OL 19 (m/z 699) (Figure 5D) shows that the lipid carries additional hydroxyl groups on both the amide-linked [C16:0$(OH)_2$] and ester-linked [C19:0(OH)] fatty acids. Similarly, OL 18 (m/z 697) was shown to be a hydroxylated version of OL 16 and a doubly hydroxylated version of lipid 2.

OlsD Dioxygenase Modifies the Amidified Fatty Acyl Residue of OL in Vitro. To obtain more OlsD-modified OL for structural analysis, we developed an enzymatic assay for the OlsD activity. Different *E. coli* cell-free extracts were incubated with ornithine-containing lipid, and the products were analyzed. When $[^{14}C]$OL was incubated with a cell-free extract of *E. coli*

harboring OlsD-expressing pNG40, the formation of a compound (*N*-acyl-OH-OL) that migrated like NL or NL1 was observed (Figure 6, lane 1). When [^{14}C]OL was incubated with a cell-free extract of *E. coli* harboring the empty pET16b plasmid, no formation of *N*-acyl-OH-OL was observed (Figure 6, lane 4), as when the incubation was performed with buffer only (Figure 6, lane 5). Separation of the cell-free extract by ultracentrifugation showed that most of the OlsD dioxygenase was associated with the membrane fraction (Figure 6, lane 3) and minor activity with the soluble protein fraction (Figure 6, lane 2). Analysis of the OlsD protein sequence suggests that, in contrast to LpxO,[9] the OlsD enzyme is devoid of membrane-spanning α-helices (data not shown) that might explain the only loose association of OlsD with the membrane fraction. The OlsD dioxygenase has a pH optimum of ~7.5 and works best at a detergent concentration of 0.04% Triton X-100 (Figure S4 of the Supporting Information). Even in the absence of detergent, significant OlsD activity is detectable (Figure S4B of the Supporting Information), again suggesting that a significant part of OlsD might be in the soluble protein fraction. The OlsD enzyme activity is strictly dependent on the addition of the second substrate α-ketoglutarate (Figure S5A of the Supporting Information), whereas in the absence of ascorbate, OlsD activity is only reduced (Figure S5A). Omission of DTT surprisingly causes an increase in OlsD activity (Figure S5A). Omission of Fe^{2+} from the complete assay does not cause a reduction in OlsD activity (Figure S5A), and the dependence on Fe^{2+} is detected only after this metal ion is chelated with 2,2′-bipyridyl (Figure S5B of the Supporting Information). Therefore, we conclude that OlsD is a Fe^{2+}/α-ketoglutarate-dependent mixed-functional dioxygenase.

DISCUSSION

Lipid A-containing lipopolysaccharides (LPS) usually occupy the outer surface of the outer membrane in Gram-negative bacteria and pose a major permeability barrier for hydrophilic and hydrophobic compounds. Hydrocarbon regions of the outer membrane are thought to be in a gel-like state of very low fluidity under physiological conditions[31] because of strong interactions between the lipid molecules. Different environments and stresses require adjustments in the outer membrane that are realized by certain chemical modifications of LPS and/or other lipids composing the outer membrane,[31,34] i.e., PE, sphingolipids, sulfonolipids,[35] and OLs.[11,34] Introduction of an additional hydroxyl group into fatty acyl residues of membrane lipids increases the likelihood of hydrogen bonding with neighboring molecules and thus reduces the fluidity of the membrane. With all of the outer membrane lipids mentioned above, the strategy of decorating their fatty acyl residues with hydroxyl groups at the 2 and/or 3 position, and thereby stabilizing the membrane, is used. In addition to these non-glycerol-based membrane lipids, the glycerophospholipid PE, which is enriched in the outer membrane, can be 2-hydroxylated on its *sn*-2-located fatty acyl residue in *Burkholderia*.

In *B. cepacia* NCTC 10661, the levels of the 2-hydroxylated lipids 2-OH-OLs and 2-OH-PE are increased at high temperatures,[3] conditions that could be unfavorable for the formation and stabilization of monolayers of LPS, due to electrostatic repulsion between highly acidic molecules of LPS, probably altering the permeability of the external membrane.[31] The hydroxylated OLs could substitute for LPS, with the hydroxylations allowing the establishment of lateral interactions such as hydrogen bond formation to stabilize the external membrane.[31] There is evidence that the presence of OLs in membranes allows bacteria to survive in unfavorable environments. For example, OLs contribute to increased cell yields under phosphorus-limiting growth conditions[36] and to increased resistance to acidic conditions.[10,11] In *Rhodobacter capsulatus*, OLs are required for optimal steady-state amounts of *c*-type cytochromes.[37] Although we found that the OlsD-dependent modification occurs under acidic growth conditions, the exact roles of the distinct OLs in *B. cenocepacia* are not clear. Transcriptome studies suggest that the *olsB* (*bcal1281*) transcript is upregulated 4.3-fold after growth of *B. cenocepacia* J2315 in cystic fibrosis sputum instead of in a minimal medium,[38] whereas *olsD* (*bcam2401*) is downregulated in *B. cenocepacia* J2315 biofilms by oxidative stress (2.2-fold by H_2O_2 or 3.4-fold by NaOCl treatment).[39] Another study reports 3-fold increased *olsD* and 4-fold increased *olsB* transcript levels after growth of *B. cenocepacia* J2315 in a soil extract-containing medium when compared to cystic fibrosis sputum-containing medium.[40]

Previous reports have indicated that members of the *Burkholderia* genus produce two different classes of OLs, standard nonhydroxylated OL and OL hydroxylated at the 2 position of their external ester-linked fatty acyl residue (2-OH-OL). Like *Burkholderia*, members of the genera *Flavobacterium*,[41,42] *Thiobacillus*,[4] *Gluconobacter*,[43] *Ralstonia*,[44] and *Rhizobium*[11] produce 2-OH-OLs. We now report on an additional possible modification of OL that is caused by OlsD and to date has not been known to occur in nature. Expression of OlsD in *S. meliloti* or in *B. cenocepacia* causes hydroxylation of the amide-linked OL fatty acyl residue. Introduction of the *olsD* gene into *S. meliloti* 1021 caused the formation of one new lipid (NL) and introduction into *B. cenocepacia* J2315 two new lipids (NL1 and NL2). The results suggest that OlsD might use the preexisting OLs of *S. meliloti* 1021 and *B. cenocepacia* J2315 as substrates. This is consistent with a model based on the results obtained via MS. The predicted dioxygenase encoded by *olsD* converts the standard OL form (protonated molecule at *m*/*z* 693) to NL (*m*/*z* 709) in *S. meliloti* 1021, and in *B. cenocepacia* J2315, OlsD converts the standard OL (*m*/*z* 667) to NL1 (*m*/*z* 683) and 2-OH-OL (*m*/*z* 683) to NL2 (*m*/*z* 699). The MS data also show that spot NL2 contains dihydroxylated OLs with one additional hydroxyl group on the ester-linked fatty acid residue and the other on the amide-linked residue.

The introduction of *bcam2401* (*olsD*) into *S. meliloti* or *B. cenocepacia* causes hydroxylation of the amide-linked fatty acyl residue of OLs, and OlsD is not required for the formation of 2-OH-OL. Introduction of *bcam1214* into *S. meliloti* or *B. cenocepacia* did not lead to the formation of any additional extractable lipid by the method used.[27] This suggests that the *B. cenocepacia* J2315 BCAM1214 with more similarity to the LpxO-encoded dioxygenase from *S. typhimurium* does not conduct the 2-hydroxylation on the ester-linked fatty acid residues of OLs to yield 2-OH-OLs. Although the reported structure of lipid A of *B. cenocepacia* J2315 does not indicate a 2-hydroxy modification at any of its acyl residues,[45] it seems likely that the *bcam1214* gene is involved in modifying lipid A of *B. cenocepacia* J2315, because of its high degree of similarity to the LpxO enzyme from *S. typhimurium*. However, as in the case of LpxO-catalyzed hydroxylation in *S. typhimurium*, such a BCAM1214-catalyzed modification of burkholderial lipid A might occur only under certain stress conditions. Although our postgenomic approach of predicting biochemical functions based on protein similarities has led to the discovery of this new pathway for OL

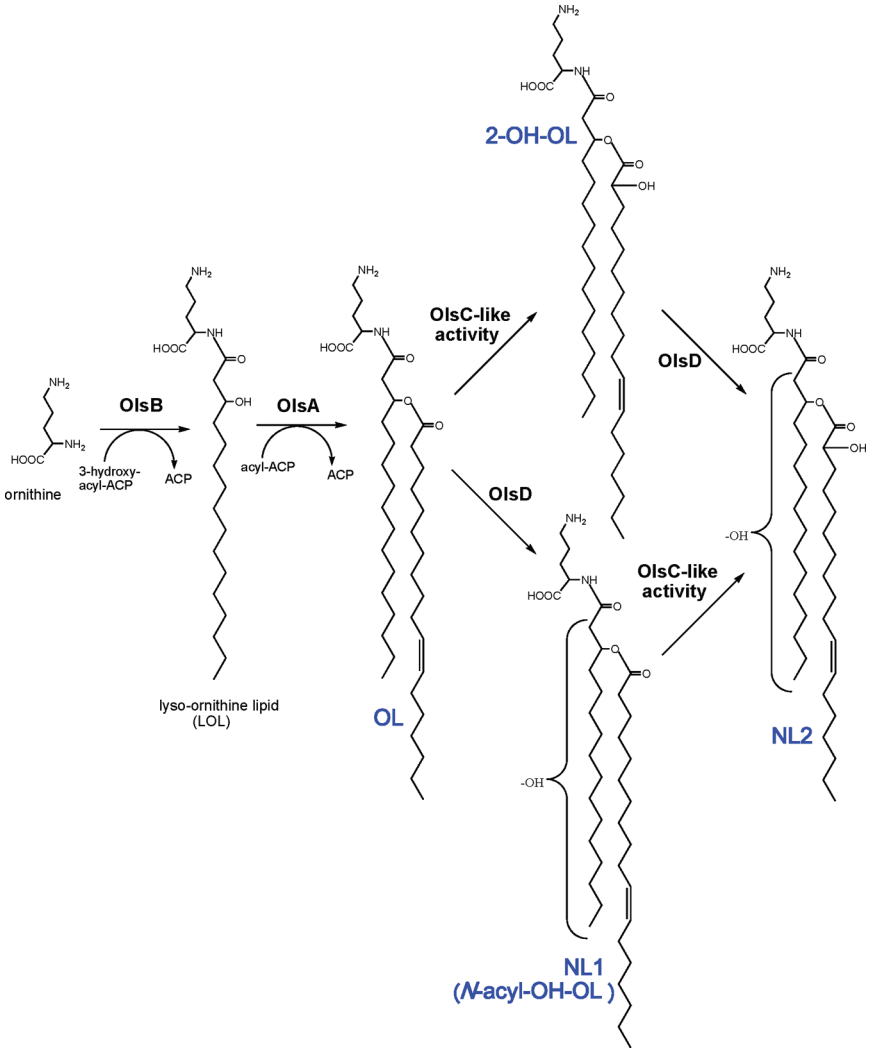

Figure 7. Biosynthesis of OLs in *B. cenocepacia* J2315. The genes encoding OlsB and OlsA have first been identified in *S. meliloti*, whereas the gene encoding the OL 2-hydroxylase OlsC has been described in *R. tropici*. We show that *bcal1281* from *B. cenocepacia* encodes the OlsB *N*-acyltransferase that has an acyl chain preference for a 3-hydroxypalmitoyl. The *O*-acyltransferase OlsA is probably encoded by *bcal3137* in *B. cenocepacia*. There is no homologue to the rhizobial OlsC in *B. cenocepacia* that hydroxylates OL at the 2 position of the esterified acyl residue; however, an OlsC-like activity must exist in *B. cenocepacia*. Here we describe that the hydroxylation introduced by the *bcam2401*-encoded OlsD occurs on the internal, amidified fatty acid.

modification (hydroxylation of the amidified acyl residue of OLs), we have not yet found the gene(s) or enzyme(s) responsible for the formation of 2-OH-OLs and 2-OH-PE. Therefore, at this point, the *B. cenocepacia* genes and/or enzymes that produce the 2-hydroxy group on the *sn*-2-linked acyl chain of PE or on the ester-linked fatty acyl chain of OL are still unknown; however, in *B. cenocepacia*, a close LpxO homologue is probably not involved in this process. Notably, in *R. tropici* CIAT899, the enzyme generating the 2-hydroxylation of 2-OH-OLs is the OlsC dioxygenase,[11] but OlsC does not cause the formation of 2-OH-PE. Recently, a search with the iron-binding motif of LpxO-like dioxygenases led to the identification of the Kdo 3-hydroxylase KdoO that hydroxylates the outer Kdo unit of lipopolysaccharide in *Burkholderia ambifaria* and *Yersinia pestis*.[46] However, the KdoO protein shows no overall sequence similarity to LpxO, OlsC, or OlsD and therefore is a distant relative within the $Fe^{2+}/O_2/\alpha$-ketoglutarate-dependent dioxygenase superfamily.

Currently, it is not known how the different LpxO-like dioxygenases (LpxO, OlsC, and OlsD) recognize their substrate acyl chain on distinct membrane lipids. LpxO of *S. typhimurium* (LpxOSt) hydroxylates exclusively the myristate moiety at position 2 that is linked to the 3′-(*R*)-3-hydroxymyristate chain of lipid A.[9] *Pseudomonas aeruginosa* contains two LpxO orthologs (LpxO1PAO1 and LpxO2PAO1), and both secondary acyl chains of its lipid A are modified with 2-OH groups.[47] Probably each of these orthologs is responsible for hydroxylating one of the two secondary acyl chains of *P. aeruginosa* lipid A.[9] LpxOSt, LpxO1PAO1, LpxO2PAO1, BCAM1214 of *B. cenocepacia*, and many other LpxO-like dioxygenases form a clearly distinct subfamily (Figure S6 of the Supporting Information) of dioxygenases that probably hydroxylate lipid A at the 2 position of distinct acyl chains. Two other LpxO-like dioxygenase subfamilies exist (Figure S6 of the Supporting Information), one represented by OlsC and the other represented by OlsD. The OlsC-like dioxygenase subfamily exists in several α-proteobacteria (Figure S6 of the Supporting Information) and modifies OLs by hydroxylation of the 2 position of the esterified fatty acyl residue.[11] In contrast, the OlsD-like dioxygenase subfamily exists

exclusively in species of the genera *Burkholderia* and *Serratia* (Figure S6 of the Supporting Information) and was shown in this work to cause hydroxylation of the amide-linked fatty acyl residue of OLs.

Spot NL1 produced by *B. cenocepacia* (Figure 2F) shows the same mobility on 2D-TLC as spot NL observed from *S. meliloti* after expression of *olsD* and $olsB_{Sm}$ (Figure 1B). However, the mass spectrometric data indicate that in the case of *B. cenocepacia* it is always a C16:0-amidified acyl residue that is hydroxylated by OlsD (Figure 7), whereas in the case of *S. meliloti*, the amidified acyl residue hydroxylated by OlsD is a C18:0. Analysis of the standard OL fractions from both organisms also indicated that standard OLs from *B. cenocepacia* have 3-hydroxypalmitate and *S. meliloti* 3-hydroxystearate as amidified acyl residues. In contrast, there is significant heterogeneity with respect to the esterified acyl residues in the two organisms. This observation might imply that OlsA-encoded *O*-acyltransferases[7] are not highly selective with regard to the chain lengths of the acyl residues linked to acyl carrier proteins (ACPs) in acyl-ACP acyl donor substrates. OlsA from *Rhodobacter capsulatus* seems to be able to acylate 1-acyl-*sn*-glycerol-3-phosphate in addition to lyso-ornithine lipid[48] and therefore exhibits relaxed substrate specificity toward the acyl acceptor substrate, as well. In contrast, OlsB *N*-acyltransferases, which catalyze the first step in the biosynthesis of ornithine-containing lipids (Figure 7), might be highly selective with regard to their acyl residues linked to ACPs. We suggest that OlsB from *B. cenocepacia* might use predominatly 3-hydroxypalmitoyl-ACP as the acyl donor substrate whereas OlsB from *S. meliloti* may prefer 3-hydroxystearyl-ACP. A strict selectivity for chain length of the acyl residue has been reported for some LpxA *O*-acyltransferases performing the initial acylation step during lipid A biosynthesis.[49]

■ ASSOCIATED CONTENT

Supporting Information. Cloning of burkholderial genes, construction of *B. cenocepacia* J2315 mutants, additional MS analysis of membrane lipids from *B. cenocepacia* J2315 and *Sinorhizobium meliloti* 1021, and enzymatic and phylogenetic characterization of the OlsD dioxygenase. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +52-7773-131697. Fax: +52-7773-175581. E-mail: otto@ccg.unam.mx.

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■ ABBREVIATIONS

BCC, *B. cepacia* complex; CL, cardiolipin; DGTS, diacylglyceryltrimethylhomoserine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Homopipes, homopiperazine-*N*,*N*′-bis(2-ethanesulfonic acid); LB, Luria-Bertani; MS, mass spectrometry; NL, new lipid; OD, optical density; OL, ornithine-containing lipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; P_i, inorganic phosphate; TLC, thin layer chromatography; TY, tryptone/yeast extract; 2D, two-dimensional; 2-OH, hydroxylation at the 2 position of an acyl residue.

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