Supporting Information for:

The dioxygenase-encoding *olsD* gene from *Burkholderia cenocepacia* causes the hydroxylation of the amide-linked fatty acyl moiety of ornithine-containing membrane lipids

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Supporting Experimental Procedures

Cloning of genes from B. cenocepacia J2315 bcam1214, bcam2401 (olsD), and bcal1281 (olsB$_{Bc}$). The oligonucleotide primers oLOP23 (AGGAATACATATGCGCTGGGTCCTGCTG) and oLOP24 (AAAGGATCCTCAGATACAGAAGATCGCGAC), introducing *Nde*I and *Bam*HI sites (underlined), respectively, were used in the PCR to amplify the gene *bcam1214*. The oligonucleotide primers oLOP25 (AGGAATACATATGCCCGTCGCGCTTCG) and oLOP26 (AAAAGATCTCTACACCGGAATCGACCG) introducing *Nde*I and *Bgl*II sites (underlined), respectively, were used in the PCR to amplify the *olsD (bcam2401)* gene. In all PCR reactions, genomic DNA from *B. cenocepacia* J2315 was used as a template. After digestion of the PCR products with the corresponding enzymes, the fragments obtained were cloned in the plasmids of the pET vector family that had been digested with the same enzymes. The gene *bcam1214* was cloned in pET9a resulting in plasmids pSphx01 whereas *olsD* was cloned in pET9a and pET16b resulting in plasmids pSphx02 and pNG40, respectively. The gene *olsB$_{Bc}$* was previously cloned in pET17b to yield the plasmid pNG23 (*1*). Sequencing analysis confirmed that pSphx01 carries the DNA fragment coding for BCAM1214, pSphxO2 and pNG40 carry the DNA fragment coding for OlsD. The pET9a- and pET17b-derived plasmids were linearized with *Bgl*II and each was cloned in the broad-host-range plasmid pRK404 that had been digested with *Bam*HI to obtain plasmids pSphx03, pSphx04, and pNG24 and they were verified to carry *bcam1214*, *olsD*, and *olsB$_{Bc}$*, respectively. A plasmid (pNG28) that was lacking the *olsB$_{Bc}$* gene was constructed by cloning *Bam*HI-restricted pET17b into pRK404.

Inactivation of olsD (bcam2401) by a chloramphenicol resistance-conferring cassette. The chloramphenicol acetyltransferase (CAT) gene of pBBR1MCS was amplified with the oligonucleotide primers oLOP27 (AAAGGATCCACGCGTATCCTGGTGTCCCTG) and oLOP28 (AAAGGATCCACGCGTCCACACAACATACGAG) that introduced *Bam*HI

restriction sites and cloned into pUC18 to yield the plasmid pCAT. The oligonucleotide primers oLOP31 (AAATCTAGAAGATCGTCGGCAGCTG) and oLOP32 (AAAGGATCCGGATGAAGTGCTGGTC) were used in the PCR to amplify about 1.2 kb of genomic DNA upstream of *olsD* of *B. cenocepacia* J2315, introducing *Xba*I and *Bam*HI sites (underlined) into the PCR product. Similarly, the primers oLOP33 (GCTGGGGATCCGGATCC) and oLOP34 (AAACTAAGCTTCCGCCGTGACGCGC) were used to amplify about 1.1 kb of genomic DNA downstream of the *olsD* gen, introducing a *Hind*III site (underlined) in oLOP34, while oLOP33 matches the *Bam*HI sites (underlined) present in the genomic DNA. The PCR product amplified with oligonucleotides oLOP33 and oLOP34 was digested with *Bam*HI/*Hind*III and cloned into pBluescriptSK+ that had been digested with the same two enzymes to yield the plasmid pRRL01. Then, plasmid pRRL01 was digested with *Bam*HI and *Xba*I and was ligated together with the *cat* gene obtained with *Bam*HI digestion from pCAT and with the PCR product resulting from the amplification with oLOP31 and oLOP32 that have been digested with *Xba*I and *Bam*HI. The resulting plasmid containing the flanking regions of *olsD* interrupted by a chloramphenicol resistance gene was named pSphx06. The plasmid pSphx06 was digested with *Xba*I/*Hind*III to reclone the regions flanking *olsD* and the chloramphenicol resistance gene located between those regions as a *Xba*I/*Hind*III fragment into the suicide vector pK18*mobsacB* (*2*) to yield pSphx08. Via diparental mating using as donor strain *E. coli* S17-1, pSphx08 was introduced into the wild type *B. cenocepacia* J2315. Transconjugants were selected on LB medium containing chloramphenicol and piperacillin to counterselect against *E. coli*. Genetic analysis by PCR showed that the transconjugants were the result of a single event of recombination and in order to select for colonies in which the pK18*mobsacB* was lost, a selected transconjugant was grown in the presence of sucrose. A single recombinant was first grown under nonselective conditions in complex medium until an OD_{620} of 1.0 was achieved, and was then plated onto LB medium containing 5% (wt/vol) sucrose and chloramphenicol. Several large colonies among a background of small colonies appeared after 8 days and 25 of the biggest colonies were toothpicked on 5 and 10% sucrose and 7 of them grew well on 10% sucrose. The genetic organization of the 7 candidates was analyzed using the oligos oLOP25 and oLOP26 and only two of them were double recombinants. The clone AQ3 was selected for further studies. Southern hydridization analysis also showed that in the clone AQ3, *olsD* has been replaced by a chloramphenicol resistance cassette (data not shown).

Inactivation of $olsB_{Bc}$ (bcal1281) by a chloramphenicol resistance-conferring cassette. The oligonucleotide primers oLOP90 (ATGTTGATATCGCGTGTTCCAGCAAGTTTCG) and oLOP85 (AAAGGATCCTAGGCGTCGGCAGTTCTCG) were used in the PCR to amplify about 1.1 kb of genomic DNA upstream of *$olsB_{Bc}$*, introducing *Eco*RV and *Bam*HI sites (underlined) into the PCR product. Similarly, the primers oLOP86 (AAGGGATCCCGACTTCAACTGC) and oLOP87 (ACTCTCTAGACTGTTCGCGCTCGTTTATTGG) were used to amplify about 1.2 kb of genomic DNA downstream of the *$olsB_{Bc}$* gen, introducing a *Bam*HI site (underlined) in oLOP86 and a *Xba*I site (underlined) in oLOP87. The PCR product amplified with oligonucleotides oLOP90 and oLOP85 was digested with *Eco*RV/*Bam*HI and cloned into pBluescriptSK+ that had been digested with the same two enzymes to yield the plasmid pNG10. The PCR product amplified with oligonucleotides oLOP86 and oLOP87 was digested with *Bam*HI/*Xba*I and cloned into pBluescriptSK+ that had been digested with the same two enzymes to yield the plasmid pNG11. Then, plasmid pNG10 was digested with *Bam*HI and *Xba*I and was ligated with the 1.2 kb DNA fragment obtained after *Bam*HI/*Xba*I digestions of pNG11 to yield plasmid pNG14. Later, plasmid pNG14 was linearized with *Bam*HI and was

ligated with the *Bam*HI fragment from pCAT containing the *cat* gene. The resulting plasmid, containing the flanking regions of $olsB_{Bc}$ interrupted by a chloramphenicol resistance gene, was named pNG15. Plasmid pNG15 was digested with *Eco*RV/*Xba*I to reclone the regions flanking $olsB_{Bc}$ and the chloramphenicol resistance gene located between those regions as a *Eco*RV/*Xba*I fragment into the suicide vector pK18*mobsacB* (*2*) that had been digested with *Sma*I/*Xba*I to yield pNG16. Plasmid pNG16 was introduced into the wild type *B. cenocepacia* J2315 via diparental mating using as donor strain *E. coli* S17-1. Transconjugants were selected on LB medium containing chloramphenicol and piperacillin to counterselect against *E. coli*. A single recombinant was first grown under nonselective conditions in complex medium until the OD_{620} reached 1.0 and then was plated on LB medium containing 10% (wt/vol) sucrose and chloramphenicol. After 8 days of growth in selective medium, the three fastest growing colonies were chosen for genotypic and phenotypic analysis. Southern hydridization analysis showed that in the 3 strains $olsB_{Bc}$ had been replaced by a chloramphenicol resistance cassette (data not shown) and one of them, NG1, was chosen for further studies.

Supporting Results

Glycerophospholipids of B. cenocepacia J2315. The *B. cenocepacia* wild type strain produces two forms of PE differing in the presence or absence of ester-linked 2-hydroxyfatty acids. Furthermore, for PE lipids, the incorporation of the 2-hydroxyfatty acid is known to be specific to the *sn*-2 position in *B. cenocepacia* strains (*3, 4*). In an effort to determine whether the presence of an extra copy of *olsD* alters the observed PE composition and the site of hydroxylation within these lipids, both forms of PE were extracted from the 2D-TLC plates obtained from the *B. cenocepacia* J2315 (pSphx04) lipids. Negative ion mass spectrometric data indicated the presence of a range of PE species (Figure S1A). The PE species giving more abundant MS signals were shown using tandem MS to carry C16:0, C17:1, C18:1, or C19:0 on *sn*-1 and C16:0, C16:1, C17:1, C18:1, or C19:1 on *sn*-2. In the fraction corresponding to 2-OH-PE (Figure S1B), the 2-OH-PE species (PE lipids 16, 19, 20, 21, and 22) giving the more abundant signals were similarly shown to carry C16:0, C17:1, C18:1, or C19:0 on *sn*-1 and C16:0(OH), C17:1(OH), C19:0(OH), or C19:1(OH) on *sn*-2. In some cases, hydroxylated versions of the corresponding PE lipids can be clearly assigned (Figure S1C) and as expected, hydroxylation is specific to the *sn*-2 fatty acyl moiety. However, the hydroxylated versions of PE found are not due to OlsD. Negative ESI mass spectra of PG and CL indicated that the acyl substitutions were similar to those on PE (data not shown). We did not detect any hydroxylated fatty acyl residues in PG or CL.

Supporting References

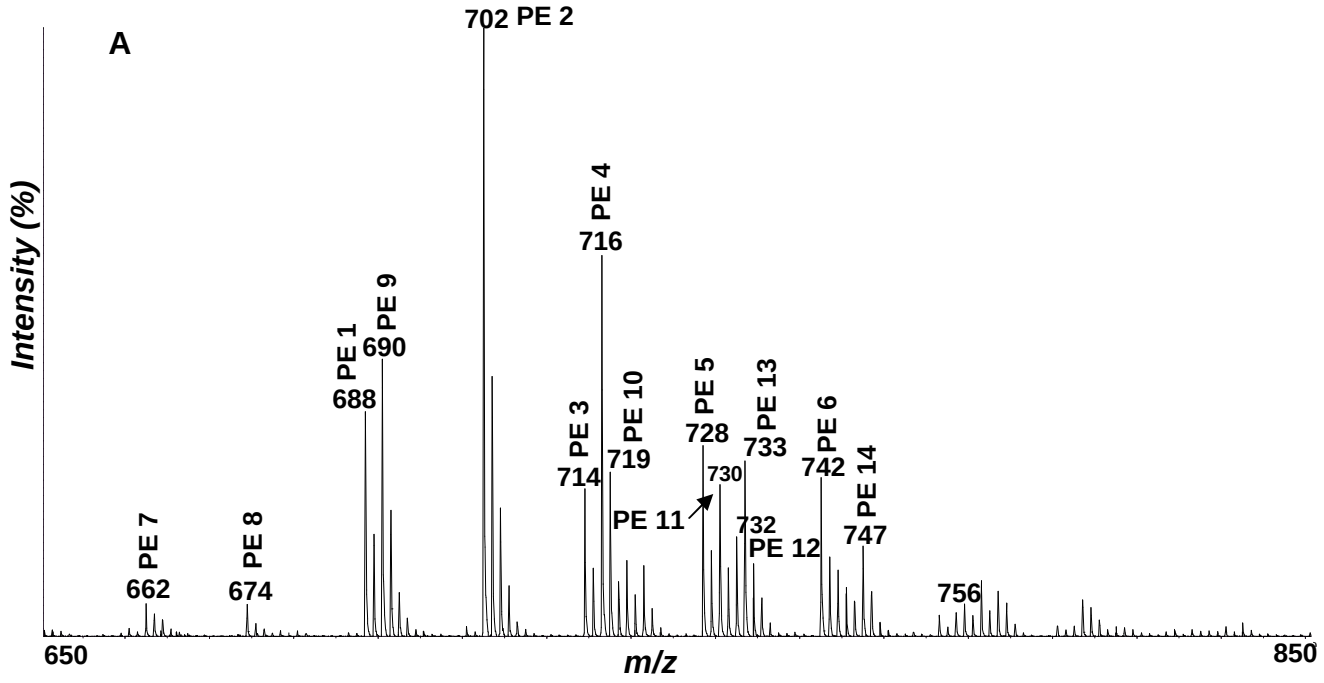
1. Vences-Guzmán, M. A., Guan, Z., Ormeño-Orrillo, E., González-Silva, N., López-Lara, I. M., Martínez-Romero, E., Geiger, O., and Sohlenkamp, C. (2011) Hydroxylated ornithine lipids increase stress tolerance in *Rhizoboium tropici* CIAT899. *Mol. Microbiol. 79,* 1496-1514.

2. Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., and Pühler, A. (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene 145*, 69-73.

3. Phung, L. V., Chi, T. T. B., Hotta, H., Yabuchi, E., and Yano, I. (1995) Cellular lipid and fatty acid compositions of *Burkholderia pseudomallei* strains isolated from human and environment in Viet Nam. *Microbiol. Immunol. 39*, 105-116.

4. Taylor, C. J., Anderson, A. J., and Wilkinson, S. G. (1998) Phenotypic variation of lipid composition in *Burkholderia cepacia*: a response to increased growth temperature is a greater content of 2-hydroxy acids in phosphatidylethanolamine and ornithine amide lipid. *Microbiology 144*, 1737-1745.

Figure S1. Negative ion mass spectra of the extracts of the PE spot (A) or the 2-OH-PE spot (B) from *B. cenocepacia* J2315 x pSphx04. Peak intensity is normalized to the most intense signal in the region displayed. Comparison of some PE and 2-OH-PE structures (C).



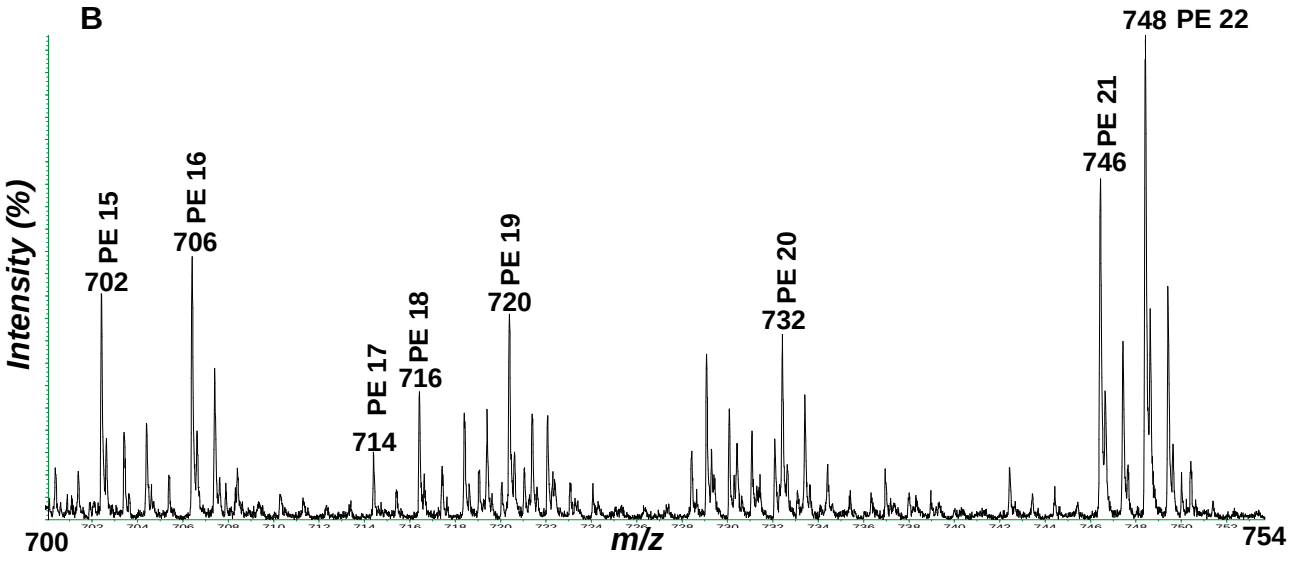


Figure S1 continued:

C

PE lipid #	$[M-H]^-$	Structure	
		sn-2	*sn*-1
9	690	16:0	16:0
16	706	16:0(OH)	16:0
4	716	16:0	18:1
20	732	16:0(OH)	18:1
11	730	19:1	16:0
21	746	19:1(OH)	16:0
12	732	16:0	19:0
22	748	16:0(OH)	19:0

Figure S2. Positive ESI mass spectra of the extracted spots assigned OL (A), NL1 (B), 2-OH-OL/NL2 (C). Peak intensity is normalized to the most intense signal in the region displayed.

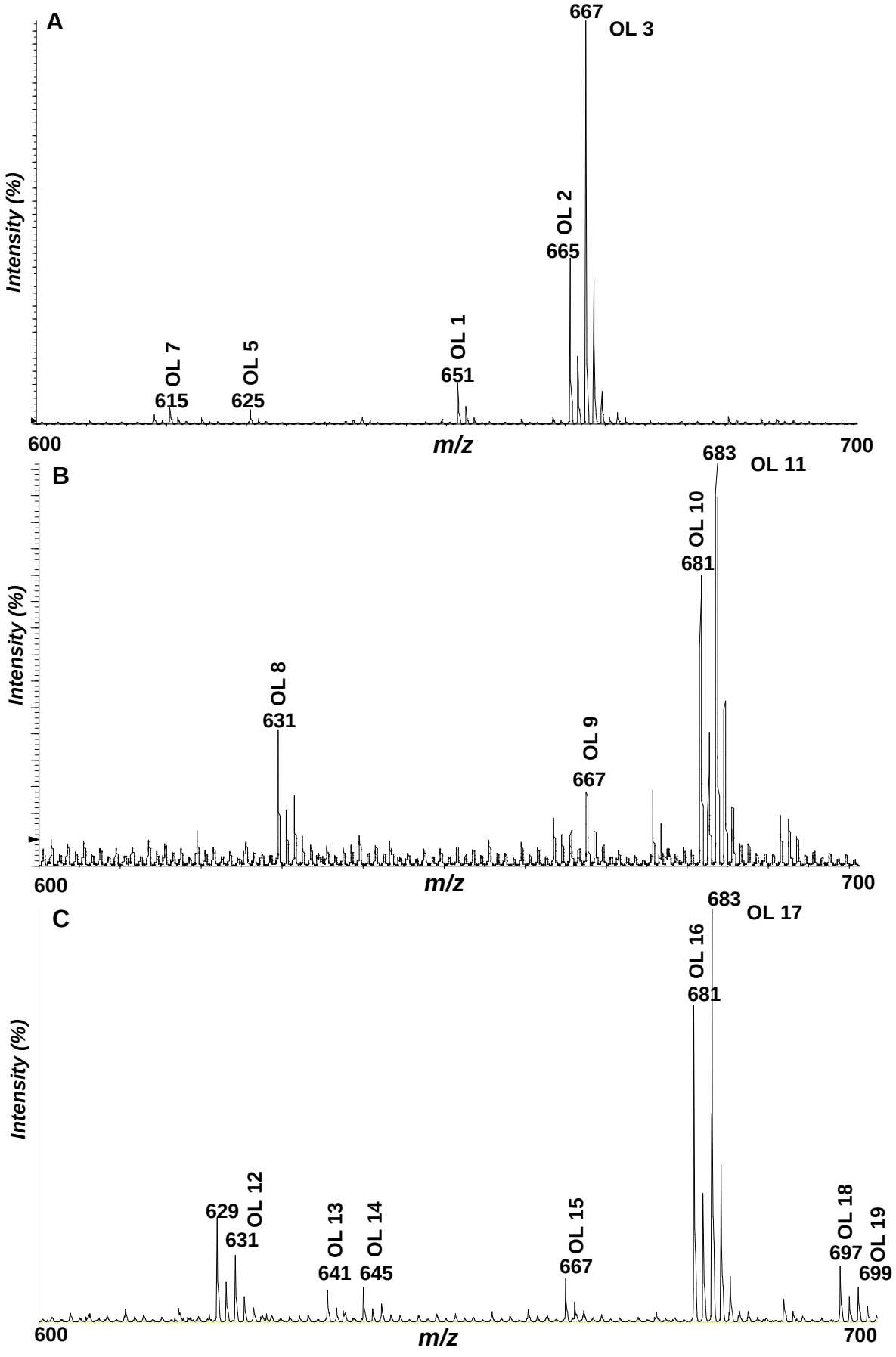


Figure S3. (A) Electrospray mass spectrum of the total lipids from *Sinorhizobium meliloti* x pSphx04 that contains the *olsD* gene from *Burkholderia cenocepacia* J2315. Peak intensity is normalized to the most intense signal in the region displayed. (B) Positive ion mode CID spectrum of the hydroxylated OL at *m/z* 709 from *S. meliloti* x pSphx04. Peak intensities are normalized to the most intense fragment ion.

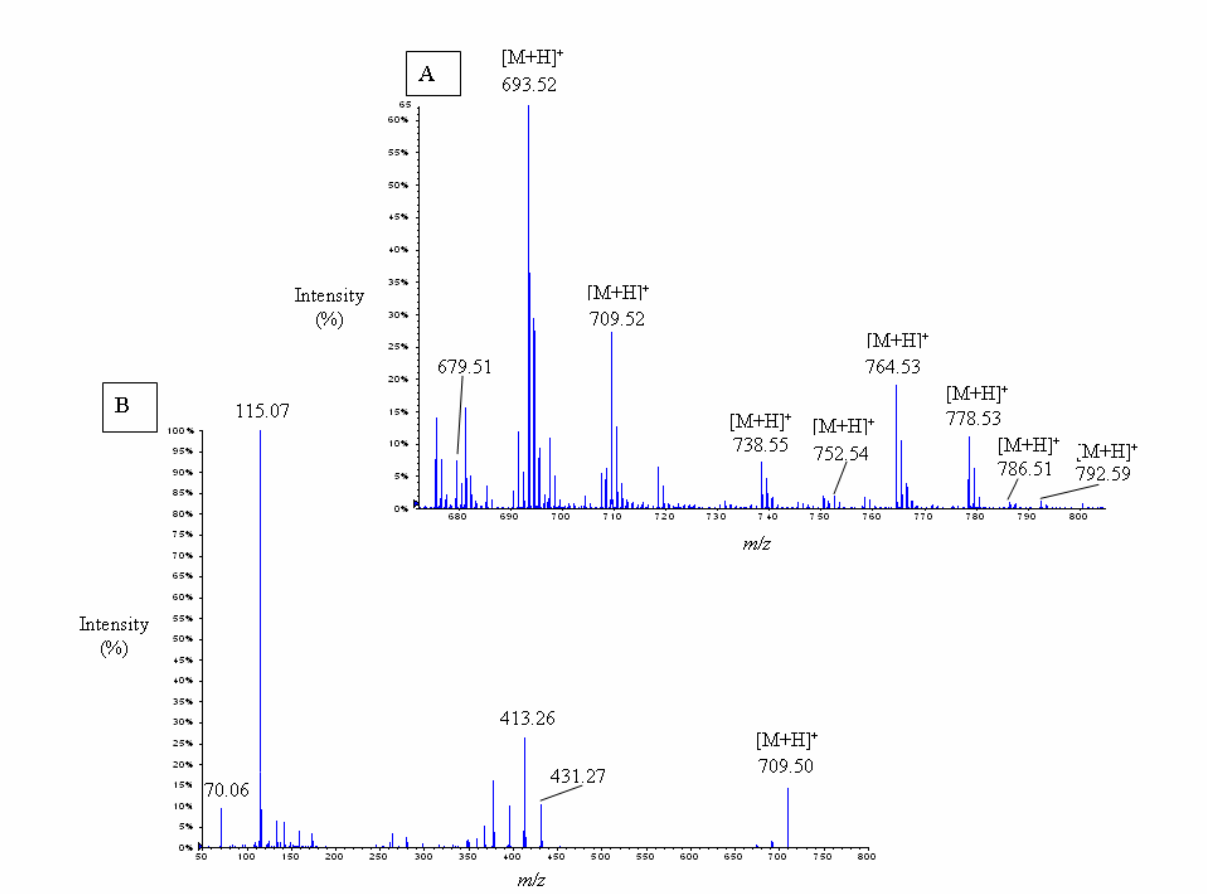
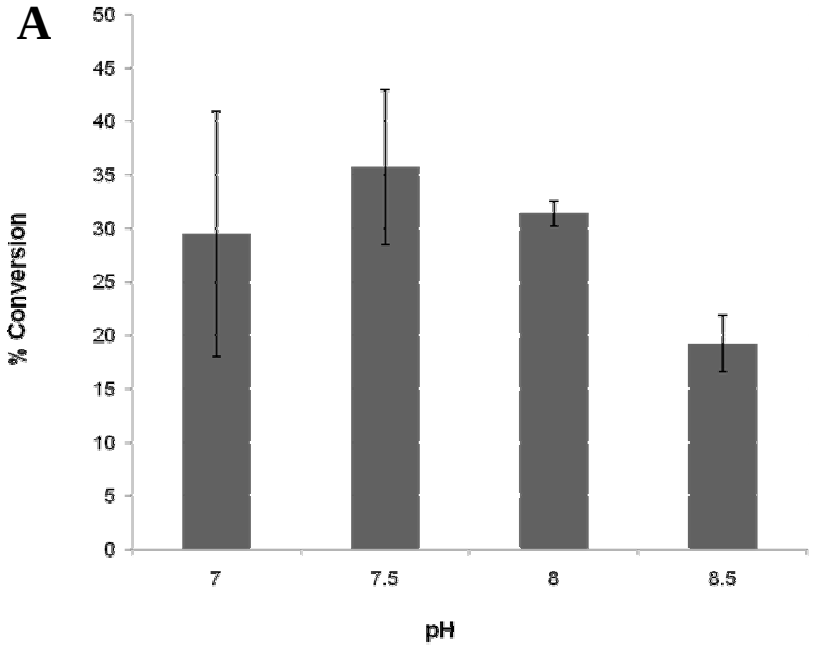


Figure S4. pH and detergent dependence of OlsD activity. (A) OlsD-catalyzed conversion of OL to its hydroxylated derivative *N*-acyl-OH-OL was monitored after incubation in 50 mM Hepes/KOH buffer at the pH values 7.0, 7.5, 8.0, and 8.5. (B) OlsD-catalyzed conversion of OL to its hydroxylated derivative *N*-acyl-OH-OL was monitored in the presence of increasing amounts of Triton X-100 (% v/v). OlsD enzyme assays were performed at a final protein concentration of 1 mg/mL and for 120 min. The values shown are mean values ± standard deviation derived from three independent experiments.



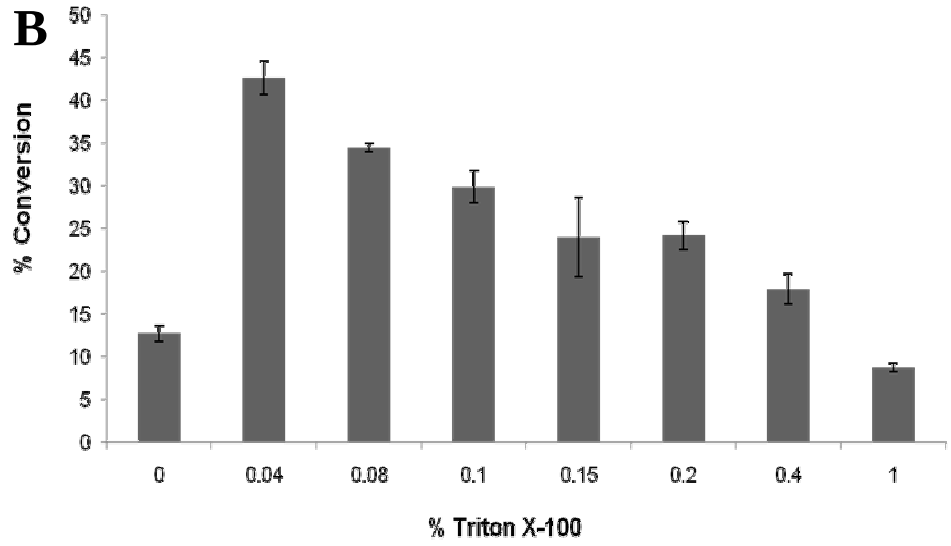
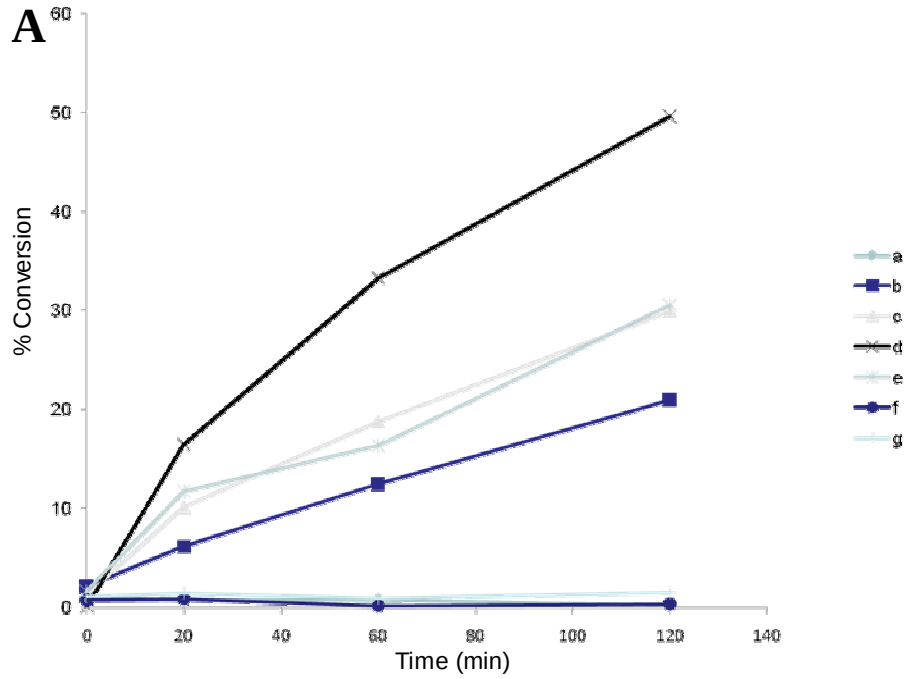


Figure S5. Dependence of OlsD activity on soluble cofactors. (A) A standard OlsD assay mixture containing 1 mg/mL of protein was prepared in which either α-ketoglutarate (a), ascorbate (b), iron (c), or dithiothreitol (d) was omitted. The complete assay (e) is described in Experimental Procedures and was also performed with cell-free extract of *E. coli* BL21 (DE3) x pET16b in which no OlsD had been expressed (f) or with buffer only (g). The values shown are mean values derived from three independent experiments. (B) Inhibition of OlsD activity by increasing concentrations of 2,2'-bipyridyl, an iron chelator. The standard incubation mixture containing 1 mg/mL protein was supplemented with the indicated concentrations of 2,2'-bipyridyl. [^{14}C]OL and the OlsD product *N*-acyl-OH-[^{14}C]OL are indicated. NE indicates the no-enzyme control.



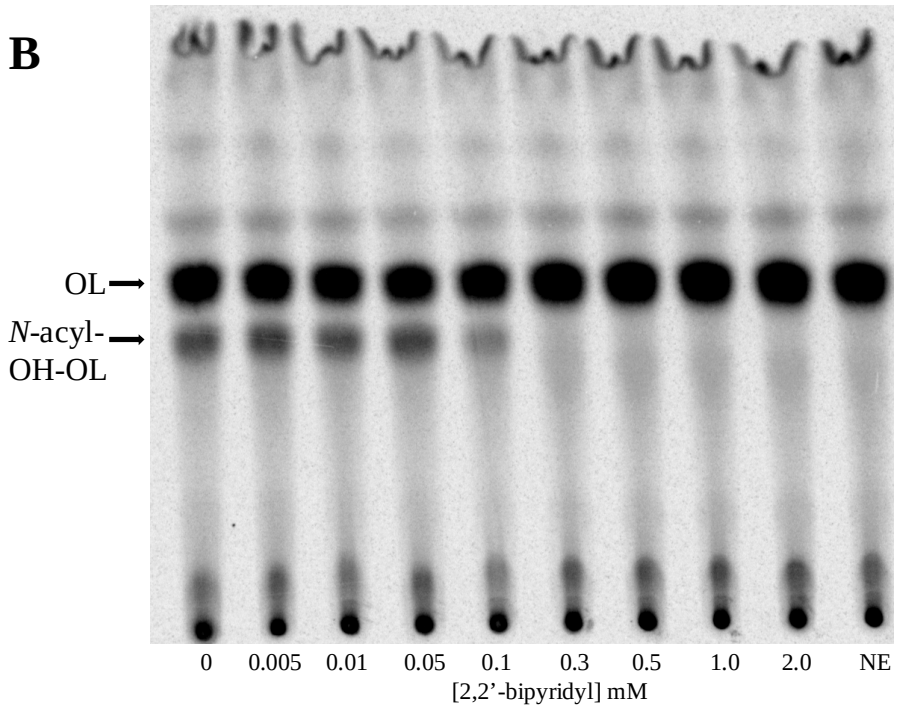


Figure S6. Unrooted phylogenetic tree of LpxO-like bacterial dioxygenases. The tree was constructed using the program CLUSTAL W (http://www.expasy.ch/). Distances between the sequences are expressed as 10 changes per amino acid residue. Accession numbers and locus tags are as follows: ORFBm (*Brucella melitensis* 16M; NP_539381,BMEI0464), OlsCRt(*Rhizobium tropici* CIAT899; AAY28727, OlsC), ORFMsp (*Mesorhizobium* sp. BNC1; YP_674289, Meso_1730), ORFSpr (*Serratia proteomaculans* 568; YP_001479424), ORFBt (*Burkholderia thailandensis* MSMB43; ZP_02466722, Bpse38_010100025414), ORFBma (*Burkholderia mallei* ATCC23344; YP_105332, BMAA0571), ORFBam (*Burkholderia ambifaria* IOP40-10; ZP_02888624, BamIOP4010DRAFT_0686), OlsD (*Burkholderia cenocepacia* J2315; BCAM2401 [OlsD]), LpxO2PAO1 (*Pseudomonas aeruginosa* PAO1; NP_249627, PA0936), BCAM1214 (*Burkholderia cenocepacia* J2315; BCAM1214), LpxOSt (*Salmonella typhimurium* LT2; NP_463151, STM4286 [LpxO]), ORFXoo (*Xanthomonas oryzae* pv. oryzae; AAW73486, XOO0232), LpxO1PAO1 (*Pseudomonas aeruginosa* PAO1; NP_253202,PA4512).