UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

CENTRO DE CIENCIAS GENÓMICAS
DOCTORADO EN CIENCIAS BIOMÉDICAS

BIOSÍNTESIS Y FUNCIONES DE LOS LÍPIDOS DE ORNITINA

T E S I S

QUE PARA OBTENER EL GRADO DE

DOCTOR EN CIENCIAS

P R E S E N T A:

NAPOLEÓN GONZÁLEZ SILVA

DIRECTOR DE TESIS: DR. OTTO GEIGER

CUERNAVACA, MOR. **NOVIEMBRE DEL 2011**

Universidad Nacional Autónoma de México
UNAM
Biblioteca Central
Dirección General de Bibliotecas de la UNAM

UNAM – Dirección General de Bibliotecas
Tesis Digitales
Restricciones de uso

DERECHOS RESERVADOS ©
PROHIBIDA SU REPRODUCCIÓN TOTAL O PARCIAL

Todo el material contenido en esta tesis esta protegido por la Ley Federal del Derecho de Autor (LFDA) de los Estados Unidos Mexicanos (México).

El uso de imágenes, fragmentos de videos, y demás material que sea objeto de protección de los derechos de autor, será exclusivamente para fines educativos e informativos y deberá citar la fuente donde la obtuvo mencionando el autor o autores. Cualquier uso distinto como el lucro, reproducción, edición o modificación, será perseguido y sancionado por el respectivo titular de los Derechos de Autor.

UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
CENTRO DE CIENCIAS GENÓMICAS

Tesis Doctoral:

"Biosíntesis y funciones de los lípidos de ornitina"

que para obtener el grado de Doctor en Ciencias presenta: Napoleón González Silva

Este trabajo se realizó en el laboratorio del Programa de Ecología Genómica del Centro de Ciencias Genómicas/UNAM, bajo la tutoría del Dr. Otto Geiger,

El comité tutor que evaluó el avance del presente proyecto de investigación estuvo integrado por el Dr. Otto Geiger, la Dra. Gloria Soberón Chávez y la Dra. Isabel Ma. López Lara.

El jurado de la réplica oral de este trabajo de investigación se integró por el Dr. Michael F. Dunn, el Dr. Jorge Luis Folch Mallol, la Dra. Ma. Del Carmen Gómez Eichelmann, el Dr. Dimitris Georgellis y el Dr. Otto Geiger.

El trabajo experimental de esta tesis lo financió la Dirección General de Asuntos del Personal Académico/UNAM (200806 y 218009) y el Consejo Nacional de Ciencia y Tecnología de México (CONACyT 82614).

Durante la realización de esta tesis Napoleón González Silva recibió una beca de doctorado otorgada por CONACyT del periodo de Febrero de 2005 a Enero de 2010.

Agradecimientos

Académicos

Un especial y sincero agradecimiento al Dr. Otto Geiger por compartir su conocimiento y experiencia, por guiarme durante el desarrollo de este proyecto y por todo el tiempo dedicado.

De igual manara agradezco a la Dra. Ma. Isabel Ma. López Lara por sus sugerencias, aportaciones y enseñanzas.

Hago extenso mi agradecimiento al Dr. Christian Sohlenkamp por sus sugerencias, por su disponibilidad para resolver dudas amablemente y por ser amigo y maestro.

También agradezco a la Dra. Gloria Soberón Chávez por sus cuestionamientos, por el esfuerzo que realizó de venir a Cuernavaca semestralmente a discutir los avances de este proyecto y por asumir con gran responsabilidad y seriedad su papel de tutora.

Al doctor Dr. Michael F. Dunn, a la Dra. Ma. Del Carmen Gómez Eichelmann, el Dr. Dimitris Georgellis y al Dr. Jorge Luis Folch Mallol por la cuidadosa revisión de esta tesis y por sus sugerencias para mejorarla.

A la Dra. Esperanza Martínez Romero por proveer lo que se necesita en el laboratorio y por propiciar la interacción académica en el Programa de Ecología Genómica.

A José Espíritu, a Martin, a Lucila, a Gladys y a Ma. Dolores por todo el apoyo brindado.

Al CONACyT, a la UNAM y al CCG por todos los recursos invertidos en mi formación.

Personales

A mis padres, hermanos, tíos, primos, sobrinos y especialmente a mi tío Víctor y a mis tías María Trinidad y Eduviges por todos sus consejos y el apoyo brindado.

A la Familia Romero-Gallardo por su amistad por el apoyo que me brindo antes y durante el desarrollo de este proyecto. Especialmente a Mario M. Romero Tinoco por motivarme con su optimismo a seguirme superando.

A mis amigos Abel Vázquez, David Martínez y a sus familias, por sus consejos y amistad.

A mis amigos del CCG J. Luis acosta, Miguel, Ángel, Rosa, Mario, Diana Sahonero, Uriel, Daniela, Roberto, Diana Vera, Emilio, Ramón, Ernesto, Marco, Aline, Martha, Tania, Tabita, Luis E. Servín, Luis M. Bolaños y Wendy, así como a todos mis excompañeros y amigos del CCG por generar un ambiente de trabajo agradable.

Dedicatoria

A toda mi familia y a la memoria de mis abuelos Josefina y Severo.

Contenido

Índice de figuras

Índice de tablas

Abreviaturas

ACP: acyl carrier protein (proteína acarreadora de acilos)

AcpS: acyl carrier protein synthase (ACP sintasa)

CL: cardiolipin (cardiolipina)

CM: cytoplasmic membrane (membrana citoplasmática)

CoA: coenzyme A (coenzima A)

cpm: counts per minute (cuentas por minuto)

DGTS: diacylglyceryl-*N,N,N*-trimethylhomoserine (diacilgliceril *N*,*N*,*N*-trimetilhomoserina)

DMPE: dimethylphosphatidylethanolamine (dimetilfosfatidiletanolamina)

GL: glycine-containing lipid (lípido de glicina)

IM: Inner membrane (membrana interna)

LL: lysine-containing lipid (lípido de lisina)

LOL: lyso-ornithine lipid (lisolípido de ornitina)

LPS: lipopolysaccharide (lipopolisacárido)

MDO: membrane-derived oligosaccharides (oligosacáridos derivados de membrana)

MMPE: monomethylphosphatidylethanolamine (monometilfosfatidiletanolamina)

OL: ornithine-containing lipid (lípido de ornitina)

OM: outer membrane (membrana externa)

PC: phosphatidylcholine (fosfatidilcolina)

PCR: the polymerase chain reaction (la reacción en cadena de la polimerasa)

PE: phosphatidylethanolamine (fosfatidiletanolamina)

PG: phosphatidylglycerol (fosfatidilglicerol)

SDS-PAGE: polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (electroforesis en gel de poliacrilamida con dodecilsulfato sódico)

SGL: serineglycine-containing lipid (lípido de glicinaserina)

TLC: thin layer chromatography (cromatografía en capa fina)

TLR: toll-like receptor (receptor tipo toll)

TOLs: tauro-ornithine-containing lipid (lípido de ornitinataurina)

2D: two-dimensional (en dos dimenciones)

2-OH: a hydroxyl group at C2 of an esterified fatty acyl residue (un grupo hidroxilo en el C2 de un acilo esterificado)

2-OH-OL: OL hidroxilado en el C2 de un acilo esterificado

2-OH-PE: PE hidroxilada en el C2 de un acilo esterificado

1. Resumen

Burkholderia cenocepacia J2315 es una bacteria Gram negativa conocida como un patógeno oportunista de humanos. Una característica distintiva del género *Burkholderia* es su repertorio de lípidos polares de membrana, entre ellos fosfatidiletanolamina (PE) y los lípidos de ornitina (OLs), así como sus derivados 2-hidroxilados (2-OH-PE y 2-OH-OLs, respectivamente), los cuales difieren de las versiones típicas por poseer un grupo hidroxilo en el C2 (2-OH), que sustituye a un átomo de hidrógeno, en un residuo de acilo esterificado. Similarmente, residuos de miristato esterificados del lípido A de *Salmonella typhimurium* pueden tener la modificación 2-OH, la cual es realizada por la enzima LpxO. Sin embargo, la enzima responsable de hacer la sustitución 2-OH en los 2-OH-OLs de *B. cenocepacia* J2315 todavía no se conoce. Nosotros postulamos que la 2-hidroxilación de los 2-OH-OLs podría ser realizada por una nueva dioxigenasa homóloga a LpxO. En el genoma de *B. cenocepacia* J2315 identificamos dos marcos abiertos de lectura (BCAM1214 y BCAM2401) homólogos a LpxO de *S. typhimurium*.

Al introducir *bcam2401* (nombrado *olsD*) en *Sinorhizobium meliloti* 1021 se formó un nuevo lípido y en *B. cenocepacia* J2315 dos nuevos lípidos. Sorprendentemente, los datos obtenidos por espectrometría de masas sugieren que OlsD modifica el acilo amidificado a la ornitina de los OLs con un grupo hidroxilo. Lo anterior es consistente con la menor movilidad en cromatografía en capa fina (TLC) de los lisolípidos (LOLs) derivados de OLs modificados por OlsD con respecto a los LOLs derivados de los OLs no modificados. Esto sugiere que los LOLs derivados de OLs modificados por OlsD son más polares porque probablemente están hidroxilados en el ácido graso amidificado, el cual forma parte de los LOLs. Este es el primer reporte de una enzima que hidroxila el acilo amidificado de los OLs. La hidroxilación por OlsD de los OLs en el ácido graso amidificado ocurre cuando *B. cenocepacia* J2315 se crece en estrés ácido. Hemos desarrollado un ensayo para la dioxigenasa OlsD y presentamos una caracterización inicial de la enzima. Con los ensayos enzimáticos demostramos que OlsD puede modificar in vitro a las dos clases de lípidos ornitina (OLs y 2-OH-OLs) que produce *B. cenocepacia* J2315.

En contraste, la expresión del *bcam1214* en *S. meliloti* 1021 y en *B. cenocepacia* J2315 no formó ningún lípido adicional y la mutante en este gen tiene un perfil de lípidos de membrana similar a la cepa silvestre. Este resultado indica que la proteína BCAM1214 no está involucrada en hidroxilar a los OLs ni a PE. Al no encontrar el gen que codificara para la enzima responsable de la actividad de hidroxilar a los OLs ni a PE en el C2, estudiamos otros genes candidatos que codifican para productos homólogos a la 2-hidroxilasa PHYH de humano. En el genoma de *B. cenocepacia* J2315 encontramos dos homólogos a PHYH de humano: *bcal0511* y *bcam2775*, los cuales se clonaron y se expresaron en cepas de *Escherichia coli*, *S. meliloti* y *B. cenocepacia*. Ninguno de los dos genes alteró el perfil de lípidos de membrana en las cepas en las que se expresaron. Lo que sugería que estos genes no están involucrados en hidroxilar a los OLs ni a PE.

El gen *olsB* codifica para la enzima que participa en el primer paso de la biosíntesis de los OLs. Una mutante en ese gen (NG1) derivada de *B. cenocepacia* J2315 no forma ninguna clase de OLs, aún cuando se introdujo el gen *olsD* en esa mutante. Lo que sugiere que la formación de los OLs hidroxilados ocurre solamente cuando la ruta para biosíntesis de los OLs no modificados está integra. Sorprendentemente, la mutante NG1 no restauró la formación de OLs al introducir independientemente los genes *olsB* de *S. meliloti* 1021 y de *R. tropici* CIAT899, ni al coexpresar a *olsB* y *acpP* de *S. meliloti* 1021. Sin embargo, la mutante en *olsB* (AAK1) derivada de *S. meliloti* 1021 si restauró la producción de OLs al complementarla con el *olsB* de *B. cenocepacia* J2315. Actualmente desconocemos porqué los genes *olsB* rizobiales no complementan a la mutante NG1.

También realizamos una evaluación de la sensibilidad a antibióticos de las cepas mutantes AQ3 (mutante en *olsD*) y NG1, así como de la cepa silvestre. Pero no observamos diferencias en la sensibilidad entre las mutantes y la cepa silvestre.

Recientemente se demostró que el gen *olsC* de *R. tropici* CIAT899 codifica para la enzima que está involucrada en hidroxilar el C2 del ácido graso esterificado de los OLs de esta bacteria. El gen *olsC* tiene dos probables codones de inicio de la traducción lo que teóricamente podría producir dos versiones (la corta y la larga) de la enzima OlsC. La coexpresión de *olsC* (versión corta) con *olsB* de *B. cenocepacia* J2315 en la cepa CS111

formó un nuevo lípido, lo que no ocurrió al expresar la versión larga de *olsC* con *olsB* de *B. cenocepacia* J2315. Eso sugiere que la versión corta de OlsC es la funcional. Mostramos indicios experimentales que indican que OlsC, al igual que LpxO, podría ser una 2-hidroxilasa dependiente de $Fe^{2+}/O_2/\alpha$-cetoglutarato.

Existe el antecedente de que la coexpresión de *olsB* y *olsA* de *S. meliloti* 1021 en *E. coli* no produce OLs. Debido al resultado anterior se especuló que otra proteína podría estar involucrada en la biosíntesis de los OLs. En *S. meliloti* 1021 el gen *smc02490* podría ser el que codifica para dicha enzima, ya que homólogos a este gen coexiste o se encuentran fusionados con homólogos del gen *olsB* en muchas bacterias. Actualmente, hemos construido una mutante en el gen *smc02490* y se harán experimentos para determinar si este gen está involucrado en la biosíntesis de los OLs.

2. Abstract (resumen en inglés)

Burkholderia cenocepacia is an important opportunistic pathogen, and one of the most striking features of the *Burkholderia* genus is the repertoire 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* (named *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 in the amide-linked fatty acyl chain. This is consistent with the lower mobility in thin layer chromatography of lyso-ornithine lipids (LOLs) derived from OLs modified by OlsD, when compared to the LOLs derived from unmodified standard OLs. OlsD-modified LOLs are more polar than standard OLs due to an extra hydroxyl group on the amide-linked fatty acyl moiety. The hydroxyl modification of OLs on the amide-linked fatty acyl moiety due to OlsD occurs under acid stress conditions. An assay has been developed for the OlsD dioxygenase, and an initial characterization of the enzyme is presented. With enzyme assays we show that OlsD can modify in vitro the two classes of ornithine lipids (OLs and 2-OH-OLs), which produces *B. cenocepacia* J2315.

In contrast, expression of *bcam1214* in *S. meliloti* 1021 or *B. cenocepacia* J2315 did not cause the formation of any additional lipid. The *B. cenocepacia bcam1214*-deficient mutant has a membrane lipid profile similar to wild type. These results suggest that BCAM1214 does not hydroxylate OLs or PE. Also, two *B. cenocepacia* J2315 homologues (*bcal0511* and *bcam2775*) of the human phytanoyl-CoA hydroxylase PHYH, which hydroxylates at the 2-position of fatty acids, were studied for their capacity to hydroxylate

OL- or PE-bound fatty acyl residues at the 2-position. After cloning and expressing either *bcal0511* or *bcam2775*, in *Escherichia coli*, *S. meliloti*, or *B. cenocepacia* no changes in membrane lipid profiles could be observed, suggesting that these genes are not involved in hydroxylating OLs or PE.

The *N*-acyltransferase-encoding OlsB catalyzes the first step of OL biosynthesis and the OlsB-deficient mutant NG1 of *B. cenocepacia* J2315 does not form any kind of OLs. Therefore, formation of hydroxylated OLs occurs only when the biosynthesis pathway for nonmodified standard OLs is intact. Surprisingly, the NG1 mutant cannot be complemented for the formation of OLs by introducing the *olsB* gene from *S. meliloti* 1021 or from *R. tropici* CIAT899 or by combined expression of *olsB* and *acpP* from *S. meliloti* 1021 in NG1. In contrast, the *olsB* mutant AAK1 of *S. meliloti* 1021 can be complemented for OL production upon expression of the *olsB* gene from *B. cenocepacia* J2315. Currently, we don't know why rhizobial *olsB* genes cannot complement the *olsB*-deficient mutant NG1 of *B. cenocepacia*.

B. cenocepacia J2315 mutants deficient in *olsD* (AQ3) or *olsB* (NG1) were not more sensitive than the wild type to a wide range of antibiotics tested.

The gene *olsC* from *R. tropici* CIAT899 encodes the enzyme that hydroxylates esterified fatty acyl residues of OLs at the 2-position. The *olsC* gene has two potential start codons for translation which theoretically could produce two versions (a short and a long version) of the enzyme OlsC. The combined expression of the short version rhizobial *olsC* and *olsB* from *B. cenocepacia* J2315 in *S. meliloti* strain CS111 caused the formation of a new lipid, which did not occur when the long version of *olsC* was coexpressed with *olsB* from *B. cenocepacia* J2315. This result suggests that the short version of OlsC is the functional one. Also, we have developed an assay for the OlsC dioxygenase and, like LpxO, it seems to be a 2-hydroxylase dependent on Fe^{2+}, O_2, and α-ketoglutarate.

Surprisingly, the combined expression of *olsB* and *olsA* from *S. meliloti* 1021 in *E. coli* does not cause the formation of OLs leading to the suggestion that more rhizobial genes/proteins might be required for a complete OL biosynthesis. Homologues of the *smc02490* gene are fused to or coexist with *olsB.* Therefore, we speculated that the

smc02490 product might be required for OL biosynthesis. We have constructed a mutant in the *smc02490* gene and currently perform experiments to determine whether this gene is involved in OL biosynthesis.

3. Introducción

3.1. Envolturas celulares de las bacterias

Las envolturas celulares de las bacterias consisten de las estructuras celulares que envuelven al citoplasma. La membrana citoplasmática (CM) o membrana interna (IM), el espacio periplasmático con la pared celular y la membrana externa (OM) son las envolturas celulares de las bacterias Gram negativas (fig. 1). En contraste, las envolturas celulares de las bacterias Gram positivas consisten solamente de una CM y la pared celular. Las envolturas celulares de las bacterias están constituidas por lípidos, proteínas, péptidos, carbohidratos o combinaciones de estas moléculas.

La CM es muy similar entre bacterias Gram negativas y Gram positivas, y consiste de una bicapa lipídica formada por dos monocapas lipídicas constituidas principalmente de glicerofosfolípidos, así como de proteínas de membrana integrales y periféricas.

El espacio periplasmático es el espacio que existe entre la membrana interna y externa de las bacterias Gram negativas. Este es un ambiente oxidante donde la estructura de las proteínas puede ser estabilizada por enlaces disulfuro. Dependiendo del microorganismo, el espacio contiene enzimas hidrolíticas que degradan alimentos y algunas sustancias tóxicas, proteínas de unión, las cuales inician el proceso de transporte de sustratos, quimiorreceptores, las cuales son proteínas involucradas en la quimiotaxis, así como enzimas involucradas en diversas vías bioquímicas incluyendo la síntesis de peptidoglicano [Madigan y Martinko, 2006]. En el espacio periplasmático de las Gram negativas también se encuentra la pared celular. La pared celular en las bacterias Gram negativas es mucho más delgada respecto a la de bacterias Gram positivas, esta previene de la lisis osmótica [Madigan y Martinko, 2006]. La pared celular está compuesta principalmente de peptidoglicano que está constituido por polímeros lineales de β-(1,4)-*N*-acetilglucosamina y ácido *N*-acetilmurámico unidos por pequeños péptidos [Vollmer y Bertsche, 2008]. En el espacio periplasmático también hay oligosacáridos derivados de membrana (MDO), y los MDO de *Escherichia coli* están compuestos de seis a ocho residuos de glucosa unidos por enlaces β-(1-2) y β-(1-6) [Rumley et al., 1992].

La OM es exclusiva de las bacterias Gram negativas y es la envoltura más externa que forma la superficie celular de este grupo de bacterias. La OM también está formada por dos monocapas. La monocapa interna de la OM al igual que las monocapas de la IM está constituida principalmente de glicerofosfolípidos. En contraste, la monocapa externa de la OM está constituida principalmente por monómeros de lipopolisacárido (LPS) [Raetz et al., 2007].

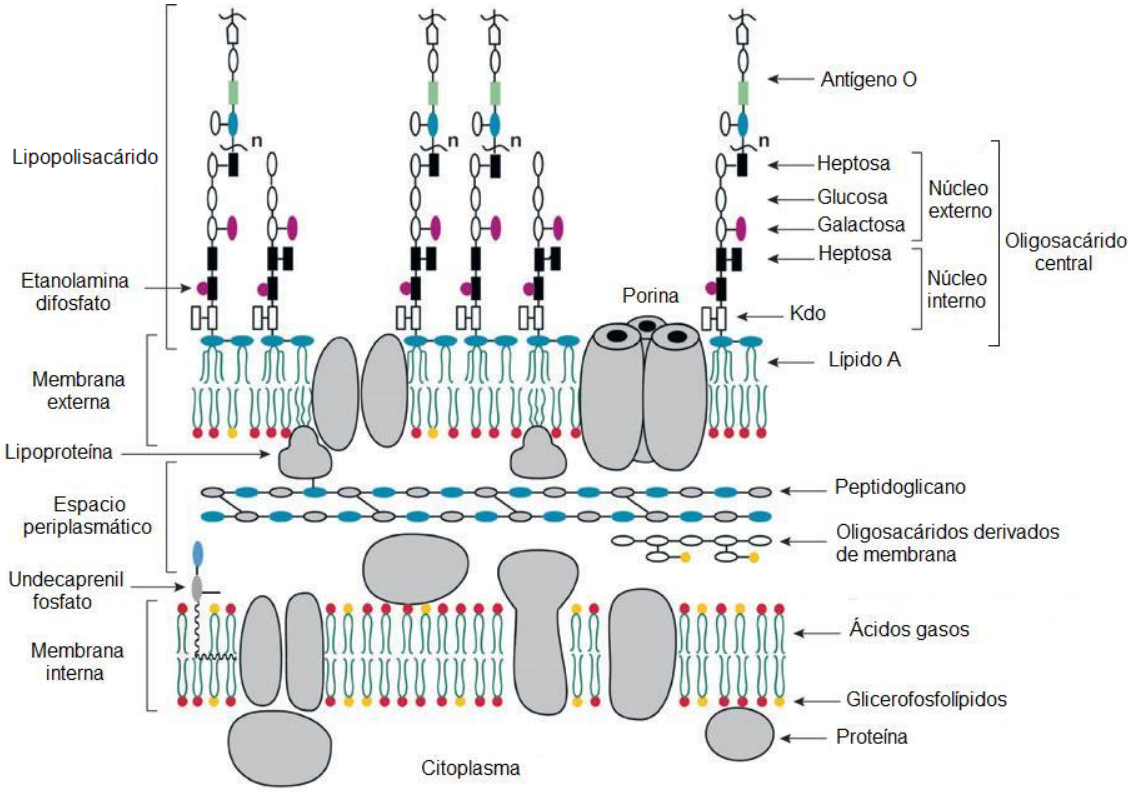


Figura 1. Estructura esquemática de las envolturas celulares de la bacteria Gram negativa *E. coli* K-12. La membrana interna, el espacio periplasmático y la membrana externa se indican, así como sus subcomponentes. Kdo (ácido 2-ceto-3-desoxi-D-mano-octulosónico). Tomada y traducida de Raetz et al. (2007).

3.1.1. Membranas celulares de las bacterias

Las membranas celulares de las bacterias son estructuras dinámicas que permiten a estos microorganismos interactuar y comunicarse con el medio extracelular. Las membranas celulares de las bacterias son imprescindibles porque delimitan el cuerpo celular, lo cual, permite establecer gradientes químicos o potenciales de membrana entre

el citoplasma y el ambiente exterior, que a través de la respiración proveen de la energía que las células destinan a procesos esenciales. Los gradientes se generan debido a que las membranas celulares funcionan como barreras de permeabilidad selectiva muy efectivas para iones, compuestos hidrofílicos y hidrofóbicos, lo que consecuentemente regula el flujo de muchas sustancias hacia fuera y hacia dentro de las células. Aunque algunos compuestos hidrofóbicos pueden difundirse a través de las membranas con relativa facilidad.

Las membranas se forman por la incorporación de lípidos y proteínas sintetizados por las células, los cuales se insertan en las membranas preexistentes. Las membranas de las bacterias Gram negativas están constituidas por ciertas proporciones de clases de lípidos, y proteínas, los que determinan su identidad estructural y funcional [Madigan y Martinko, 2006]. Las membranas celulares consisten de una bicapa lipídica y de proteínas integrales y periféricas, algunas de estas son glicoproteínas o lipoprotínas. La estructura de bicapa de la membrana representa la conformación más estable de las moléculas lipídicas en un ambiente acuoso [Madigan y Martinko, 2006].

Los lípidos y las proteínas de membrana están unidos principalmente por interacciones no covalentes como enlaces de hidrógeno e interacciones hidrofóbicas. En adición cationes tales como Mg^{+} y Ca^{+} ayudan a estabilizar las membranas por combinación iónica con cargas negativas de los lípidos [Madigan y Martinko, 2006]. Las bicapas lipídicas están constituidas por dos monocapas de lípidos que están orientados en direcciones opuestas formando un dominio hidrofóbico con sus residuos de acilo o alquilo y dos dominios hidrofílicos determinados por los grupos cabeza polares de los lípidos, unos se dirigen hacia el citosol y otros hacia el exterior de la célula (fig. 1) [Madigan y Martinko, 2006]. Los dominios hidrofílicos y hidrofóbicos formados por el ensamble macromolecular de las bicapas son los que impiden la difusión espontánea de iones o de compuestos de naturaleza opuesta a esos dominios.

Muchos de los materiales que se mueven del citoplasma celular al ambiente extracelular, y viceversa, lo hacen a través del transporte activo, el cual usa proteínas transportadoras que consumen energía metabólica al realizar su actividad. Otros

materiales se trasladan a través de transporte pasivo mediante canales de difusión generales o específicos formados por proteínas que forman poros (porinas) en las membranas. La difusión a través de las membranas está determinada por el tamaño y la naturaleza de los materiales en cuestión.

Algunos componentes de las membranas suplen de sustratos para la biosíntesis de otros componentes celulares y de moléculas señales. Mientras tanto, las proteínas de membrana están involucradas en funciones esenciales para las células tales como el trasporte selectivo de iones o moléculas, en la conversión y generación de energía a través de la cadena respiratoria, división celular, motilidad, en la señalización celular funcionando como receptores censando señales intracelulares y extracelulares, y en el metabolismo participando como enzimas en diversos procesos del catabolismo y anabolismo [Facey y Kuhn, 2010; Madigan y Martinko, 2006].

3.1.1.1. Membrana interna y externa de bacterias Gram negativas

Las bacterias Gram negativas están rodeadas por dos membranas diferentes: la IM y la OM. Las dos membranas están separadas por el espacio periplasmático y se componen principalmente de una mezcla de glicerofosfolípidos pero difieren considerablemente en su estructura y composición. Se asume que en *E. coli* la IM está compuesta por una bicapa asimétrica con la siguiente composición de glicerofosfolípidos: alrededor de 80 % de fosfatidiletanolamina (PE), 15 % de fosfatidilglicerol (PG) y 5 % de cardiolipina (CL) [Kadner, 1996]. La OM, también es una bicapa asimétrica y su monocapa interna tiene una composición lipídica similar a la membrana citoplasmática; la monocapa externa de esta membrana en la mayoría de las especies de las bacterias Gram negativas está compuesta de LPS y en unas pocas especies también por sulfonolípidos o glicoesfingolípidos, además del LPS o en su lugar [Nikaido, 2003]. Aunque glicerofosfolípidos también han sido detectados en esa monocapa en casos extraordinarios.

Ambas membranas contienen muchas proteínas que son esenciales para muchas funciones celulares. Estas proteínas desempeñan funciones protagónicas en la transducción de energía, también están involucradas en el censado y en la transducción de

señales de estímulos ambientales, así como en el influjo y eflujo de sustancias [Facey y Kuhn, 2010].

La biogénesis de estas proteínas requiere de el direccionamiento coordinado a la membrana, la inserción y el desplazamiento a través de la membrana, y subsecuentemente el ensamble en complejos multiprotéicos. Las proteínas de la membrana tienen que incorporarse en la IM o en la OM y ser plegadas correctamente para funcionar en la célula. Para facilitar el direccionamiento se requiere del plegamiento y ensamble, muchas chaperonas moleculares, de la translocación y de maquinaria de inserción [Facey y Kuhn, 2010]. La información de porqué algunas proteínas son dirigidas a las membranas como destino final está contenida dentro de la secuencia de aminoácidos de esas proteínas. En general, las proteínas con hélices α con segmentos que atraviesan la membrana se localizan en la IM, mientras que proteínas anfipáticas con la forma de barril β (llamadas proteínas de membrana externa u Omps) están localizadas en la OM. En *E. coli* aproximadamente de 20-30 % de todas las proteínas codificadas en su genoma (alrededor de 4000) son proteínas de la IM y aproximadamente 2 % son proteínas de la OM [Facey y Kuhn, 2010].

Las membranas de las células eucariontes albergan microdominios conocidos como balsas lipídicas que contienen proteínas involucradas en el transporte y en la señalización. Las balsas lipídicas de eucariontes son enriquecidas en lípidos tales como esteroles y esfingolípidos. Recientemente López y Kolter (2011) mostraron evidencia de que las bacterias también contienen microdominios que son similares funcionalmente a los reportados en eucariontes. En eucariontes la proteína flotilina-1 se ha localizado en balsas lipídicas y reportes previos indican que las proteínas de bacterias homólogas a la flotilina-1 de eucariontes están distribuidas heterogéneamente en la membrana citoplasmática en un patrón punteado [Zhang et al. 2005; Donovan y Bramkamp 2009]. El ácido zaragocico en *Bacillus subtilis* inhibe la formación de biofilm y la secreción de proteínas pero no viabilidad de las células [López y Kolter, 2011]. El ácido zaragocico es un conocido inhibidor de la escualeno sintasa y debido al efecto que causa en *B. subtilis* se ha sugerido que los lípidos asociados con las balsas lipídicas bacterianas probablemente son poliisopreniodes

[López y Kolter, 2011]. Otra evidencia que López y Kolter (2011) mostraron es que una mutante de *B. subtilis* en las proteínas homólogas a la flotilina-1 de eucariontes se vio afectada en la ruta de traducción de señal que conduce a la formación de biofilm, en la que está involucrada la censora cinasa (KinC). La proteína KinC presumiblemente está localizada en balsas lipídicas al igual que las proteínas bacterianas homólogas a la flotilina-1.

3.1.2. Lípidos de membrana de bacterias

La función estructural de los lípidos de membrana es esencial para los organismos vivos. Los lípidos de membrana normalmente son anfifílicos y cilíndricos que consisten de dos cadenas hidrofóbicas largas de acilo o alquilo y un grupo cabeza hidrofílico [Dowhan, 2008]. El área cubierta por los grupos cabeza de estos lípidos suele ser similar al área cubierta por la de sus cadenas hidrocarbonadas, lo que permite, que los monómeros lipídicos interactúen, favoreciendo la formación de la bicapa en lugar de micelas [Dowhan, 2008].

En las bacterias los glicerofosfolípidos comprenden alrededor del 10 % del peso seco de la célula, y cada mol de lípido requiere para su biosíntesis de alrededor de 32 moles de adenosín trifosfato [Nelson y Cox, 2010]. En la bacteria modelo *E. colí* los principales lípidos de membrana son los glicerofosfolípidos: PE, PG y CL [Rock, 2008]. Otros glicerofosfolípidos tales como: fosfatidilserina, fosfatidilcolina (PC) y fosfatidilinositol también están presentes en grupos específicos de bacterias. Algunas bacterias tienen la capacidad de modificar ciertos glicerofosfolípidos con residuos de aminoácidos o con grupos hidroxilo [Geiger et al., 2010]. Formas deaciladas de CL se han encontrado en la membrana celular de especies del género *Streptomyces* [Hoischen et. al., 1997; Sandoval-Calderón et al., 2009].

Los glicolípidos también están presentes en las membranas de las bacterias, así como lípidos esteroides y hopanoides [López-Lara et al., 2003; Geiger et al., 2010]. Otros lípidos sin fósforo en su estructura como el sulfolípido sulfoquinovosildiacilglicerol y diacilgliceril *N*,*N*,*N*-trimetilhomoserina (DGTS) son sintetizados principalmente en grandes cantidades

cuando algunas bacterias se crecen en condiciones limitadas en fósforo [López-Lara et al., 2003; Geiger et al., 2010].

E. coli y casi todas las bacterias Gram negativas contienen LPS (fig. 2). El lípido A es una parte del LPS y forma la monocapa lipídica externa de la OM de estas bacterias, la biosíntesis y el sistema de modificación del lípido A ha sido descrito recientemente [Raetz et al., 2007]. En algunas bacterias Gram negativas el LPS es remplazado por esfingolípidos [Kawahara et al., 1991]. Los sulfonolípidos también se han reportado que están presentes en la OM de bacterias Gram negativas [Pitta et al., 1989].

Además de los lípidos mencionados en algunas bacterias, se ha reportado la presencia de lípidos con la estructura aciloxi-acil que contienen aminoácidos, entre ellos, los lípidos que contienen lisina, glicina, glicinaserina, ornitina u ornitinataurina. La estructura aciloxi-acil también está presente en lípido A. El lípido A de las Enterobacteriacea es un potente inmunoactivador de la inmunidad innata de mamíferos y se ha demostrado que algunos de los lípidos con la estructura aciloxi-acil que contienen aminoácidos también lo son.

3.1.2.1. Lípidos de membrana con la estructura aciloxi-acil de bacterias

3.1.2.1.1. Lipopolisacárido

Los LPSs son glucolípidos anfipáticos complejos que forman la mayor parte de la superficie (75 %) de la mayoría de las bacterias Gram negativas. Los LPSs son inmunogénicos e interaccionan con organismos hospederos eucariotas y con el ambiente exterior [Raetz y Whitfield, 2002; Raetz *et al*., 2007]. Una molécula típica de LPS consiste de tres partes (fig. 1): 1) el lípido A, 2) el oligosacárido central, el cual se divide conceptualmente en núcleo interno y externo, y 3) un polisacárido distal (antígeno O).

El lípido A es la parte del LPS que ancla a estas moléculas a la OM. El lípido A de *E. coli* K-12 está compuesto por un disacárido de glucosamina unido por enlaces β−(1’,6), el cual es típicamente fosforilado en las posiciones 1 y 4’, y acilado con ácido (*R*)-3-hidroximirístico en las posiciones 2, 2’, 3 y 3’, mediante enlaces tipo amida (2, 2’) y éster (3,3’) (fig. 2) [Raetz y Whitfield, 2002]. Los ácidos grasos de las posiciones 2´ y 3´ también son acilados en su C3 formando enlaces éster y produciendo la estructura aciloxi-acil (fig.

2). El lípido A de *E. coli* y de muchas otras bacterias difiere de un glicerofosfolípido por tener seis ácidos grasos saturados en lugar de dos saturados o insaturados. Esta característica hace a la OM mucho más hidrofóbica [Nikaido, 1996] que una bicapa típica de glicerofosfolípidos. La hidrofóbicidad de esta membrana también es promovida por las interacciones laterales fuertes entre las moléculas de LPS produciendo baja fluidez [Nikaido, 2003].

El núcleo interno del oligosacárido central contiene típicamente residuos de Kdo (ácido 2-ceto-3-desoxi-D-mano-octulosónico) y uno normalmente está unido al disacárido de glucosamina y por otro lado a un residuo de L-glicero-D-manoheptosa (heptosa). Además, la estructura del núcleo interno frecuentemente es decorada con otros azúcares, grupos fosfato o residuos de etanolamina difosfato (fig. 1). Los múltiples grupos aniónicos (entre ellos, grupos fosfato) del esqueleto del disacárido de glucosamina y del núcleo interno son conocidos por interactuar fuertemente con cationes divalentes, los cuales compensan la repulsión electrostática entre las moléculas adyacentes de LPSs, debida a esos grupos aniónicos. El núcleo interno de *E. coli* K-12 se conecta con el núcleo externo a través de un residuo de heptosa conservado. El núcleo externo del oligosacárido central muestra una mayor diversidad estructural respecto al núcleo interno y contiene residuos de azúcar entre ellos heptosa y hexosas como glucosa, galactosa, *N*-acetilglucosamina, [Raetz y Whitfield, 2002].

El antígeno O está unido al núcleo externo y es la parte más variable del LPS, es un polisacárido constituido por unidades repetitivas de monosacáridos u oligosacáridos. La longitud del antígeno O difiere aún en un mismo organismo, pudiendo estar ausentes en unos y en otros consistir de más de 60 monosacáridos. Los monosacáridos que componen las unidades repetitivas son generalmente, azúcares neutros y acídicos, aminoazúcares, además de los azúcares inusuales, tales como 6-desoxihexosas ó 3,6-didesoxihexosas [Raetz y Whitfield, 2002].

Algunos ácidos grasos esterificados del lípido A de ciertas bacterias son modificados con un grupo hidroxilo. En *S. typhimurium* existen esos ácidos grasos S-2-hidroxilados y están esterificados al C3 del acilo unido a la posición 3´ del segundo residuo de

glucosamina del lípido A y se ha pensado que son importantes para la patogénesis de este organismo (fig. 2) [Gibbons et al., 2000; Gibbons et al., 2008]. La S-2-hidroxilación es realizada después de que el residuo de acilo es unido a la molécula del lípido A y es catalizada por la dioxigenasa LpxO dependiente de $Fe^{2+}/O_2/\alpha$-cetoglutarato para producir el 2-OH-lípido A-Kdo_2 (fig. 2) [Gibbons et al., 2000; Gibbons et al., 2008]. La expresión del gen *lpxO* se incrementó cuando S. *typhimurium* se creció en bajas concentraciones de Mg^{2+} [Gibbons et al., 2005]. Se ha especulado que los grupos hidroxilos extras podrían incrementar los enlaces de hidrógeno entre las moléculas adyacentes de lípidos que los poseen aumentando la estabilidad e impermeabilidad de la membrana externa a ciertos compuestos en algunas condiciones de crecimiento [Nikaido, 2003; Gibbons et al., 2008].

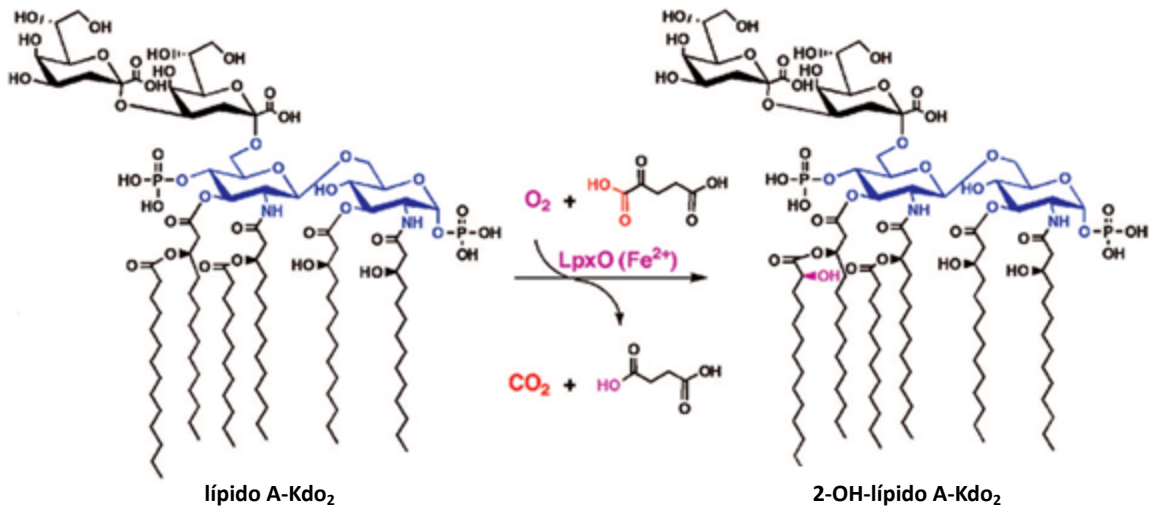
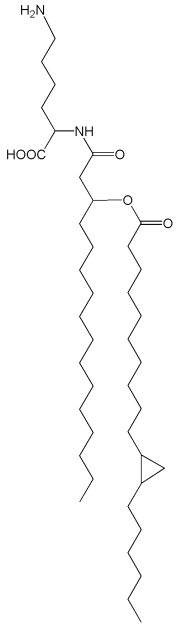


Figura 2. Estructura del lípido A-Kdo_2 de *E. coli* y la reacción catalizada por LpxO propuesta por Gibbons et al. (2008). LpxO cataliza la 2-hidroxilación del residuo de miristato unido al C3 del acilo que está unido a la posición 3´ del lípido A-Kdo_2 hexaacilado para producir el 2-OH-lípido A-Kdo_2. Tomada de Gibbons et al. (2008).

3.1.2.1.2. Lípidos de lisina

Los lípidos de lisina (LLs) se describieron en una cepa de *Agrobacterium tumefaciens* [Tahara et al., 1976a]. La estructura de los LLs se reportó como α-*N*-(aciloxi-acil)-lisina [Tahara et al., 1976a]. En esta estructura el grupo α-amino de la lisina está *N*-acilado con un residuo de palmitato y un segundo acilo está esterificado al C3 del palmitato formando

la estructura aciloxi-acil (fig. 3) [Tahara et al., 1976a]. La configuración del carbono asimétrico del palmitato no se ha determinado. No se han hecho ensayos sobre la bioactividad de estos lípidos y tampoco se conocen los genes involucrados en su biosíntesis.



LL

Figura 3. Lípido de lisina (LL) de *Agrobacterium tumefaciens*. Tomada de Geiger et al. (2010).

3.1.2.1.3. Lípidos de glicina

Los lípidos de glicina (GLs) se identificaron en la bacteria que se mueve por "gliding" *Cytophaga johnsonae* C21 y en la bacteria marina *Cyclobacterium marinus* WH [Kawazoe et al., 1991; Batrakov et al., 1999]. Los GLs son análogos a los LLs descritos anteriormente pero en lugar de la lisina contienen una glicina. La estructura de la especie mayoritaria de GL de *C. marinus* WH se determinó como *N*-[3-D-(13-metiltetradecanoiloxi)-15-metilhexadecanoil]glicina [Batrakov et al., 1999]. En esta estructura (fig. 4) el grupo iso-acilo amidificado está esterificado en el C3 por otro grupo iso-acilo. La configuración absoluta del C3 asimétrico del acilo amidificado se determinó como D [Batrakov et al., 1999].

Los GLs también son llamados citolipinas porque se identificaron inicialmente en el género *Cytophaga* [Batrakov et al., 1999]. Estos constituyen alrededor del 6 y 5 % del total de lípidos en *C. johnsonae* C21 [Kawazoe et al., 1991] y en *C. marinus* WH [Batrakov et al., 1999], respectivamente. Basados en análisis por métodos cromatográficos de varias cepas se asumió que lipoaminoácidos estructuralmente similares a los GLs están presentes en varias bacterias del género *Cytophaga* que se mueven por "gliding" [Kawazoe et al., 1992], y en algunas bacterias Gram negativas de agua dulce pertenecientes a los géneros *Arcocella* [Nikitin et al., 1994] y *Flectobacillus* [Raj y Maloy, 1990] relacionados al género *Cyclobacterium*. Los últimos tres géneros son distintos sistemáticamente de la familia *Cytophagaceae*. Debido a eso se sugirió que los GLs pudieran estar ampliamente distribuidos entre bacterias acuáticas Gram negativas [Batrakov et al., 1999]. Sin embargo, una explicación alternativa podría ser que los genes estructurales que codifican para las proteínas que participan en la biosíntesis de los GLs hayan sido transferidos horizontalmente.

Hasta la fecha los genes involucrados en la biosíntesis de los GLs no se conocen aunque nosotros esperamos que esos genes sean similares a los genes involucrados en la biosíntesis de los lípidos de ornitina, debido a la similitud estructural entre ambas moléculas [Geiger et al., 2010]. Interesantemente, fusiones homólogas del gen *olsB*, que se describirán más adelante, que se han detectado en especies de *Alteromonadales*, también están en los genomas de varios miembros del género *Cytophaga*. Actualmente de los GLs solamente conocemos que funcionan como unidades estructurales de las membranas celulares de algunas bacterias.

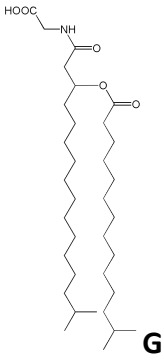


Figura 4. Lípido de Glicina (GL) de *Cyclobacterium marinus* WH. Tomada de Geiger et al. (2010).

3.1.2.1.4. Lípidos de glicinaserina

El lípido de glicinaserina (SGL) se aisló del patógeno oportunista *Flavobacterium meningosepticum* y se le llamo "flavolipina", debido al nombre del género de la bacteria de la cual se aisló por primera vez [Kawai et al., 1988]. La flavolipina se caracterizó por métodos químicos y fisicoquímicos, e inicialmente se propusó la estructura *N*-(3-aciloxi-acil)serina, la cual es incorrecta [Kawai et al., 1988; Shiozaki et al., 1998a] porque le falta un residuo de glicina. Intentos por sintetizar químicamente la flavolipina para estudiar su actividad biológica llevó a la reinvestigación estructural y se determinó que la flavolipina (fig. 5) contenía los aminoácidos serina y glicina condensados y dos grupos acilos, uno amidificado a la glicina y el otro esterificado al C3 del acilo amidificado [Shiozaki et al., 1998b]. La configuración absoluta del C3 asimétrico del acilo amidificado se determinó como (*R*) [Shiozaki et al., 1998b; Gomi et al., 2002]. Flavolipina comparte la estructura básica con los GLs pero tiene un residuo adicional de serina (figs. 4 y 5), lo cual, sugiere que los GLs pudieran ser precursores biosintéticos directos para la formación de flavolipinas en *C. marinus* WH [Batrakov et al., 1999]. A diferencia de *C. marinus* WH, siete especies analizadas del género *Cytophaga* no sintetizan flavolipinas. Hasta la fecha los genes involucrados en la biosíntesis de flavolipina no han sido identificados.

Por cierto tiempo se pensó que flavolipina era exclusiva de especies del género *Flavobacterium*, posteriormente se encontró en *C. marinus* WH [Batrakov et al., 1998] y por lo tanto, es probable que también esté presente en otras bacterias de agua marina. Flavolipina constituye alrededor de 21 y 11 % del total de lípidos de *F. meningosepticum* [Kawai et al., 1988] y de *C. marinus* WH [Batrakov et al., 1998], respectivamente.

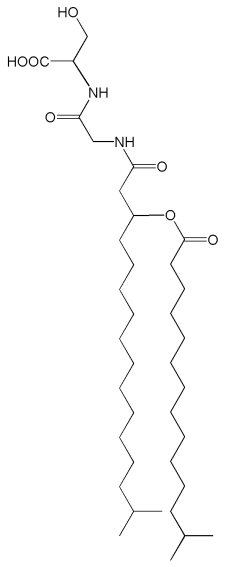


Figura 5. Lípido de glicinaserina (SGL) de *Flavobacterium meningosepticum*. Tomada de Geiger et al. (2010).

3.1.2.1.5. Lípidos de ornitina

3.1.2.1.5.1. Distribución y estructura de los lípidos de ornitina

Los lípidos de ornitina (OLs) están ampliamente distribuidos entre las bacterias Gram negativas y también han sido reportados en algunas Gram positivas, entre ellas especies de *Mycobacterium* y *Streptomyces* [López-Lara et al., 2003] pero están ausentes en arqueas y eucariontes. La estructura de los OLs de diferentes bacterias ha sido reportada como α-*N*-(aciloxi-acil)-ornitina [Knoche y Shively, 1972; Geiger et al., 1999]. Los OLs son análogos a los LLs y a los GLs, descritos anteriormente, pero en lugar de la lisina o glicina contienen un residuo de ornitina. Su estructura consiste de un grupo acilo que está unido al grupo α-amino de la ornitina por enlace amida. Un segundo grupo acilo está ligado por enlace éster al C3 del acilo amidificado (fig. 6). La estructura de los OLs que se ha elucidado por resonancia magnética nuclear es consistente con la que se ha descrito anteriormente [Okuyama y Monde, 1996; Maneerat et al., 2006; Keck et al., 2011]. En algunas bacterias los grupos acilos unidos por enlace éster están hidroxilados en los carbonos de las posiciones 2 o 3 [Asselineau, 1991].

Recientemente también se han reportado acilos amidificados hidroxilados en los OLs de *B. cenocepacia* J2315 [González-Silva et al., 2011] y OLs hidroxilados en el residuo de

ornitina en *R. tropici* CIAT899 [Vences-Guzmán et al., 2011]. Los acilos amidificados de los OLs de diferentes bacterias normalmente son de C16 o de C18 y saturados, mientras que los esterificados son muy variables en su longitud y suelen tener una insaturación o un grupo ciclopropano, y en algunas bacterias ambos acilos son iso-acilos [Palacios-Chaves et al., 2011; González-Silva et al., 2011]. La configuración del C3 asimétrico del acilo amidificado de los OLs es D o (*R*) [Kawai et al., 1999]. Aunque los OLs están presentes en ambas membranas de las bacterias Gram negativas, estos se han encontrado mayoritariamente en la membrana externa [Dees y Shively, 1982; Palacios-Chaves et al., 2011; Vences-Guzman et al., 2011].

3.1.2.1.5.2. Biosíntesis de los lípidos de ornitina

La biosíntesis de un OL estándar se da en dos pasos. En el primer paso, participa la enzima OlsB que es una *N*-aciltransferasa y cataliza la transferencia de un grupo acilo 3-hidroxilado de una proteína acarreadora de acilos (ACP) al grupo α-amino de la ornitina formando el lisolípido de ornitina (LOL) [Gao et al., 2004]. En el segundo paso, la enzima OlsA que es una *O*-aciltransferasa cataliza la transferencia de otro grupo acilo de una ACP al grupo 3-hidroxilo del LOL formando el OL (fig. 6) [Weissenmayer et al., 2002]. La transcripción del gen *olsB* en *S. meliloti* 1021 se incrementa cuando se crece en condiciones limitadas en fósforo y está regulada por el regulador transcripcional PhoB [Krol y Becker, 2004]. La expresión constitutiva de *olsB* en *S. meliloti* 1021 incrementó la formación de OLs independientemente de si se crece en concentraciones altas o bajas de fósforo, sugiriendo que en *S. meliloti* 1021 la formación de OlsB suele ser limitante para la cantidad de OL que se forma en esta bacteria [Gao et al., 2004]. Sin embargo, muchas otras bacterias sintetizan OLs en cantidades relativamente altas aún cuando se crecen en medios de cultivo ricos en fósforo [Palacios-Chaves et al., 2011; Vences-Guzman et al., 2011; González-Silva et al., 2011].

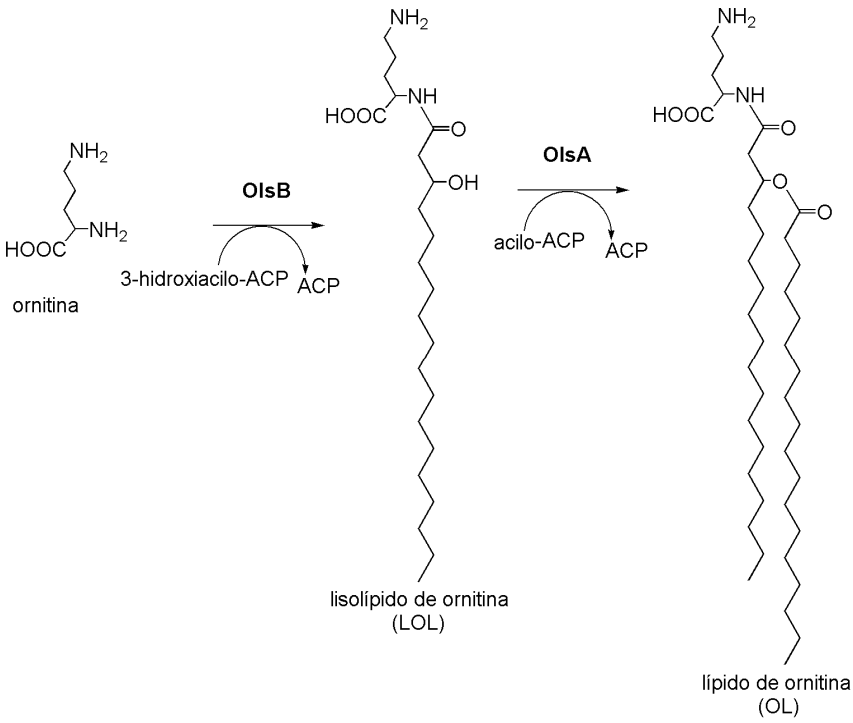


Figura 6. Biosíntesis y estructura del lípido de ornitina de *S. meliloti* 1021. Tomada y traducida de Geiger et al. (2010).

3.1.2.1.5.2.1. La *N*-aciltransferasa OlsB

Mutantes deficientes en el gen *olsB* derivadas de *S. meliloti* [Gao et al., 2004], *Rhodobacter capsulatus* [Aygun-Sunar et al., 2006], *Brucella abortus* [Palacios-Chaves et al., 2011] y *B. cenocepacia* [González-Silva et al., 2011] son incapaces de formar LOLs, OLs y cualquier derivado de estos. La expresión de *olsB* de *S. meliloti* 1021 en *E. coli* causó la formación de LOL [Gao et al., 2004]. El resultado anterior sugiere que OlsB es una *N*-aciltransferasa que condensa ornitina con un grupo acilo 3-hidroxilado para formar el LOL, y por lo tanto, cataliza el primer paso de la biosíntesis de los OLs. Las predicciones indican que la enzima OlsB es de 296 aminoácidos y que es soluble en agua. La caracterización funcional de OlsB definió una función concreta para un grupo completo de proteínas ortólogas (COG3176) previamente asignadas como hipotéticas o como hemolisinas putativas [Gao et al., 2004]. OlsB pertenece a la superfamilia de acil-CoA *N*-aciltransferasas [Gough et al., 2001].

Heath y Rock [1998] describieron un motivo consenso (H(X)4D) común de aciltransferasas de glicerolípidos y demostraron que un cambio en la H conservada de este motivo por una A eliminó la actividad de la glicerol-3-fosfato *O*-aciltransferasa PlsB de *E. coli*. En OlsB, H87 y D92 podría ser ese motivo, el cual, está conservado en las proteínas homólogas a OlsB [Gao et al., 2004]. Una búsqueda en PhyloFacts con OlsB de *B. cenocepacia* J2315 identificó a las sintasas de acil homoserina lactona EsaI [Watson et al., 2002] y LasI [Gould et al., 2004], y las predicciones sugieren que la estructura terciaria de OlsB es similar a la de estas sintasas de autoinductores. Como OlsB, las sintasas de acil homoserina lactona son *N*-aciltransferasas que usan acil-ACPs y un derivado de un aminoácido (SAM) como sustrato (fig. 7). La sintasa FeeM de *N*-acil aminoácido de un microbio de suelo no cultivado se une a la proteína portadora de grupos acilos FeeI catalizando la formación de *N*-acil tirosina y su estructura se asemeja a la de sintasas de acil homoserina lactona EsaI y LasI [Wagoner y Clardy, 2006].

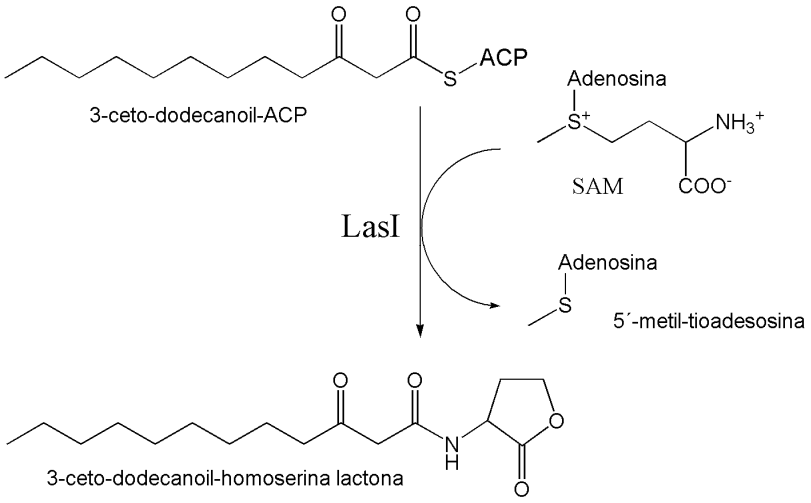


Figura 7. Síntesis de 3-ceto-dodecanoil-homoserina lactona, molécula señal del quórum sensing de *Pseudomonas aeruginosa*. Tomada y traducida de Geiger et al. (2010).

Análisis genómico indica que como muchos otros grupos de bacterias, el orden Rhodobacterales tienen un operón *olsBA* (contiene el *olsB1*) y además tienen otro gen que codifica para otro producto homólogo a OlsB (*olsB2*) (fig. 8). El gen *olsB2* no está localizado

físicamente cercano al operón *olsBA* pero está presente en su genoma. Los homólogos OlsB2 de diferentes Rhodobacterales están más cercanamente relacionados entre ellos que con los verdaderos homólogos OlsB1 (fig.8). Interesantemente, *Rhodobacter sphaeroides* forma lípidos con la estructura aciloxi-acil que contienen glutamina, además, de los OLs conocidos [Zhang et al., 2009]. El primer paso de la biosíntesis de los lípidos que contiene glutamina podría ser catalizado por el homólogo OlsB2 encontrado exclusivamente en Rhodobacterales [Geiger et al., 2010].

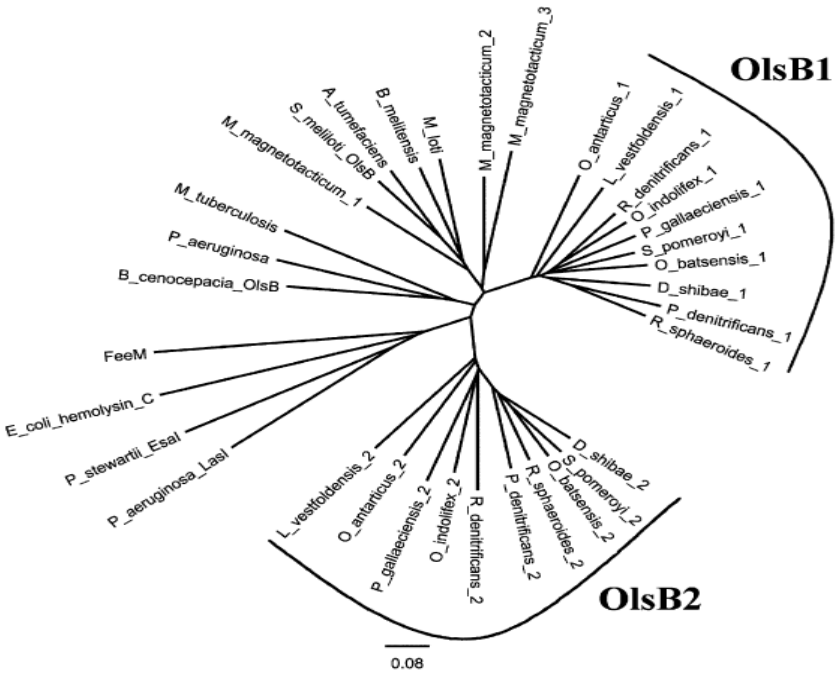


Figura 8. Árbol filogenético sin raíz de OlsB de *Sinorhizobium meliloti*, OlsB de *Burkholderia cenocepacia* y ORFs similares a OlsB. El árbol fue construido usando el programa CLUSTALW (http://www.expasy.ch/). Las distancias entre las secuencias están expresadas como 0.08 cambios por aminoácido. Los números de acceso son los siguientes: OlsB de *Sinorhizobium meliloti* 1021 (NP_384499), OlsB de *Burkholderia cenocepacia* J2315 (YP_002230419), HlyC de *Escherichia coli* CFT073 (NP_755444), EsaI de *Pantoea stewartii* subsp. *stewartii* (AAA82096), LasI de *Pseudomonas aeruginosa* PAO1 (NP_250123), la *N*-aciltransferasa FeeM de una bacteria no cultivada (AAM97306), *Agrobacterium tumefaciens* str. C58 (NP_353376), *Brucella melitensis* 16M (NP_540717), *Dinoroseobacter shibae* DFL 12 (YP_001532815 y YP_001533050), *Loktanella vestfoldensis* SKA53 (ZP_01003503 y ZP_01003690), *Magnetospirillum magnetotacticum* MS-1 (ZP_00050271, ZP_00055184, y ZP_00053729), *Mesorhizobium loti* MAFF303099 (NP_104372), *Mycobacterium tuberculosis* H37Rv (NP_217543), *Oceanicola batsensis* HTCC2597 (ZP_00999281 y ZP_00999194), *Oceanobulbus indolifex* HEL-45 (ZP_02153706 y ZP_02153839), *Octadecabacter antarticus* 307 (EDY79302 y EDY80135), *Paracoccus denitrificans* PD1222 (ZP_00631241 y ZP_00629627), *Phaeobacter gallaeciensis* 2.10 (ZP_02148428 y ZP_02148788), *Pseudomonas aeruginosa* PAO1 (NP_253040), *Rhodobacter sphaeroides* 2.4.1 (YP_354511 y YP_352676), *Roseobacter denitrificans* OCh 114 (YP_682889 y YP_683344), and *Silicibacter pomeroyi* DSS-3 (YP_167215 y YP_167705). OlsB1 y OlsB2 son los dos subgrupos diferentes de homólogos OlsB presentes en las bacterias del orden Rhodobacterales. Tomada y traducida de Geiger et al. (2010).

Los únicos otros ejemplos donde múltiples están presentes homólogos de *olsB* en un mismo genoma son las α-proteobacterias *Magnetospirillum magneticum* y *Magnetospirillum magnetotacticum* (fig. 8). Además de los OLs, otros aminolípidos desconocidos han sido descritos en la membrana del magnetosoma de *Magnetospirillum* los cuales podrían ser sintetizados por el segundo homólogo [Schüler, 2004]. Notablemente, la expresión heteróloga de DNA microbiano extraído directamente de muestras ambientales condujo a la identificación de *N*-acilos de cadena larga derivados de tirosina, fenilalanina, triptófano y arginina, todos ellos tienen actividad antibiótica [Brady et al., 2002; Brady y Clardy, 2005; Clardy y Brady, 2007]. Es posible que algunas de las *N*-aciltransferasas que transfieren acilos cadena larga involucradas en la formación de estos compuestos catalicen el paso inicial en la biosíntesis de otros lípidos de membrana con la estructura aciloxi-acil [Geiger et al., 2010].

Geiger et al. (2010) han especulado que la biosíntesis de OLs pudiera ser más complicada de lo que se ha descrito, debido a que en un análisis de los genomas de bacterias secuenciadas encontraron que varias bacterias del orden *Alteromonadales* y en la ε-proteobacteria *Arcobacter butzleri* la proteína OlsB está fusionada en su *N*-terminal a un dominio de una proteína hipotética de función desconocida.

3.1.2.1.5.2.2. La *O*-aciltransferasa OlsA

Mutantes deficientes en *olsA* derivadas de *S. meliloti* 1021 [Weissenmayer et al., 2002], *R. capsulatus* [Aygun-Sunar et al., 2006], *Brucella abortus* [Palacios-Chaves et al., 2011] y *Pseudomonas aeruginosa* [Lewenza et al., 2011] también fueron incapaces de formar OLs. La sobreexpresión *olsB* en una mutante deficiente de *olsA* derivada de *S. meliloti* 1021 provocó la acumulación del LOL [Gao et al., 2004]. Las predicciones sugieren que OlsA de *S. meliloti* es una proteína de 292 aminoácidos con una probable hélice transmembranal cercana al *N*-terminal.

Un alineamiento de OlsA con algunas enzimas procariotas que exhiben las actividades de aciltransferasas de ácido lisofosfatídico [Aygun-Sunar et al., 2006] muestra que hay principalmente dos regiones bien conservadas (los aminoácidos 67-83 y 139-154) donde OlsA tiene la mayor similitud con otros miembros de ese grupo. En aciltransferasas de

ácido lisofosfatídico, dos motivos, NHQS y PEGTR, están conservados [West et al., 1997], y se encuentran en formas modificadas (NHVS, aminoácidos 72-75; PEGTT, aminoácidos 143-147) en la secuencia de OlsA [Weissenmayer et al., 2002]. Basados en el motivo (H(X)4D) común a aciltransferasas de glicerolípidos en OlsA, H73 y D78 podrían formar ese motivo. Es sorprendente, sin embargo, que las secuencias que codifican para las enzimas con actividades de aciltransferasas de ácido lisofosfatídico en general son muy diferentes. Por ejemplo, en la bacteria *Neisseria meningitidis* hay tres enzimas (NlaA, NlaB, y la tercera actividad que se detectó en la mutante doble deficiente en *nlaA* y *nlaB*) con actividad de aciltransferasas de ácido lisofosfatídico in vitro [Shih et al., 1999]. Aunque NlaA y NlaB son del mismo organismo, ellas son muy diferentes y es interesante observar que mutantes deficientes en *nlaA* y mutantes deficientes en *nlaB* muestran diferentes fenotipos sugiriendo que NlaA y NlaB realizan diferentes reacciones bioquímicas in vivo en *N. meningitidis*.

Por su secuencia, OlsA claramente se agrupa dentro de las aciltransferasas de ácido lisofosfatídico caracterizadas. Sin embargo, mutantes deficientes en *olsA* de *S. meliloti* son incapaces de formar OLs, y no muestran acumulación de ácido lisofosfatídico ni debilitamiento en la biosíntesis de glicerofosfolípidos. Por lo tanto, debe de haber una actividad idéntica a la de PlsC en *S. meliloti* responsable de estas últimas funciones, presumiblemente SMc00714 [Basconcillo et al., 2009]. OlsA es requerida para la actividad de *O*-aciltransferasa dependiente de acil-AcpP y LOL, que convierte el LOL en OL [Weissenmayer et al., 2002]. Además de OlsA, *Pseudomonas fluorescens* posee dos homólogas a aciltransferasas de ácido lisofosfatídico, HdtS y PatB [Cullinane et al., 2005]. HdtS y PatB son capaces de complementar el crecimiento de una mutante deficiente en PlsC de *E. coli*, mientras que OlsA de *P. fluorescens* no complementa. Aunque HdtS o PatB pueden realizar la función de PlsC in vivo ellas no son idénticas funcionalmente. Mutantes carentes de PatB muestran crecimiento reducido en altas temperaturas mientras que mutantes deficientes en HdtS están afectadas en crecimiento, motilidad y tienen reducida cantidad de ácido *cis*-vaccénico [Cullinane et al., 2005].

También *Rhodobacter capsulatus* posee tres homólogos a aciltransferasas de ácido lisofosfatídico, OlsA, PlsC316 y PlsC3498 [Aygun-Sunar et al., 2007]. OlsA y PlsC316 son capaces de complementar el crecimiento de una mutante deficiente de PlsC de *E. coli*, mientras que PlsC3498 no complementa. Una mutante deficiente de PlsC316 tiene cantidades reducidas de ácidos grasos C16 [Aygun-Sunar et al., 2007]. Se ha observado que OlsA de *R. capsulatus* también es capaz de acilar a 1-acilo-*sn*-glicerol-3-fosfato además de al LOL, exhibiendo especificidad relajada hacia el sustrato aceptor de acilos [Aygun-Sunar et al., 2007]. Se espera que estudios futuros más detallados con el grupo actual de "aciltransferasas de ácido lisofosfatídico" revelaran numerosos subgrupos con actividades bioquímicas ligeramente diferentes [Geiger et al., 2010].

Actualmente, se conoce poco sobre las funciones que desempeñan los OLs en las bacterias que los poseen. Las mutantes de *S. meliloti* [Weissenmayer et al., 2002; López-Lara et al., 2005] y de *P. aeruginosa* [Lewenza et al., 2011] deficientes en la biosíntesis de OLs no muestran ninguna alteración sobresaliente en su fenotipo macroscópico cuando se compara con la cepa silvestre. Solamente en una doble mutante de *S. meliloti* deficiente en la biosíntesis de OLs y de DGTS se observó reducida producción celular cuando se creció en condiciones limitadas en fósforo [López-Lara et al., 2005]. En *R. capsulatus*, los OLs se requieren para el óptimo estado de equilibrio de las cantidades de citocromos tipo-*c* en la membrana [Aygun-Sunar et al., 2006]. Estudios en otras bacterias han correlacionado un incremento en la resistencia a péptidos antimicrobiales con la producción OLs [Minnikin y Abdolrahimzadeh, 1974; Dorrer y Teuber, 1977]. Pero en estudios recientes han demostrado que en *Brucella abortus* y *P. aeruginosa* los OLs no se requieren para incrementar esa resistencia [Palacios-Chaves et al., 2011; Lewenza et al., 2011].

3.1.2.1.5.2.3. Las hidroxilasas de lípidos de ornitina OlsC y OlsE

En algunas especies de los géneros *Burkholderia*, *Flavobacterium* [Asselineau, 1991; Kawai et al., 1988], *Thiobacillus* [Knoche y Shively, 1972], *Gluconobacter* [Tahara et al., 1976b], *Streptomyces* [Asselineau, 1991], *Ralstonia* [Galbraith et al., 1999] y *Rhizobium* [Vences-Guzmán et al., 2011], existen OLs en los cuales el grupo acilo unido por enlace

éster posee un grupo hidroxilo en el C2 (2-OH-OLs). Estos acilos esterificados son similares a los que existen en el lípido A de algunas bacterias, mencionados anteriormente (fig. 2). Los residuos de acilo 2-OH no se forman mediante la biosíntesis normal de ácidos grasos y una actividad enzimática específica es necesaria para formar el grupo hidroxilo en el C2 de un residuo de ácido graso.

Rhizobium tropici CIAT899 es un simbionte de plantas de frijol, tolerante a pHs ácidos y a altas temperatura [Martínez-Romero et al., 1991; Vences-Guzmán et al., 2011] forma cuatro clases moleculares de OLs (nombrados S1, S2, P1 y P2) [Rojas-Jiménez et al., 2005]. Una mutante deficiente en *olsC* (899-*olsCΔ1*) no forma P1 ni P2. El producto codificado por *olsC* es una putativa dioxigenasa similar a LpxO que puede convertir las dos formas menos polares de los OLs (sustratos, S1 y S2) a las dos formas más polares (productos, P1 y P2) [Rojas-Jiménez et al., 2005]. Recientemente se demostró que la conversión de S1 y S2 a P1 y P2, respectivamente, es por una hidroxilación en el C2 del acilo esterificado [Vences-Guzmán et al., 2011].

Una predicción indica que OlsC de *R. tropici* CIAT899 es una proteína soluble en agua de 281 aminoácidos [Rojas-Jiménez et al., 2005]. Recientemente también se identificó el gen *olsE* que codifica para la enzima que modifica a los OLs S1 para convertirlo en los OLs S2 [Vences-Guzmán et al., 2011]. OlsE presumiblemente hidroxila a los OLs S1 en el residuo de ornitina y se ha especulado que probablemente OlsE también modifica a los OLs P1 para convertirlos en los OLs P2 [Vences-Guzmán et al., 2011]. Las predicciones indican que la proteína OlsE (331 aminoácidos) es muy hidrofóbica y puede formar entre 4 y 6 hélices transmembranales [Vences-Guzmán et al., 2011]. La mutante en *olsE* (MAV04) no formó los OLs S2 y P2, y la doble mutante *olsC*/*olsE* (MAV05) no formó a los OLs S2, P1 y P2 [Vences-Guzmán et al., 2011]. En *R. tropici* CIAT899 las cuatro clases de OLs se localizan principalmente en la membrana externa [Vences-Guzmán et al., 2011].

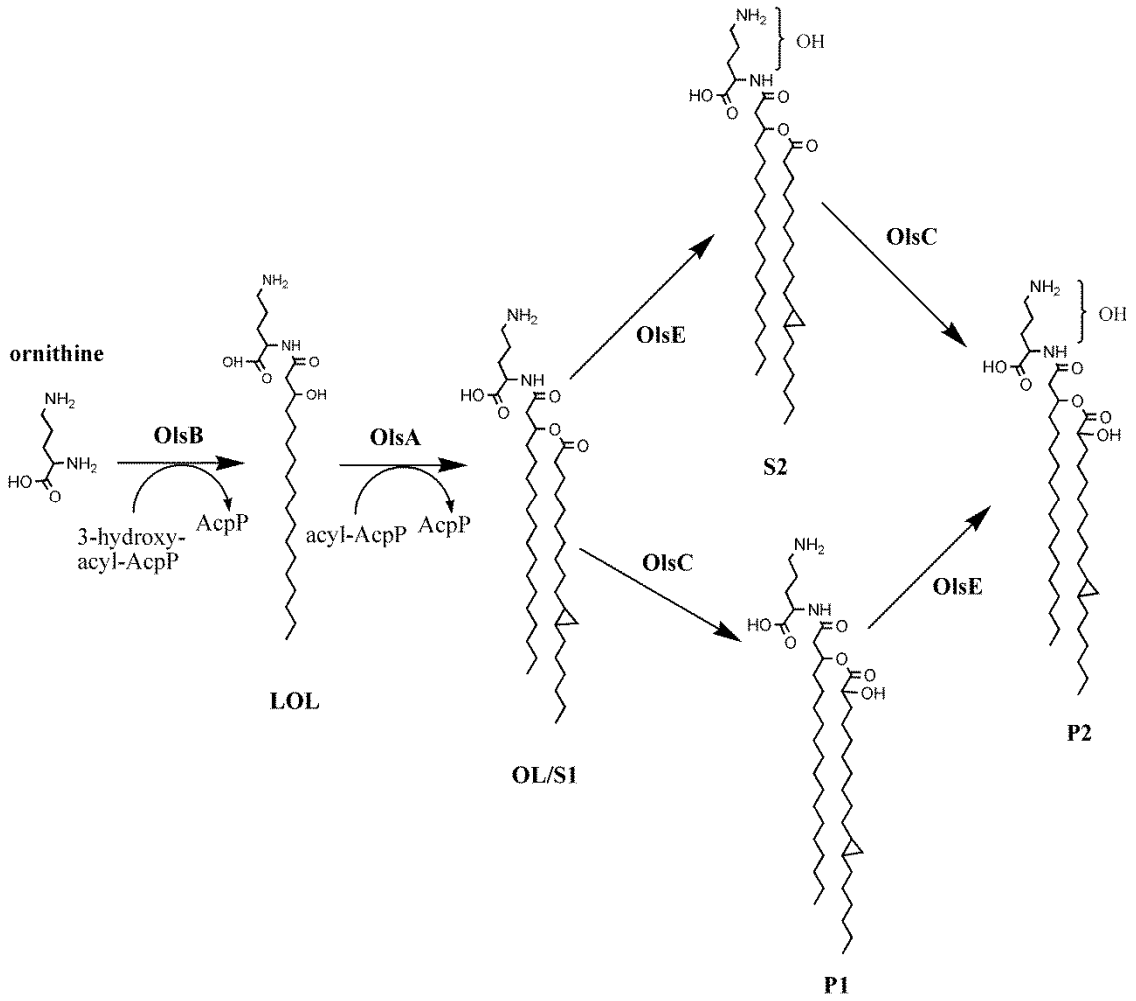


Fig. 9. Biosíntesis de los lípidos de ornitina en *Rhizobium tropici* CIAT899. Los genes que codifican para las enzimas OlsB y OlsA se identificaron por primera vez en *S. meliloti* 1021 [Weissenmayer et al., 2002; Gao et al., 2004], mientras que el gen que codifica para la hidroxilasa OlsC de OLs se describió por primera vez en *R. tropici* CIAT899 [Rojas-Jiménez et al., 2005]. Vences-Guzmán et al. (2011) determinaron que OlsC hidroxila al C2 del ácido graso secundario y también identificaron el gen que codifica para la hidroxilasa OlsE, la cual introduce un grupo hidroxilo en el residuo de ornitina de los OLs. Lisolípido de ornitina (LOL), lípido de ornitina (OL). Tomada y traducida de Vences-Guzman et al. (2011).

Notablemente, grupos 2-OH en acilos esterificados han sido reportados en los esfingolípidos y PE de bacterias, otros componentes mayoritarios de la membrana externa de bacterias Gram negativas. Especies moleculares de PE hidroxilada en el C2 del acilo unido a la posición *sn*-2 (2-OH-PE) se forman en miembros del género *Burkholderia* [Phung et al., 1995; Taylor et al., 1998]. En *Burkholderia cepacia* NCTC 10661 los lípidos 2-hidroxilados (2-OH-OLs y 2-OH-PE) se incrementaron cuando esa cepa creció en altas temperaturas [Taylor et al., 1998]. La bacteria *Thiobacillus thiooxidans* crece en ambientes

muy ácidos y debido a la localización de OLs en la membrana externa se especuló que esos lípidos podrían estar involucrados en la resistencia a acidez [Dees y Shively, 1982].

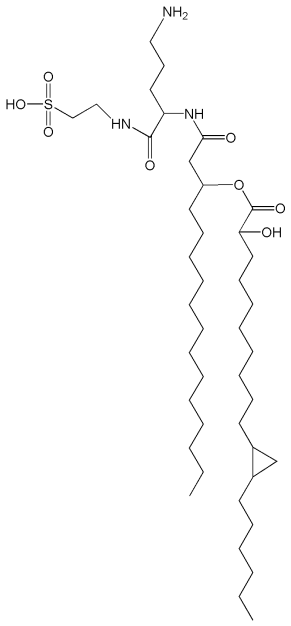
Rojas-Jiménez et al. (2005) reportaron que la cepa 899-*olsCΔ1* creció a pH 4.5 de una forma similar a la cepa silvestre, ambas cepas alcanzaron una OD a 600 nm de aproximadamente 1.6. Sin embargo, la complementación de la mutante en *olsC* a pH 4.5 con un fragmento que contenía a *olsC* en un vector de alto número de copias, afectó seriamente el crecimiento de dicha cepa alcanzando una OD a 600 nm menor a 0.4 [Rojas-Jiménez et al., 2005]. Al crecer en medio TY y marcar los lípidos radiactivamente de la cepa 899-*olsCΔ1* complementada, Rojas-Jiménez et al. (2005) reportaron que esta cepa formó mayoritariamente los OLs P1 y P2, y una ausencia casi total de S1 y S2. La cepa 899-*olsCΔ1* derivada de *R. tropici* CIAT899 formó nódulos en las plantas de frijol poco desarrollados 21 días después de la inoculación con la bacteria, los nódulos carecían de lenticelas y fijaron dos veces menos nitrógeno [Rojas-Jiménez et al., 2005].

Recientemente Vences-Guzmán et al. (2011) reportaron estudios más detallados del perfil lipídico de *R. tropici* CIAT899 y de las cepas mutantes (899-*olsCΔ1*, MAV04 y MAV05) crecidas en medio TY a diferentes temperaturas (30 ^{o}C, 37 ^{o}C y 42 ^{o}C) y pHs (7.0, 4.5 y 4.0). Mutantes en *olsC* que no hidroxilan OLs son más sensibles a estrés térmico y ácido, y las tres mutantes carentes de hidroxilasas de OL establecieron una simbiosis deficiente [Vences-Guzmán et al., 2011]. Las diferencias obtenidas entre los estudios de Rojas-Jiménez et al. (2005) y el de Vences-Guzmán et al. (2011) posiblemente se deben a diferencias en los medios de cultivo [Vences-Guzmán et al., 2011].

3.1.2.1.5.3. Lípidos de ornitinataurina hidoxilados

En *Gluconobacter cerinus*, los 2-OH-OLs son parcialmente modificados con un residuo de taurina el cual está ligado por enlace amida a el grupo α-carboxilo de la ornitina (fig. 10) [Tahara et al., 1978]. Se ha observado que una fracción particular de *G. cerinus* requiere ATP y Mn^{2+} para condensar taurina con los 2-OH-OLs para formar los lípidos que contienen ornitinataurina 2-hidroxilados (2-OH-TOLs). Los 2-OH-TOLs han sido nombrados cerilipina debido al nombre de la bacteria de la cual se aisló [Tahara et al., 1978].

Actualmente desconocemos el gen que codifica para la enzima que hace la actividad de condensar taurina con los 2-OH-OLs.



2-OH-TOL

Figura 10. Lípido de ornitinataurina 2-hidroxilado (2-OH-TOL) de *Gluconobacter cerinus*. Tomada de Geiger et al. (2010).

3.1.2.2. La respuesta inmune innata hacia los lípidos de membrana con la estructura aciloxi-acil de bacterias

Los lípidos de membrana con la estructura aciloxi-acil de bacterias inducen drásticamente la respuesta inmune de mamíferos al ser reconocidos por los receptores tipo toll (TLRs) como patrones moleculares asociados a patógenos. El ejemplo mejor estudiado es la inducción que ocasiona el lípido A, la parte reactiva del LPS, que estimula a las células de la inmunidad innata usando como mediadores de la señal al TLR4 y al factor nuclear κB. En la ruta de señalización del lípido A también está involucrada la proteína MD-2, la cual, se asocia físicamente con TLR4 en la superficie celular, confiriéndoles sensibilidad a los receptores TLR4s hacia las moléculas de LPSs [Shimazu et al., 1999]. Al igual que el lípido A de *E. coli*, la estructura 3-aciloxi-acilamida con la configuración (*R*) en su carbono asimétrico también está presente en los GLs [Batrakov et al., 1999], SGLs [Shiozaki et al., 1998ab; Gomi et al., 2002] y en OLs [Kawai et al., 1999] (figs. 2, 4, 5 y 6).

Sin embargo, solamente se ha evaluado la capacidad de inducir la respuesta inmune innata de los SGLs y de los OLs. La respuesta inmune innata se midió por la producción de prostaglandina E2, interleucina 1β y del factor α de la necrosis tumoral cuando los lípidos se expusieron a los macrófagos [Kawai y Akagawa 1989]. Un estudio reciente sugiere que el estado físico en el que se aplican el lípido A y el OL a los macrófagos, afecta la actividad biológica de estos lípidos [Okemoto et al., 2008]. Los OLs y los SGLs pueden ser usados como coadyuvantes [Kato y Goto, 1997; Kawai et al., 1999; Kawai et al., 2002] y cuando se inyectan en ratones antes que el lípido A puede prevenir los efectos letales de este último, aunque el efecto protector de SGLs fue más débil y debido a eso se especuló que los OLs y SGLs podrían funcionar como antagonistas bloqueadores de los eventos provocados por el agonista lípido A [Kawai et al., 1992].

Como el lípido A, la señal que causa la respuesta inmune innata por SGLs es traducida vía el complejo TLR4/MD-2 [Gomi et al., 2002]. La señalización mediada por el complejo TLR4/MD-2 se incremento por los SGLs cuando se expresó CD14 [Kawasaki et al., 2003]. La proteína CD14 actúa como un correceptor (junto con el TLR4 y MD-2) para la detección del LPS. Kawasaki et al. (2003) también demostró que la configuración (*R*) del residuo lipídico de los SGLs es esencial para la inducción de la señal mediada por el complejo TLR4/MD-2, y que la configuración del residuo de serina y la secuencia de aminoácidos en el dominio de aminoácidos no son importantes para la inducción de la señal. Los componentes que participan en traducción de la señal inducida por los OLs aún no han sido identificados y sugerimos que debido a la similitud estructural de los OLs con el lípido A y con los SGLs, probablemente los OLs usan los mismos componentes que el lípido A y los SGLs ya que la respuesta inmune que provocan también es similar.

4. Antecedentes

La bacteria Gram negativa *Burkholderia cenocepacia* J2315 es un miembro del complejo *Burkholderia cepacia* (BCC), un subgrupo de importantes patógenos oportunistas muy virulentos del género *Burkholderia* que infecta a humanos con fibrosis quística, con la enfermedad granulomatosa crónica y a individuos inmunosuprimidos causando una alta tasa de mortalidad [Mahenthiralingan et al., 2005]. Aunque se conocen muchos factores que contribuyen a la virulencia del BCC, frecuentemente infecciones con el BCC no son eficientemente eliminadas por los tratamientos con antibióticos comunes.

Los lípidos de membrana de *B. cenocepacia* J2315 son: CL, PG, PE y OLs, así como los derivados atípicos 2-hidroxilados de PE y OLs (2-OH-PE y 2-OH-OLs, respectivamente), los cuales difieren de las versiones estándar por poseer un grupo hidroxilo en el C2 (2-OH), que sustituye a un átomo de hidrógeno, en un residuo de acilo esterificado [Phung et al., 1995; Taylor et al., 1998]. Un OL estándar se compone de un residuo de ornitina y dos residuos de acilos sin grupos hidroxilos (fig. 6), uno de estos acilos está amidificado al grupo α-amino de la ornitina y el otro acilo está esterificado al C3 del acilo amidificado [Knoche y Shively, 1972; Geiger et al., 1999].

La ruta biosintética para los OLs sin hidroxilaciones se elucidó en *S. meliloti* 1021 y consiste de dos pasos. En el primer paso, la enzima OlsB que es una *N*-aciltransferasa cataliza la transferencia de un grupo acilo 3-hidroxilado de una ACP al grupo α-amino de la ornitina formando el lisolípido de ornitina (LOL) [Gao et al., 2004]. En el segundo paso, la enzima OlsA que es una *O*-aciltransferasa cataliza la transferencia de un segundo grupo acilo de una ACP al grupo 3-hidroxilo del LOL formando el OL (Fig. 6) [Weissenmayer et al., 2002]. *B. cenocepacia* J2315 tiene los genes ortólogos que codifican para las proteínas OlsA y OlsB. En este trabajo mostramos que *bcal1281* codifica para la *N*-aciltransferasa OlsB y probablemente *bcal3137* codifica para la *O*-aciltransferasa OlsA.

En 2-OH-PE de *B. cenocepacia* J2315 el residuo de acilo 2-OH está ligado exclusivamente a la posición *sn*-2 en ese glicerofosfolípido, mientras que en los 2-OH-OLs los residuos de acilos 2-hidroxilados son los acilos esterificados [Phung et al., 1995; Taylor et al., 1998]. Los residuos de acilo 2-hidroxilados no se forman durante la biosíntesis

estándar de ácidos grasos y se requieren actividades enzimáticas específicas para formar un grupo hidroxilo en el C2.

En *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) una proteína (LpxO) se identificó como la responsable de formar el grupo 2-OH en el residuo de miristato esterificado al ácido graso de la posición 3´del lípido A [Gibbons et al., 2000]. La reacción de hidroxilación es catalizada por la dioxigenasa LpxO dependiente de $Fe^{2+}/O_2/\alpha$-cetoglutarato (fig. 2) [Gibbons et al., 2008]. Se ha especulado que tales grupos hidroxilos extras podrían incrementar los enlaces de hidrógeno entre las moléculas adyacentes de lípido A aumentando la estabilidad de la membrana externa y consecuentemente disminuyendo la permeabilidad a ciertos compuestos en algunas condiciones de crecimiento [Nikaido, 2003; Gibbons et al., 2008]. Una enzima homóloga a LpxO, OlsC de *R. tropici* CIAT899 está involucrada en la formación de las dos clases de OLs más polares (P1 y P2), a partir de las dos menos polares (S1 y S2), existentes en ese organismo [Rojas-Jiménez et al., 2005]. Mientras se desarrollaba este trabajo, Vences-Guzmán et al. (2011) reportaron que OlsC hidroxila in vivo a los lípidos S1 y S2 en el C2 del acilo esterificado para convertirlos en P1 y P2, respectivamente.

Como el lípido A, los OLs están en la membrana externa [Dees y Shively, 1982; Palacios-Chaves et al., 2011; Vences-Guzmán et al., 2011] y por lo tanto en la superficie celular. Cuando las bacterias entran a un mamífero, sus patrones moleculares asociados a patógenos de la superficie celular activan el sistema inmune de los mamíferos y normalmente las bacterias son fagocitadas por los macrófagos y dirigidas al compartimento lisosomal para digerirlas. En ese compartimento los nutrimentos son restrictivos para las bacterias y en esas condiciones teóricamente se favorece la producción de OLs. Por lo tanto, los OLs podrían desempeñar papeles protagónicos para la sobrevivencia en el interior de macrófagos de algunas bacterias que poseen la capacidad de sintetizarlos.

La función que desempeña la modificación 2-OH en PE y OLs en *B. cenocepacia* J2315 no se conoce. Para estudiarla optamos por tratar de identificar los genes que potencialmente podrían hacer la modificación 2-OH en los residuos de acilo 2-

hidroxilados, generar mutantes en esos genes y crecerlas en diferentes condiciones. El genoma de *B. cenocepacia* J2315 contiene dos marcos abiertos de lectura (BCAM1214 y BCAM2401 [OlsD]) homólogos a LpxO de *S. typhimurium*, anotados como putativas β-hidroxilasas.

5. Hipótesis:

Los 2-OH-OLs en *Burkholderia cenocepacia* J2315 se forman a partir de OLs no hidroxilados mediante una nueva 2-hidroxilasa homóloga a LpxO de *Salmonella typhimurium*.

6. Objetivo general:

Identificar genes que codifiquen para enzimas involucradas en hidroxilar a los OLs de *B. cenocepacia* J2315 y determinar en qué parte de la molécula de los OLs los hidroxilan, así como caracterizar las enzimas que tengan esa capacidad.

6.1. Objetivos particulares:

1. Determinar si las putativas hidroxilasas de *B. cenocepacia* J2315 homólogas a LpxO, hidroxilan a los OLs.
2. Determinar la parte de las moléculas de los OLs hidroxilada por la(s) hidroxilasa(s).
3. Desarrollar un ensayo enzimático para la(s) hidroxilasa(s).
4. Construir mutantes de *B. cenocepacia* J2315 en los genes homólogos a *lpxO* de *S. typhimurium* así como en el gen *olsB* y analizar los fenotipos de las mutantes generadas.

7. Procedimientos experimentales de los resultados adicionales

7.1. Deacilación química de lípidos de ornitina

Se modificó OL radiactivo in vitro con OlsD, como se ha descrito previamente [González-Silva at al., 2011] y los lípidos modificados y no modificados se extrajeron por el método de Bligh y Dyer (1959). Después se separaron por TLC con el sistema de solventes cloroformo: metanol: ácido acético glacial (130:50:20, v/v/v). El gel de silica que contenía los lípidos se raspó y los lípidos se extrajeron del gel con el método de Bligh y Dyer (1959). Posteriormente los lípidos se secaron y se sometieron a un tratamiento alcalino suave (KOH 0.3 M en metanol a 37 ^{o}C por 3 h); las condiciones anteriores hidrolizan los enlaces éster. Después el pH del hidrolizado se neutralizó con ácido acético glacial y el metanol de las muestras se volatilizó con un flujo de nitrógeno. Posteriormente, los lípidos se extrajeron con acetato de etilo, se disolvieron en metanol: cloroformo (1:1, v/v), y una alícuota se cuantificó en el contador de centelleo. Subsiguientemente se cargaron en la capa fina alrededor de 11,000 cuentas por minuto (cpm) de los OLs no modificados y modificados, y 23,000 cpm de los LOLs no modificados y modificados. Posteriormente se corrió la capa fina con el sistema de solventes cloroformo: metanol: acido acético glacial (130:50:20, v/v/v). Por último, la capa fina se expuso a una pantalla del Phosphoimager y se escaneó 3 días después.

7.2. Ensayo in vitro con OlsD usando como sustrato a los 2-OH-OLs

Al introducir el plásmido pSphx04 que contenía al gen *olsD* en la cepa silvestre *B. cenocepacia* J2315 se formaron dos nuevos lípidos de membrana (NL1 y NL2), y el análisis por espectrometría de masas de NL1 y NL2 sugería que estos lípidos tenían sustituido un átomo de hidrógeno por un grupo hidroxilo en un carbono del acilo amidificado [González-Silva et al., 2011]. Este resultado indicaba que probablemente OlsD puede usar como sustratos a las dos clases de lípidos de ornitina (OLs y 2-OH-OLs) que produce esta cepa [González-Silva et al., 2011]. Para reafirmar lo anterior, procedimos a realizar un ensayo enzimático in vitro con OlsD usando como sustrato a los 2-OH-OLs de *B. cenocepacia* J2315. El sustrato (2-OH-OLs) se preparó a partir 10 ml de cultivo en LB de la cepa *B. cenocepacia* pNG24, marcado con 10 μCi de acetato de sodio [1-^{14}C] (60 mCi/mmol) y se

cosechó a una absorbancia de 1.0 a 620 nm. Los lípidos totales de membrana se extrajeron por el método de Bligh y Dyer (1959). La fase de cloroformo se separó por cromatografía en capa fina de dos dimensiones (2D-TLC) [González-Silva et al., 2011]. Los 2-OH-OLs radiactivos fueron localizados por autoradiografía y extraídos de la fracción de gel de la silica, y los 2-OH-OLs se diluyeron en una mezcla de metanol: cloroformo (1:1, v/v). Después una alícuota se cuantificó en el contador de centelleo. Posteriormente el ensayo se realizó similarmente a como lo reportaron previamente [González-Silva et al., 2011]. La diferencia consistió en usar como sustrato a los 2-OH-OLs en lugar de los OLs y en hacerlo a un pH de 7.5. Después del ensayo, los lípidos radiactivos se extrajeron y se separaron por TLC usando la siguiente mezcla de solventes cloroformo: metanol: ácido acético (130:50:20, v/v/v), y los lípidos se detectaron y cuantificaron con el phosphorimager.

7.3. Inactivación de *bcam1214*

La expresión heteróloga del gen *bcam1214* en *S. meliloti* 1021 y en *B. cenocepacia* J2315 sugirió que este gen no estaba involucrado en modificar a los OLs ni a PE. Para demostrar convincentemente lo anterior se procedió a generar una mutante por remplazo génico en dicho gen. Los oligonucleótidos oLOP91 (ATGTTGATATCTGATGGTGTATCCGATGGG) y oLOP92 (AAAGGATCCTATGAAAAGCAGTTCAGCG) que introducen los sitios de corte *Eco*RV y *Bam*HI (subrayados), respectivamente, se usaron en la reacción en cadena de la polimerasa (PCR) para amplificar un fragmento de 1076 pares de bases (pb) localizado río arriba del gen *bcam1214*. Similarmente, los oligonucleótidos oLOP147 (AAGGGATCCATCGTCGTCGACGGCGAATC) y oLOP148 (ACTCTCTAGATCCTC GATCGCACGGTGGTC) que introducen los sitios de corte *Bam*HI y *Xba*I (subrayados), respectivamente, se usaron en la PCR para amplificar un fragmento de 641 pb que comprende parte del *bcam1214* y de su región río abajo. El producto de la PCR amplificado con oLOP91 y oLOP92 se digirió con *Eco*RV/*Bam*HI y se clonó en pBluescriptSK+ previamente digerido con las mismas enzimas, resultando el plásmido pNG12. El producto

de la PCR amplificado con oLOP147 y oLOP148 se digirió con *Bam*HI/*Xba*I, y se clonó en pUC18 previamente digerido con las mismas enzimas, resultando el plásmido pNG49.

Por secuenciación de los insertos de los plásmidos pNG12 y pNG49 se confirmó que se habían clonado los fragmentos deseados. El plásmido pNG49 se movilizó a la cepa JM110 (no metila el DNA) debido a que en DH5α se metila el último nucleótido (A) del sitio de corte *Xba*I, lo cual no permitía liberar el inserto. Posteriormente el plásmido pNG12 se digirió con *Bam*HI y *Xba*I y se ligó con el fragmento de DNA de 641 pb obtenido después de la digestión con *Bam*HI/*Xba*I de pNG49 derivado de JM110, resultando el plásmido pNG51. Después el plásmido pNG51 se linearizó con *Bam*HI y se ligó con el fragmento *Bam*HI del pCAT que contiene el gen para la cloranfenicol acetiltransferasa (*cat*) que confiere resistencia a cloranfenicol. El plásmido resultante contenía las regiones flanqueantes de *bcam1214* interrumpidas por un gen que confiere resistencia a cloranfenicol y se llamó pNG53. El plásmido pNG53 se movilizo a la cepa JM110. Después el pNG53 se extrajo de esa cepa, se digirió con *Eco*RV*/Xba*I para reclonar las regiones flanqueantes de *bcam1214* y el gen que confiere resistencia a cloranfenicol localizado entre las dos regiones. El fragmento *Eco*RV*/Xba*I se clonó en el vector suicida pK18*mobsacB* [Schäfer et. al., 1994] que previamente se digirió con *Sma*I/*Xba*I, produciendo el plásmido pNG55.

El plásmido pNG55 se introdujo en *B. cenocepacia* J2315 (cepa silvestre) mediante una cruza biparental, usando como donadora a la cepa de *E. coli* S17-1. Las transconjugantes se seleccionaron en el medio de cultivo LB con cloranfenicol y ácido nalidíxico, lo que también permitió contraseleccionar a *E. coli* S17-1. Posteriormente una recombinante sencilla se creció en condiciones no selectivas en LB hasta alcanzar una absorbancia de 1.0 a 620 nm, se hicieron diluciones y se sembraron en LB con sacarosa al 10 % (peso/volumen) y cloranfenicol para seleccionar recombinantes dobles. Después de 8 días de crecimiento en el medio selectivo, las 5 colonias que crecieron más rápido se seleccionaron para analizar su genotipo por hibridación tipo Southern. En la hibridación tipo Southern se usó como sonda marcada con digoxigenina el fragmento de 1076 pb, localizado río arriba del gen *bcam1214.* El DNA de la cepa silvestre y de las candidatas a

mutantes en el gen *bcam1214* se digirió con *Nco*I y *Pst*I. Se esperaba que la sonda en la cepa silvestre hibridara con un fragmento de 1654 pb y en las candidatas a mutantes con uno de 2130 pb. Después de obtener las mutantes se analizó su perfil de lípidos de membrana como se ha descrito anteriormente [González-Silva et al., 2011].

7.4. Clonación y expresión de los genes *bcal0511* y *bcam2775* de *B. cenocepacia* J2315 homólogos a PHYH

Ninguno de los dos genes de *B. cenocepacia* J2315 homólogos a *lpxO* (*bcam1214* y *olsD*) fueron responsables de codificar para una enzima que modificara con un grupo hidroxilo al C2 del acilo esterificado de los OLs o de PE de *B. cenocepacia* J2315. Nosotros también estudiamos otros genes candidatos que podrían codificar para enzimas responsables de hacer 2-hidroxilaciones en el ácido graso esterificado de los OLs. Ellos son los que codifican productos homólogos a la enzima peroxisómica fitanoil-CoA hidroxilasa (PHYH) de humano, la cual cataliza la α-oxidación (2-hidroxilación) del ácido fitánico proveniente del consumo de ciertos alimentos [Jansen et al., 1997; Jansen et al., 2000]. En el genoma de *B. cenocepacia* J2315 se identificaron dos genes que codifican para productos homólogos a PHYH de humano: *bcal0511* y *bcam2775*, y se procedió a clonarlos y a expresarlos.

Los oligonucleótidos oLOP107 (AGGAATACATATGTCGTCTCCATTGCAGTCGG) y oLOP108 (AAAGGATCCCTAGAACTGCACTTCCGGC) que introducen sitios de corte *Nde*I y *Bam*HI (subrayados), respectivamente, se usaron en la PCR para amplificar el gen *bcal0511* (774 pb). Los oligonucleótidos oLOP97 (AGGAATACATATGACTCATTCGATCGACAGG) y oLOP98 (AAATAAGATCTCAGATCGCGCCGACCTGCC) que introducen sitios de corte *Nde*I y *Bgl*II (subrayados), respectivamente, se usaron en la PCR para amplificar el gen *bcam2775* (875 pb). El gen *bcam2775* se encuentra en la hebra reversa y su primer nucleótido era G, en el oligonucleótido oLOP97 se sustituyó por A, para poderlo clonar con el sitio de corte *Nde*I y quedara direccionalmente en fase con el marco de lectura del vector de expresión pET9a. En ambas reacciones de PCR se usó como molde DNA genómico de *B. cenocepacia* J2315. Después de la amplificación los productos se digirieron con las correspondientes

enzimas y los fragmentos obtenidos se clonaron en pET9a, resultando los plásmidos pNG26 y pNG18, respectivamente.

Por secuenciación de los insertos de los plásmidos se confirmó que pNG26 porta el gen *bcal0511* y pNG18 a *bcam2775*. Con los plásmidos pNG26 y pNG18 se transformaron células competentes de la cepa de expresión de *E. coli* BL21 (DE3) pLysS. Para expresar a *bcal0511* y a *bcam2775*, cultivos en fase estacionaria de cepas derivadas BL21 (DE3) pLysS que contenían los plásmidos pET9a vacío (control negativo), pNG26 y pNG18 en LB con kanamicina y cloranfenicol, se diluyeron 1:100 en 20 ml de LB con kanamicina y cloranfenicol, y se crecieron a 30 ^{o}C hasta alcanzar una absorbancia de 0.3 a 620 nm. Posteriormente se adicionó IPTG (0.2 mM), se trasfirió 1 ml de cada cepa a un tubo tipo Falcon de 10 ml y se adicionaron 2 µCi de acetato de sodio [1-^{14}C] (60 mCi/mmol). El crecimiento de todos los cultivos se continúo por 3 h. Las células de los cultivos radiactivos se cosecharon por centrifugación a 13,400 *g* por 5 min, se desechó el sobrenadante y se resuspendieron en 100 µl de agua.

Posteriormente los lípidos se extrajeron por el método de Bligh y Dyer (1959) y se analizaron 2D-TLC [González-Silva et al., 2011]. Las células de los cultivos no radiactivos se cosecharon por centrifugación a 8,228 *g* por 15 min, se desechó el sobrenadante, las células se resuspendieron en 1.9 ml de agua y se almacenaron a -20 ^{o}C. Después la suspensión celular se congeló y descongeló 3 veces para que las células se lisaran, y después el lisado celular se centrifugó a 4,629 *g* por 15 min y se desechó el pelet. Posteriormente, 50 µl del sobrenadante (extracto crudo) de cada cepa se mezcló con 5 µl DNAsa (2.5 unidades/µl) y se incubó por 37 ^{o}C por 15 min. Subsiguientemente, a las muestras anteriores se les adicionó 55 µl del bufer 2X treatment (0.125 M de Tris/HCl pH 6.8, 4 % de SDS, 20 % de glicerol y 10 % de mercaptoetanol) y se incubaron a 95 ^{o}C por 5 min. Después se les dió un pulso en la centrifuga. Por último, se cargaron 5 µl de cada muestra en geles desnaturalizantes de poliacrilamida al 10 %.

Los plásmidos pNG26 y pNG18 se linearizaron con *Bam*HI y *Bgl*II, respectivamente, y se ligaron al vector de amplio rango de hospedero pRK404, previamente linearizado con *BamHI*. Resultando los plásmidos pNG27 y pNG20 y se verificó por digestión que portaran

a los genes *bcal0511* y *bcam2775*, respectivamente. El plásmido pNG27 se movilizó por conjugación a la cepa CS111 x pNG25 y el pNG20 a la cepa *S. meliloti* 1021 x pILAS03, ambas cepas receptoras sobreproducen OLs. Los plásmidos pNG27 y pNG20 también se movilizaron por conjugación a las cepas *B. cenocepacia* J2315 y NG1.

7.5. Complementación con genes *olsB*s rizobiales de la mutante deficiente en *olsB* derivada de *B. cenocepacia* J2315 (NG1)

Para conocer si la mutante NG1 se complementaba con los genes *olsB* de *S. meliloti* 1021 o de *R. tropici* CIAT899, se introdujeron los plásmidos pJG21 [Gao et al., 2004] y pERMAV32 (construido por Miguel A. Vences-Guzmán, datos no publicados) el cual resultó de clonar el pEMAV31 (que contiene a *olsB* de *R. tropici* CIAT899 clonado en pET9a como *Nde*I-*Bam*HI) en el pRK404 con *Bam*HI, a *B. cenocepacia* NG1, respectivamente. Ambos plásmidos se movilizaron a la mutante NG1 y a la cepa silvestre *B. cenocepacia* J2315, mediante una cruza biparental, usando como donadora a la cepa de *E. coli* S17-1. Para comprobar fehacientemente que el plásmido pJG21 contenía el gen funcional después de haberlo movilizado a la mutante NG1, el plásmido se extrajo por minipreparaciones de esa cepa y se movilizó a la mutante deficiente en *olsB* derivada de *S. meliloti* 1021 (AAK1), mediante una cruza biparental, usando como donadora a la cepa de *E. coli* S17-1. También se construyó el plásmido pNG33 que se produjo al digerir el pJG20 con *Bam*HI/*Bgl*II y clonar el fragmento liberado ($olsB_{Sm}$ + promotor T7) en el plásmido pIML21 (contiene a *acpP* de *S. meliloti* 1021 ligado al pRK404 como *Bam*HI/*Bgl*II) construido por Isabel M. López-Lara (datos no publicados) previamente digerido con *Bam*HI. Los plásmidos pIML21 y pNG33 se movilizaron a las cepas NG1 y AAK1, y los plásmidos pNG28 y pNG24 sólo se movilizaron a la cepa AAK1, mediante una cruza biparental, usando como donadora a la cepa de *E. coli* S17-1. Todas las transconjugantes se seleccionaron en el medio de cultivo LB o PY $CaCl_2$ [Beringer, 1974] con tetraciclina y ácido nalidíxico. La presencia de ácido nalidíxico, permitió contraseleccionar a *E. coli* S17-1. Posteriormente se analizó el perfil de lípidos de todas las transconjugantes mencionadas en este párrafo como ha sido descrito anteriormente [González-Silva et al., 2011].

7.6. Ensayo de sensibilidad a antibióticos con cepas derivadas de *B. cenocepacia* J2315

Mutaciones que causan cambios en la composición lipídica de la membrana de las bacterias frecuentemente alteran la sensibilidad a antibióticos. Para comparar la sensibilidad a antibióticos de las cepas J2315, AQ3, AQ3 x pJG16, AQ3 x pSphx04, NG1, NG1 x pNG28 y NG1 x pNG24 se crecieron por 3 días en cajas de LB, se adicionó tetraciclina si contenían plásmido. Posteriormente una colonia aislada de cada cepa se inoculó en 10 ml de LB líquido, con tetraciclina para las cepas que contenían plásmido y se dejaron crecer hasta una absorbancia de 0.3 a 620 nm. Después se distribuyeron uniformemente 50 µl de cada cepa sobre la superficie de LB de cajas petri (todas sin antibióticos). Posteriormente se colocaron los discos que contenían los antibióticos (tabla 1) del kit (Oxoid), se incubaron a 30 ^{o}C por 4 días y finalmente se comparó la sensibilidad mediante la medición del diámetro de las zonas transparentes (halos de inhibición) alrededor de los discos.

Tabla 1. Antibióticos usados en los ensayos de sensibilidad.

Código de los antibióticos	Nombre	Unidades/µg	Blanco
TE30	tetraciclina	30	subunidad del ribosoma 30S
C30	cloranfenicol	30	subunidad del ribosoma 50S
VA30	vancomicina	30	pared celular
PB300	polimixina B	300	membranas celulares
B10	bacitracina	10	membranas celulares
E15	eritromicina	15	subunidad del ribosoma 50S
F300	nitrofurantoína	300	DNA y metabolismo energético
TOB10	tobramicina	10	subunidad del ribosoma 30S
W5	trimetoprima	5	metabolismo del ácido fólico
N30	neomicina	30	subunidad del ribosoma 30S
CN10	gentamicina	10	subunidad del ribosoma 30S
S10	estreptomicina	10	subunidad del ribosoma 30S
AMP10	ampicilina	10	pared celular
FOX30	cefoxitina	30	pared celular
DA2	clindamicina	2	subunidad del ribosoma 50S
K30	kanamicina	30	subunidad del ribosoma 30S
PRL1000	piperacilina	1000	pared celular
NA30	ácido nalidíxico	30	DNA girasa

7.7. Clonación de las dos versiones de *olsC* en pNG23 para cooexpresarlas con *olsB* de *B. cenocepacia* J2315

Rojas-Jiménez et al. (2005) construyeron una mutante en el gen *olsC* por deleción parcial de 211 pb que comprendía la región predicha que codificaba para el dominio catalítico de la enzima OlsC. Rojas-Jiménez et al. (2005) también demostraron que OlsC está involucrada en convertir las dos clases de OLs menos polares (S1 y S2) en las dos clases de OLs más polares (P1 y P2) y complementó la mutante con el plásmido pBBR1,6BE, el cual contenía un fragmento de 1660 bp *Bam*HI-*Eco*RI clonado en pBBR-MCS5. El fragmento clonado contenía al gen *olsC* y su región rio arriba que incluye una región promotora predicha. Basados en análisis con diferentes programas computacionales para predecir ORFs, Rojas-Jiménez et al. (2005) determinaron que el gen estructural es de 846 pb en el marco +1 y que comprendía de la posición 2,611 a la 3456 de la secuencia de 3,761 b depositada en GeneBank con el número de acceso AY954450. Sin embargo, Rojas-Jiménez et al. (2005) no mostraron evidencia experimental con el marco individual predicho para *olsC*. Extraordinariamente, algunos programas computacionales para la predicción de ORFs también indican que el gen *olsC* es de 900 pb y que inicia en posición 2557 en el marco +1. Por lo tanto, se predicen teóricamente dos versiones una corta (846 pb) y una larga (900 pb) de *olsC*.

Para determinar qué versión es funcional o si ambas lo son, Christian Sohlenkamp amplificó y clonó ambas versiones de *olsC* en pET9a como *Nde*I-*Bam*HI (datos no publicados). Los plásmidos pCCS98 y pCCS82 que contienen las dos versiones corta y larga, respectivamente, se digirieron con *Bam*HI-*Bgl*II para liberar los insertos y se reclonaron en el pNG23, previamente digerido con *Bam*HI, produciendo los plásmidos pNG29 y pNG30, respectivamente. Los plásmidos pNG29 y pNG30 se linearizaron con *Bam*HI y se ligaron al pRK404, previamente digerido *Bam*HI, produciendo los plásmidos pNG31 y pNG32, respectivamente. La ligación de los plásmidos pNG29 y pNG30 al pRK404 se verificó por digestión de pNG31 y pNG32, respectivamente. Los plásmidos pNG24, pNG31 y pNG32 se introdujeron por transformación en células competentes de *E. coli* S17-1 y de esa cepa se

movilizaron por conjugación a la cepa CS111. Posteriormente se analizó el perfil de lípidos de membrana de las cepas CS111 x pNG24, CS111 x pNG31 y CS111 x pNG32.

7.8. Clonación de *olsC* en pET16b, su expresión, y los ensayos enzimáticos para OlsC

El fragmento liberado por la digestión del plásmidos pCCS98 con *Nde*I/*Bam*HI (*olsC*) se ligó al pET16b, previamente digerido con *Nde*I/*Bam*HI, produciendo el plásmido pNG42. Posteriormente el plásmido pNG42 se linearizó con *Bam*HI y se ligó al pRK404, previamente digeridó con la misma enzima, produciendo el plásmido pNG43. El pNG43 se movilizó por conjugación a la cepa mutante en *olsC* 899-*olsCΔ1* derivada de *R. tropici* CIAT899, usando como cepa donadora a la *E. coli* S17-1. Después se analizó el perfil de lípidos de membrana de la cepa 899-*olsCΔ1* con el plásmido pNG43. El plásmido pNG42 también se introdujo por transformación a la cepa BL21 (DE3) x pLysS, se indujo su expresión y se prepararon los extractos crudos, una parte del extracto crudo se usó para separar por ultracentrifugación la fracción soluble y la insoluble. Se cuantificó una alícuota del extracto crudo y de las fracciones soluble e insoluble para estimar la cantidad de proteína total que contenían. Después se realizaron los ensayos enzimáticos con los extractos crudos y con las fracciones soluble e insoluble. A partir de la expresión de *olsC* todos los procedimientos posteriores se realizaron similarmente a como los describió González-Silva et al. (2011) para OlsD; como control negativo se usaron los extractos crudos de la cepa BL21 (DE3) x pLysS x pET16b. Las únicas diferencias entre los ensayos de OlsD y OlsC es que en los ensayos de OlsC pusimos una menor cantidad de proteína total (0.4 mg/ml) del extracto crudo, de la fracción soluble y de membranas, así como 0.2% de Triton X-100 en lugar de 0.1%. Después del ensayo los lípidos radiactivos se extrajeron y se separaron por TLC usando la mezcla de solventes cloroformo: metanol: ácido acético (130:50:20, v/v/v), y los lípidos se detectaron y cuantificaron con el phosphorimager.

7.9. Análisis in silico y antecedentes bibliográficos del gen *smc02490* de *S. meliloti* 1021

Los genes *olsA* y *olsB* se identificaron por primera vez en *S. meliloti* 1021 [Gao et al., 2004; Weissenmayer et al., 2002]. Sin embargo, la expresión simultanea de ambos genes en la cepa de expresión BL21 (DE3) x pLysS de *E. coli*, no produjo OLs (Jun-Lian Gao, datos no publicados). Una posible explicación, es que otro gen aún no identificado esté

involucrado en la biosíntesis de los OLs. Geiger et al. (2010) analizaron las interacciones físicas y funcionales del gen *olsB* con el software "string database" disponible en la dirección electrónica del genoma de *S. meliloti* 1021 (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi?docid=SMc01127). Geiger et al. (2010) encontraron que genes homólogos a los genes *olsB* y *smc02490* de *S. meliloti* 1021 coexiste en varias bacterias o se encuentran fusionados en *Arcobacter butzleri* y en especies del orden *Alteromonadales*. Sin embargo, en *S. meliloti* 1021 esos genes no se encuentran fusionados, ni tampoco lo están en otros miembros de las Alfaproteobacterias y de las Betaproteobacterias. Debido a lo anterior procedimos a repetir el análisis realizado por Geiger et al. (2010) para actualizar los datos y también hicimos un blastp con la proteína SMc02490 (307 aminoácidos) de *S. meliloti* 1021 con el número de acceso NP_387151.1. También procedimos a buscar los antecedentes bibliográficos de *smc02490* y a inactivarlo.

7.10. Inactivación del gen *smc02490* de *S. meliloti* 1021

Los oligonucleótidos oLOP163 (ATGTTGATATCGATGCGTCGCGAAAGAGTGG) y oLOP164 (AAAGGATCCCAGCGTATCGACGATATGGC) que introducen los sitios de corte *Eco*RV y *Bam*HI (subrayados), respectivamente, se usaron en la PCR para amplificar un fragmento de 1072 pb localizado río arriba del gen *smc02490*. Similarmente, los oligonucleótidos oLOP165 (AAAGGATCCCCGATGGAAGTGACACGTGC) y oLOP166 (ACTCTCTAGAGAAGACTGGTCTCCGGTTCG) que introducen los sitios de corte *Bam*HI y *Xba*I (subrayados), respectivamente, se usaron en la PCR para amplificar un fragmento de 1045 pb localizado río abajo del gen *smc02490*. En todas reacciones de PCR se usó como molde DNA genómico de *S. meliloti* 1021. El producto de la PCR amplificado con los oligonucleótidos oLOP163 y oLOP164 se clonó en el vector pCR®2.1-TOPO®, como lo indicó el fabricante de éste vector, resultando el plásmido pNG50. El producto de la PCR amplificado con oLOP165 y oLOP166 se digirió con *Bam*HI/*Xba*I y se clonó en pBluescriptSK+ previamente digerido con las mismas enzimas, resultando el plásmido pNG45.

Por secuenciación de los insertos de los plásmidos se confirmó que pNG50 y pNG45 portan la región río arriba y río abajo del gen *smc02490*, respectivamente. Posteriormente el plásmido pNG45 se digirió con *Eco*RV/*Bam*HI y se ligó con el fragmento de DNA de 1072 pb obtenido después de la digestión con *Eco*RV/*Bam*HI de pNG50, resultando el plásmido pNG52. Después, el plásmido pNG52 se linearizó con *Bam*HI y se ligó con el fragmento *Bam*HI del pCAT que contiene el gen *cat* que confiere resistencia a cloranfenicol. El plásmido resultante contenía las regiones flanqueantes del gen *smc02490* interrumpidas por el gen *cat* y se llamó pNG54. El plásmido pNG54 se digirió con *Eco*RV/*Xba*I para reclonar las regiones flanqueantes del gen *smc02490* y el gen *cat* localizado entre las dos regiones. El fragmento *Eco*RV/*Xba*I se clonó en el vector suicida pK18*mobsacB* [Schäfer et. al., 1994] previamente digerido con *Sma*I/*Xba*I, produciendo el plásmido pNG56.

El plásmido pNG56 se introdujo en *S. meliloti* 1021 (cepa silvestre) mediante una cruza diparental, usando como donadora a la cepa de *E. coli* S17-1. Las transconjugantes se seleccionaron en el medio de cultivo PY $CaCl_2$ con cloranfenicol, neomicina y ácido nalidíxico, lo que también permitió contraseleccionar a *E. coli* S17-1. Posteriormente una recombinante sencilla se creció en condiciones no selectivas en PY $CaCl_2$ hasta alcanzar una absorbancia de 1.0 a 620 nm, se hicieron diluciones y se sembraron en PY $CaCl_2$ con sacarosa al 10 % (peso/volumen) y cloranfenicol para seleccionar recombinantes dobles. Después de 3 días de crecimiento en el medio selectivo, se seleccionaron 100 colonias que se resembraron simultáneamente en cajas de PY $CaCl_2$ con cloranfenicol o con neomicina. Después se seleccionaron 10 colonias que crecieron solamente en el medio de cultivo que contenía cloranfenicol y que no crecieron en el medio de cultivo que contenía neomicina. Las 10 colonias se analizaron genotípicamente por hibridación tipo Southern. En la hibridación tipo Southern se usó como sonda marcada con digoxigenina el fragmento de 1072 pb, localizado río arriba del gen *smc02490.* El DNA de la cepa silvestre y de las candidatas a mutantes en el gen *smc02490* se digirió con *Eco*RI. Se esperaba que la sonda en la cepa silvestre hibridara con un fragmento de 12477 pb y en las candidatas a mutantes con uno de 2320 pb.

Tambien hemos diseñado los oligonucleótidos oLOP161 (ACCTTATCCATGGCGAGACGTGATTCGGC) y oLOP162 (AAAGGATCCTTAGCCCGCTACAACCGCCC) para amplificar al gen *smc02490*, los cuales introducen los sitios de corte *Nco*I y *Bam*HI (subrayados), el gen se clonará en el vector de expresión pET3d. Además se construyó el plásmido que se usara como control el pNG39 que resultó de la ligación del pET3d con el pRK404, ambos digeridos previamente con *Bam*HI.

8. Resultados

Los resultados obtenidos en esta tesis doctoral se detallan en las subsecciones 8.1 en la que se encuentran los artículos publicados y en la 8.2 en la que están los resultados adicionales. El III artículo contiene una discusión de los resultados principales, la cual es ampliada en la discusión de esta tesis. En la subsección 8.2 se muestran los resultados adicionales relacionados a la temática de los artículos II y III, pero que aún no han sido publicados.

8.1. Artículos publicados

8.1.1. I Artículo

Geiger, O., González-Silva N., López-Lara, I. M., and Sohlenkamp, C. (2010) Amino acid-containing membrane lipids in bacteria. *Prog. Lipid Res. 49,* 46-60.

La bacteria modelo *Escherichia coli* solamente forma tres lípidos principales de membrana fosfatidiletanolamina, fosfatidilglicerol y cardiolipina, y todos son glicerofosfolípidos. Fosfatidilserina contiene un residuo de aminoácido y es uno de los lípidos principales en las membranas eucariontes pero en muchas bacterias éste solamente es un intermediario biosintético minoritario. En algunas bacterias, los glicerofosfolípidos aniónicos fosfatidilglicerol y cardiolipina pueden ser decorados con residuos aminoacil. Por ejemplo, fosfatidilglicerol puede ser decorado con lisina, alanina o arginina mientras que en caso de cardiolipina se conocen modificaciones con lisina o D-alanina. En pocas bacterias, lípidos derivados de diacilglicerol pueden ser sustituidos con lisina u homoserina. Lípidos aciloxi-acil en los cuales la parte lipídica está unida por enlace amida al grupo α-amino de un aminoácido están ampliamente distribuidos entre las bacterias y los lípidos de ornitina son la versión más común de este tipo de lípidos. Solamente pocos grupos bacteriales forman lípidos de glicina, lípidos de glicinaserina, esfingolípidos, o sulfonolípidos. Aunque muchos de estos lípidos de membrana bacterial que contienen aminoácidos son producidos en ciertas condiciones de estrés, se conoce poco sobre las funciones moleculares específicas de estos lípidos.

Progress in Lipid Research 49 (2010) 46–60

Contents lists available at ScienceDirect

Progress in Lipid Research

journal homepage: www.elsevier.com/locate/plipres

Review

Amino acid-containing membrane lipids in bacteria

Otto Geiger *, Napoleón González-Silva, Isabel M. López-Lara, Christian Sohlenkamp

Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Apdo. Postal 565-A, Cuernavaca, Morelos CP62210, Mexico

ARTICLE INFO

Keywords:
Bacterial membrane
Amino acid-containing lipid
Phosphatidylserine
Lysyl-phosphatidylglycerol
Betaine lipid
Ornithine-containing lipid
Acyl-oxyacyl lipid
Sulfonolipid

ABSTRACT

In the bacterial model organism *Escherichia coli* only the three major membrane lipids phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin occur, all of which belong to the glycerophospholipids. The amino acid-containing phosphatidylserine is a major lipid in eukaryotic membranes but in most bacteria it occurs only as a minor biosynthetic intermediate. In some bacteria, the anionic glycerophospholipids phosphatidylglycerol and cardiolipin can be decorated with aminoacyl residues. For example, phosphatidylglycerol can be decorated with lysine, alanine, or arginine whereas in the case of cardiolipin, lysine or D-alanine modifications are known. In few bacteria, diacylglycerol-derived lipids can be substituted with lysine or homoserine. Acyl-oxyacyl lipids in which the lipidic part is amide-linked to the α-amino group of an amino acid are widely distributed among bacteria and ornithine-containing lipids are the most common version of this lipid type. Only few bacterial groups form glycine-containing lipids, serineglycine-containing lipids, sphingolipids, or sulfonolipids. Although many of these amino acid-containing bacterial membrane lipids are produced in response to certain stress conditions, little is known about the specific molecular functions of these lipids.

© 2009 Elsevier Ltd. All rights reserved.

Contents

* Corresponding author. Tel.: +52 7773 290815; fax: +52 7773 175581.
E-mail address: otto@ccg.unam.mx (O. Geiger).

0163-7827/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.plipres.2009.08.002

1. Introduction

A primary role of lipids in cellular function is to form the lipid bilayer permeability barrier of cells. Glycerophospholipids are the primary building blocks of membranes but other lipids are important components. In the bacterial model organism *Escherichia coli* only the three major membrane lipids phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin occur [1]. In addition, *E. coli* and almost all other Gram-negative bacteria usually have the lipid A-containing lipopolysaccharide in the outer monolayer of their outer membrane and lipid A modification systems have been reviewed recently [2]. However, in other bacteria, additional and alternative membrane lipids are found and in many cases neither their biosyntheses nor their functionalities are understood. Some Gram-negative bacteria have phosphatidylcholine [3] or sphingolipids [4] in their standard repertoire, whereas many Gram-positives have glycosylated diacylglycerols [5] and lysyl-phosphatidylglycerol [6] in their membranes. Notably, phosphatidylinositol is an essential lipid for *Mycobacterium tuberculosis* [7]. Steroid and hopanoid lipids only occur in some bacteria [8]. Under certain stress conditions specific membrane lipids can be formed and in some cases existing membrane lipids can suffer modifications in order to minimize the stress exerted. For example, under phosphorus-limiting conditions of growth, some bacteria form membrane lipids lacking phosphorus such as glycolipids, sulfolipids, betaine lipids, or ornithine-containing lipids [9]. Challenge of proteobacteria with acid causes modifications of membrane lipids, such as formation of lysyl-phosphatidylglycerol [10] or hydroxylation of ornithine-containing lipids [11].

Numerous examples of bacterial lipids containing amino acids or peptides are known [12] and many of them display interesting properties as antibiotics [13] and biosurfactants [14]. However, in order to form lipidic bilayer membranes, amphiphilic lipids usually need to have at least two long-chain acyl or alkyl residues and the molecules should be roughly of cylindric shape, i.e. in cross-section, the area covered by their hydrophilic head group should be similar to the area covered by the hydrophobic acyl or alkyl chains and therefore upon assembly of monomers, bilayer instead of micelle formation is favoured [15]. In this review we will focus on membrane-forming lipids containing aminoacyl residues.

2. Amino acid-containing glycerophospholipids

2.1. Phosphatidylserine and its derivates

In bacteria, CDP-diacylglycerol is the central activated intermediate for the biosynthesis of glycerophospholipids [3] (Fig. 1). Distinct and specific enzymes belonging to the CDP-alcohol phosphotransferase family condense alcohols such as glycerol-3-phosphate, inositol, choline, or serine to CDP-diacylglycerol forming phosphatidylglycerol phosphate, phosphatidylinositol, phosphatidylcholine, or phosphatidylserine (PS), respectively, (Fig. 1). The only amino acid converted by a CDP-alcohol phosphotransferase into a phospholipid head group is serine, and the condensation of CDP-diacylglycerol with serine to form phosphatidylserine (PS) constitutes the first step for the synthesis for phosphatidylethanolamine (PE) [1,16]. In most bacteria, this condensation is catalyzed by the membrane-bound type II PS synthase (Pss) but in some Gram-negative bacteria, like the *Enterobacteriaceae*, PS is synthesized by a type I Pss, which are soluble enzymes that form part of a distinct superfamily that furthermore includes cardiolipin (CL) synthases, poxvirus envelope proteins, phospholipases D, and nucleases [17]. In a second step, the decarboxylation of PS is catalyzed by PS decarboxylase (Psd) to yield PE. Although PS accounts for 5–15% of the phospholipids in eukaryotic cells [18], in most bacteria, PS is a biosynthetic intermediate and is a very minor membrane lipid. In some bacteria, however, the pool of PS seems to be larger and in *Bacillus megaterium* PS comprises some 5–10% [19] of the total membrane lipids. Also, in *Bdellovibrio bacteriovorus*, which parasitizes larger Gram-negative bacteria, PS is a major membrane phospholipid [20]. Psd-deficient bacterial mutants are unable to form PE, and as PS is not consumed they accumulate significant amounts of PS. In such mutants, PS can comprise up to 34% in *E. coli* [21], or up to 29% in *Bacillus subtilis* [22] of the total membrane lipids. A Psd-deficient mutant of *Sinorhizobium meliloti* lacks PE but forms up to 18% PS [23]. This sinorhizobial Psd-deficient mutant resembles in many vegetative aspects a Pss-deficient mutant of *S. meliloti* [16] which also lacks PE but does not form PS either. Surprisingly, Pss-deficient mutants lacking PE form nitrogen-fixing root nodules on alfalfa host plants nearly as efficiently as the wild type. In contrast, the Psd-deficient sinorhizobial mutant accumulates significant amounts of PS and only few empty nodules that are unable to fix nitrogen are formed by this mutant with much delay on the host plant [23]. In animal systems, PS plays a key role in physiological and pathological events. For example, PS exposed on activated platelets promotes the blood coagulation cascade and the aggregation of platelets, and the externalization of PS to the cell surface is a hallmark of apoptotic cells [24]. Although PS is a major membrane lipid in plants [25] its specific roles or functions are unknown. However, the presence of PS in the Psd-deficient sinorhizobial mutant interferes with the accommodation of this mutant bacterium inside the nodule, possibly due to a plant-mediated mechanism. In *E. coli*, the PS formed is distributed equally between the inner and outer membrane [26], but it is not clear whether bacteria expose their PS on the outer surface of the outermost membrane.

2.2. Aminoacyl modifications of phosphatidylglycerol

Phosphatidylglycerol (PG) and cardiolipin (CL) are the major anionic membrane lipids in most bacteria and their synthesis is well understood [1]. Modified forms of PG and CL have been described in different bacteria. Lysyl-phosphatidylglycerol (lysyl-PG) and other aminoacyl esters of PG, such as alanyl-PG, or ornithyl-PG, are major membrane lipids in several Gram-positive bacteria (firmicutes) [28]. Lysyl-PG constitutes a major membrane lipid in *Staphylococcus aureus* [29], *B. subtilis* [30], *Bacillus anthracis* [31], *Listeria monocytogenes* [32], and *Lactococcus plantarum*. In addition to lysyl-PG, some bacteria form ornithyl-PG (*M. tuberculosis*) or alanyl-PG (*Clostridium perfringens*) [6]. Moreover, aminoacylation of PG with arginine [33] or glycine [34] has been described. A large variety of PG-derived lipids are present in *Enterococcus faecalis* (formerly known as *Streptococcus faecalis*) which probably has alanyl-PG, 2′-lysyl-PG, 3′-lysyl-PG, 2′,3′-dilysyl-PG, arginyl-PG, and a diglucosyl derivative of PG [35]. As with other acylated glycerol derivatives, a 2′-lysyl-PG can undergo acyl migration to yield 3′-lysyl-PG [36]. The protein MprF ("multiple peptide resistance factor"), responsible for lysyl-PG formation, was first described in *S. aureus* during a screen for transposon mutants more susceptible to cationic peptides of the innate immune response than the wild type [37]. MprF from *S. aureus* is able to transfer lysine from charged lysyl-tRNA to PG forming lysyl-PG [38,39] (Fig. 1). Also in *B. subtilis* [40] or in *B. anthracis* [31], MprF is required for the synthesis of lysyl-PG and for resistance to cationic antimicrobial peptides. It has been thought that the presence of lysyl-PG is restricted mainly to Gram-positive bacteria although lysyl-PG is present in a strain of *Pseudomonas aeruginosa* [41] and in *Caulobacter crescentus* [42]. In screens for mutants more susceptible to acidic growth conditions, Reeve et al. [43] and Vinuesa et al. [44] identified genes coding for MprF homologues in the α-proteobacteria *Sinorhizobium medicae* and *Rhizobium tropici* that are called *lpiA* ("low pH-induc-

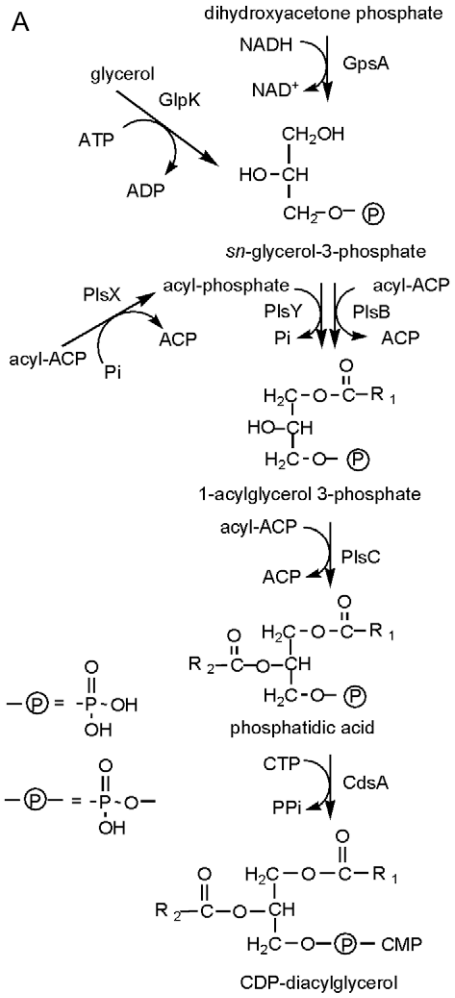


Fig. 1. Biosynthesis of glycerophospholipids in bacteria. (A) Formation of the activated intermediate CDP-diacylglycerol. Glycerol-3-phosphate forms the backbone of all glycerophospholipid molecules and it can be synthesized by two different pathways, either from glycerol directly by glycerol kinase (GlpK) or by reduction of the glycolytic intermediate dihydroxyacetone phosphate with NADH catalyzed by biosynthetic glycerol-3-phosphate dehydrogenase (GpsA) [1]. In *E. coli*, the glycerol-3-phosphate acyltransferase PlsB can use acyl-CoA or acyl-ACP as acyl donors and is the major activity for catalyzing the first acylation at position 1 of glycerol-3-phosphate thereby forming 1-acyl-glycerol-3-phosphate. However, the more widespread pathway to achieve the initial acylation of glycerol-3-phosphate among bacteria seems to involve PlsX and PlsY [27]. In this pathway, PlsX catalyzes the conversion of acyl-ACP and inorganic phosphate to acyl-phosphate and ACP. In a second step, PlsY transfers the acyl group from acyl-phosphate to glycerol-3-phosphate forming inorganic phosphate (Pi) and 1-acyl-glycerol-3-phosphate [27]. The second fatty acyl residue is added by another enzyme, the 1-acyl-glycerol-3-phosphate acyltransferase PlsC, to form phosphatidic acid. The conversion of phosphatidic acid to CDP-diacylglycerol (CDP-diglyceride) is catalyzed by CDP-diglyceride synthase CdsA [1]. (B) Diversification of phospholipid head groups. The first step in the synthesis of phosphatidylethanolamine (PE) is the condensation of CDP-diacylglycerol with serine to form phosphatidylserine (PS) catalyzed by PS synthase (Pss). In a second step, the decarboxylation of PS is catalyzed by PS decarboxylase (Psd) to yield PE. A well-known pathway for PC formation occurs by threefold methylation of PE using *S*-adenosylmethionine (SAM) as methyl donor and catalyzed by phospholipid *N*-methyltransferase (PmtA). Many PC-containing bacteria have a second pathway for PC formation, catalyzed by PC synthase (Pcs), in which choline is condensed directly to CDP-diacylglycerol forming PC and CMP [3]. In the initial step of phosphatidylglycerol (PG) and cardiolipin (CL) biosynthesis, phosphatidylglycerol phosphate synthase (PgsA) transfers glycerol-3-phosphate to CDP-diacylglycerol under the release of CMP thereby producing phosphatidylglycerol phosphate (PGP). There are at least two enzymes with PGP phosphatase activity (PgpA and PgpB) in *E. coli*, releasing inorganic phosphate from PGP to form phosphatidylglycerol (PG) [1]. Lysyl-phosphatidylglycerol (lysyl-PG) is a well-known membrane lipid in many Gram-positive bacteria and MprF can transfer lysine from charged lysyl-tRNA to PG forming lysyl-PG. The pathogen *Clostridium perfringens* has two phylogenetically distinct MprF paralogues, one responsible for the formation of lysyl-PG (MprF1) and the other causing the synthesis of alanyl-phosphatidylglycerol (MprF2) [6]. In *E. coli* and probably most other bacteria, a cardiolipin synthase (Cls) condenses two PG molecules to yield cardiolipin (CL) and free glycerol in a transesterification reaction. Although a MprF homologue is required for the lysinylation of CL, it is not known whether lysyl-CL is formed by lysinylation of CL or by Cls-catalyzed condensation of lysyl-PG with PG. The causative agent of tuberculosis *Mycobacterium tuberculosis* has phosphatidylinositol (PI) and derivatives thereof as major components in its membrane. In *M. tuberculosis*, PI is formed by condensing *myo*-inositol to CDP-diacylglyceride in a reaction catalyzed by PI synthase [7].

ible A"). In both organisms, *lpiA* is transcriptionally induced under acidic growth conditions [43–45]. Later, genes encoding for homologues of LpiA were identified in several other Gram-negative bacteria, like *Agrobacterium tumefaciens*, *Aeromonas hydrophila*, *Xanthomonas campestris*, *Xylella fastidiosa* and several species of the genera *Brucella*, *Burkholderia*, *Pseudomonas*, among others. Interestingly, most of these species interact with eukaryotic hosts as symbionts, pathogens, or commensals. Lysyl-PG formation in *R. tropici* increases the resistance to cationic peptides [10]. Notably, in most Gram-negative bacteria the *lpiA* gene probably forms an operon with the gene *atvA* ("acid tolerance and virulence A"). The biochemical function of AtvA is not known.

More recently, genes encoding proteins responsible for the formation of alanyl-PG have been identified. The pathogen *C. perfringens* has two phylogenetically distinct MprF paralogues, one responsible for the formation of lysyl-PG and the other for the synthesis of alanyl-PG [6] (Fig. 1). Alanyl-PG also occurs in *P. aeruginosa* and the responsible gene is identified [46]. Formation of

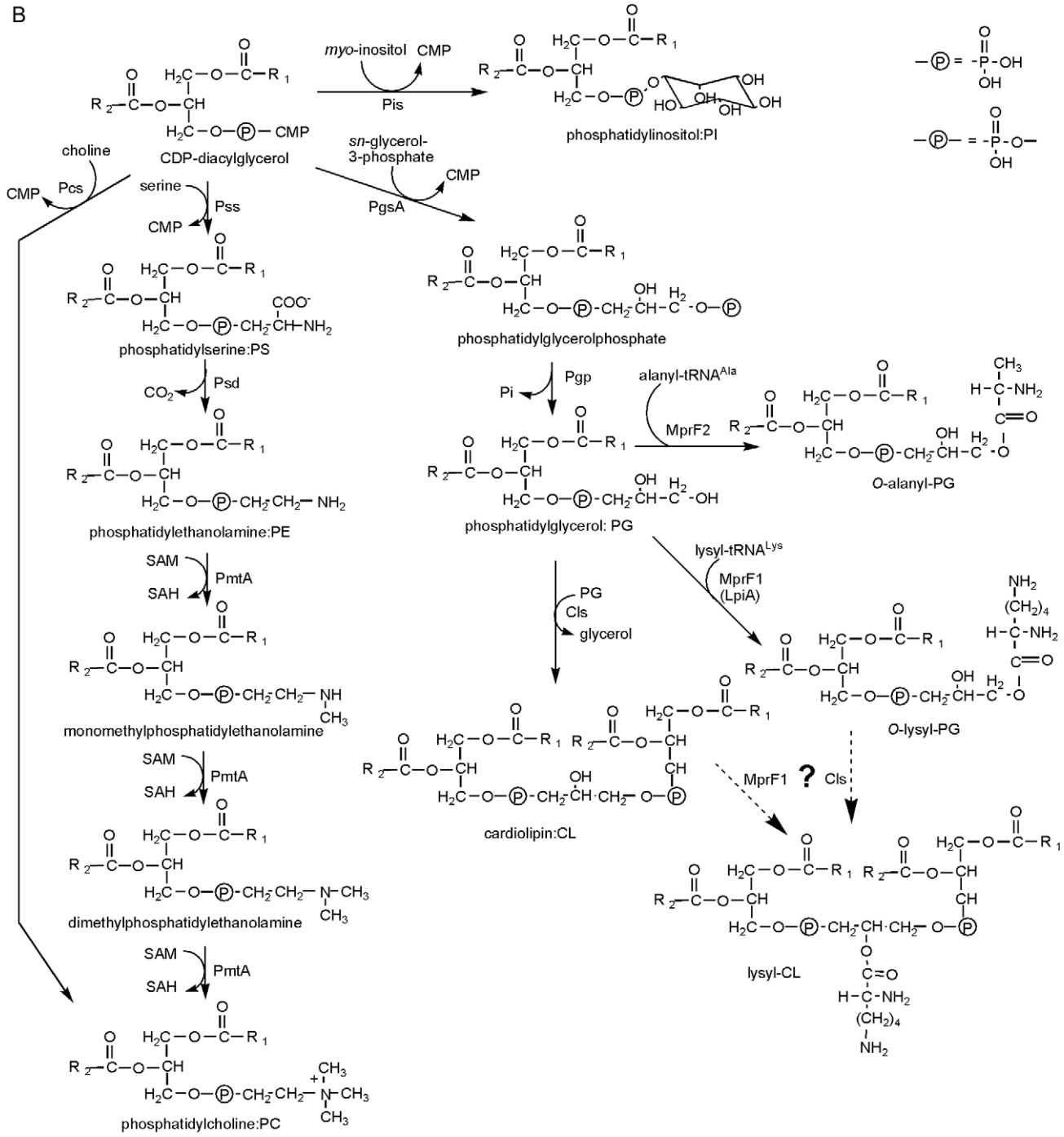


Fig. 1 (*continued*)

2′-alanyl-PG in *P. aeruginosa* is increased under acidic growth conditions. Alanyl-PG is found in the inner and the outer membrane of *P. aeruginosa* and it increases the resistance of the bacterium towards the β-lactam antibiotic cefsulodin, the heavy metal ion Cr^{3+}, the osmolyte sodium lactate, and the cationic antimicrobial peptide protamine sulphate [46].

Two genes coding for LpiA/MprF homologues are also present in *Sphingomonas wittichii* and *Enterococcus faecium* and even three LpiA/MprF homologues are encoded by the *Kineococcus radiodurans* genome, but nothing is known about their functions. Although the presence of aminoacylated PG has been described only in bacteria, a gene coding for an LpiA/MprF homologue is present in the genome of the moss *Physcomitrella patens*.

2.3. Aminoacyl modifications of cardiolipin

Cardiolipin (CL) can also be substituted on the *sn*-2 hydroxyl of the middle glycerol moiety with α-D-glucopyranosyl, D-alanyl, or L-lysyl residues [47,48] in the group N streptococcus *Vagococcus fluvialis* or with L-lysyl in *Listeria* species [32] (Fig. 1). The MprF homologue Lmo1695 of *L. monocytogenes* is required for the lysinylation of both, PG and CL [49]. However, it is not known whether lysyl-CL is formed by lysinylation of CL or by CL synthase-catalyzed condensation of lysyl-PG with PG. Therefore, the precise biosynthetic steps that form these modified versions of CL are not clear. Usually, CL is only partially ionized at physiological conditions due to the hydrogen bonding of the *sn*-2 hydroxyl group of the middle glyc-

erol moiety with the neighboring phosphate groups. Therefore, CL can function as a proton sink or a conduit for protons in transfer processes [15]. Upon derivatization of the *sn*-2 hydroxyl of the middle glycerol with the above-mentioned residues the hydrogen bonding should be impeded and the special property of CL as a proton sink will be lost.

3. Amino acid-containing diacylglycerols

3.1. Homoserine-containing betaine lipids

Although PC is known to be the major membrane lipid in eukaryotes, some lower eukaryotic organisms possess the betaine lipid diacylglyceryl-*N,N,N*-trimethylhomoserine (DGTS) instead. DGTS occurs in a wide variety of lower green plants (green algae, bryophytes and pteridophytes), chromophytes, fungi, and amoebae (reviewed in [3]). In some α-proteobacteria, DGTS acts as a phosphorus-free membrane lipid [50] that substitutes for PC under conditions of phosphate limitation [51]. There is an apparent reciprocity between the content of PC and the content of DGTS, i.e. when PC is a major membrane lipid often no DGTS is detected in the same organism, whereas in organisms where DGTS is a major lipid, PC is only found in trace levels. This correlation suggests, that DGTS and PC, both zwitterionic at physiological pH, are interchangeable at least with regard to essential functions for the respective organism. The mutual replacement of PC or DGTS occurs in culture conditions but also in natural environments [51a]. Phytoplankton communities from the phosphorus-poor Sargasso Sea have much higher betaine lipid/PC ratios than communities from the phosphorus-replete South Pacific due to the respective adjustments in eukaryotic phytoplankton [51a].

Two structural genes from *Rhodobacter sphaeroides*, *btaAB*, coding for two enzymes BtaA and BtaB involved in DGTS biosynthesis have been characterized [52,53]. The BtaA *S*-adenosylmethionine/diacylglycerol 3-amino-3-carboxypropyl transferase converts diacylglycerol (DAG) into diacylglyceryl-homoserine (DGHS) and during the formation of the ether bond, *S*-adenosylmethionine functions as donor of the homoseryl group. Subsequently, the *S*-adenosylmethionine: diacylglyceryl-homoserine-*N*-methyltransferase BtaB catalyzes threefold methylation of DGHS in order to yield DGTS (Fig. 2). Orthologues of BtaA and BtaB exist in *S. meliloti*, and a BtaA-deficient mutant of *S. meliloti* is unable to produce DGTS [54]. In the eukaryotic green alga *Chlamydomonas reinhardtii*, the betaine lipid synthase $BTA1_{Cr}$ is a bifunctional protein that can perform the homoseryl modification of diacylglycerol as well as the subsequent methylations of the homoseryl amino group [55]. In other lower eukaryotes where DGTS is present, genes coding for homologues of the bifunctional *C. reinhardtii* enzyme are present. Heterologous expression of $BTA1_{Cr}$ in *E. coli* leads to DGTS accumulation and the two domains of $BTA1_{Cr}$ are functionally equivalent to BtaA and BtaB [55].

The wealth of genome sequencing data indicates that the occurrence of DGTS-like betaine lipids is limited in bacteria. Homologues of rhodobacterial BtaA (above 42% identity and 55% similarity) and BtaB are mainly found in some orders of the α-proteobacteria, such as the Rhodobacterales (*Rhodobacter, Roseobacter, Sagittula, Stappia*), the Sphingomonadales (*Sphingomonas, Erythrobacter*), the Rhizobiales (*Rhizobium, Agrobacterium, Sinorhizobium, Ochrobactrum, Mesorhizobium, Beijerinckia, Rhodopseudomonas*), and in members of the Planctomycetes (*Planctomyces, Blastopirellula, Rhodopirellula*) (Fig. 3). More distantly related genes are present in the δ-proteobacterium *Plesiocystis pacifica* SIR-I and in *Chthoniobacter flavus* (Chlamydia/Verrucomicrobia). Genes coding for homologues of the bifunctional enzymes from *Chlamydomonas reinhardtii* are present in several lower eukaryotes such as *Candida albicans*, *Cryptococcus neoformans*, *Neurospora crassa* and *Physcomitrella patens* (Fig. 3). Planctomycetes represent a distinct bacterial phylum as they show absence of a peptidoglycan cell wall and extensive cell compartmentalization, in some cases even a

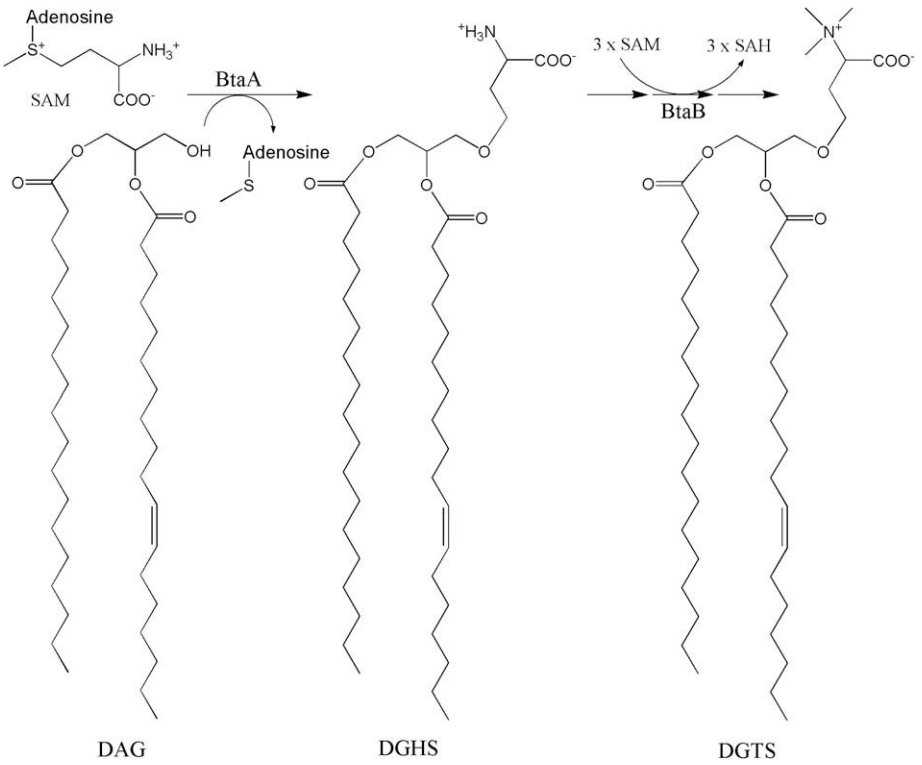


Fig. 2. Biosynthesis of diacylglyceryl-*N,N,N*-trimethylhomoserine (DGTS) [52,53]. DAG: diacylglycerol; SAM: *S*-adenosylmethionine; SAH: *S*-adenosylhomocysteine; DGHS: diacylglyceryl-homoserine.

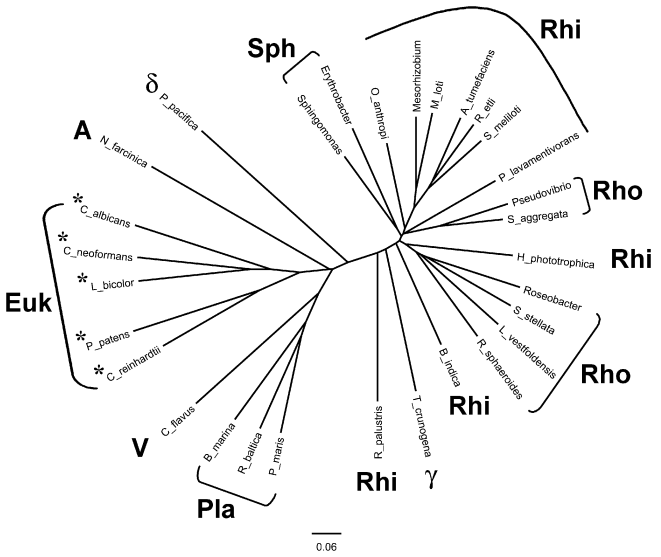


Fig. 3. Unrooted phylogenetic tree of *Rhodobacter sphaeroides* BtaA, *Sinorhizobium meliloti* BtaA, the *N*-terminal domain of *Chlamydomonas reinhardtii* $Bta1_{CR}$ and BtaA-like ORFs from other genomes. The tree was constructed using the program CLUSTALW (http://www.expasy.ch/). Distances between sequences are expressed as 0.06 changes per amino acid residue. The asterisks indicate that only the *N*-terminal domains of the respective sequences corresponding to BtaA were used for the construction of the tree. Accession numbers are as follows: *Rhodobacter sphaeroides* BtaA (ABA80038), *Sinorhizobium meliloti* 1021 BtaA (NP_386300), *Chlamydomonas reinhardtii* $Bta1_{CR}$ (XP_001700879), *Agrobacterium tumefaciens* str. C58 (NP_355081), *Beijerinckia indica* subsp. *indica* ATCC 9039 (YP_001834100), *Blastopirellula marina* DSM 3645 (ZP_01088661), *Candida albicans* SC5314 (XP_713069), *Chthoniobacter flavus* Ellin428 (ZP_03133405), *Erythrobacter* sp. NAP1 (ZP_01039410), *Hoeflea phototrophica* DFL-43 (ZP_02168757), *Laccaria bicolor* S238N-H82 (XP_001877704), *Loktanella vestfoldensis* SKA53 (ZP_01002837), *Mesorhizobium loti* MAFF303099 (NP_103130), *Mesorhizobium* sp. BNC1 (YP_674579), *Nocardia farcinica* IFM 10152 (YP_117653), *Ochrobactrum anthropi* ATCC 49188 (YP_001370273), *Parvibaculum lavamentivorans* DS-1 (ABS62442), *Physcomitrella patens* subsp. *patens* (XP_001757434), *Planctomyces maris* DSM 8797 (ZP_01856778), *Plesiocystis pacifica* SIR-1 (ZP_01909206), *Pseudovibrio* sp. JE062 (EEA94326), *Rhizobium etli* CFN 42 (YP_470361), *Rhodopirellula baltica* SH 1 (NP_863860), *Rhodopseudomonas palustris* BisB18 (YP_531155), *Roseobacter* sp. MED193 (EAQ46984), *Sagittula stellata* E-37 (EBA07014), *Sphingomonas* sp. SKA58 (ZP_01304163), *Stappia aggregata* IAM 12614 (ZP_01546622), and *Thiomicrospira crunogena* XCL-2 (YP_390494). A – Actinomycetales, Euk – Eukaryotes, V – Verrucomicrobia, Pla – Planctomycetales, Rhi – Rhizobiales, Rho – Rhodobacterales, Sph – Sphingomonadales, γ – gamma proteobacteria, δ – delta proteobacteria.

membrane-enclosed nuclear structure [56]. Interestingly, the BtaAB homologues from the order *Planctomycetales* seem to be closer related to the sequences from eukaryotic origin than to the other bacterial sequences (Fig. 3). It has been speculated that the biosynthesis of certain phosphorus-free membrane lipids such as DGTS might improve the survival of bacteria under phosphorus-depleted conditions [54]. The fact that intracellular pathogens of the genus *Brucella* apparently lack the genes needed for DGTS biosynthesis but that the closely related, and normally free-living (opportunistic) pathogen *Ochrobacter anthropi* has the respective genes supports this idea.

3.2. Mycobacterial lysine-containing lipid

A diacylglycerol-based lysine-containing lipid was isolated from *Mycobacterium phlei* strain IST [57]. Lysine is esterified to 1,2-diglyceride via an ester linkage (Fig. 4) and the major fatty acyl substitutions are palmitic and tuberculostearic acid [57]. The mycobacterial lysine-containing lipid in strain IST is not detected it in a reference strain (ATCC 19249), and it has been suggested that this lipid is involved in lysine uptake to the cell [57].

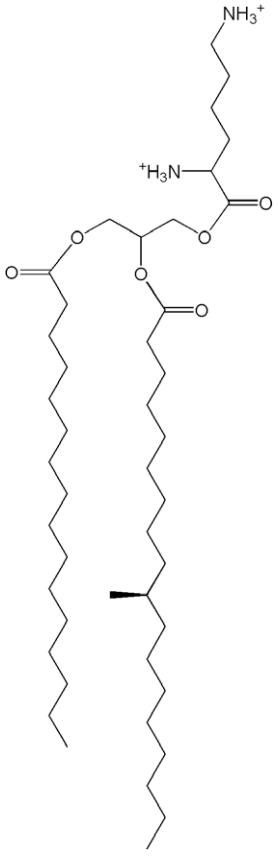


Fig. 4. Structure of mycobacterial lysyl-diacylglycerol.

4. Amino acid-containing acyl-oxyacyl lipids

4.1. Ornithine-containing lipids

4.1.1. Distribution and structure of ornithine-containing lipids

Ornithine-containing lipids (OL) are widespread among Gram-negative bacteria and have been reported in some Gram-positives, like *Mycobacterium* and *Streptomyces* species (reviewed in [9]) but are absent in *Archaea* and *Eukarya*. The α-*N*-(acyloxyacyl)-ornithines have a 3-hydroxyfatty acyl group that is attached in amide linkage to the α-amino group of ornithine [51,58]. A second fatty acyl group is ester-linked to the 3-hydroxy position of the first fatty acid (Fig. 5A). In some bacteria the fatty acyls joined by ester linkage are hydroxylated at the 2 or 3 positions [12]. The configuration of the asymmetric carbon of 3-hydoxyfatty acyls of the OLs is D or (*R*) [59]. Although OLs are found in both membranes of Gram-negative bacteria, they seem to be enriched in the outer membrane [60].

4.1.2. Biosynthesis of ornithine-containing lipids

The biosynthesis of OLs occurs in two-steps. 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 [61]. Next, the *O*-acyltransferase OlsA catalyzes the transfer of an acyl group from fatty acyl–acyl carrier protein to the hydroxy group of lyso-ornithine lipid forming OL [62] (Fig. 5A).

OlsB-deficient mutants of *S. meliloti* [61] or *Rhodobacter capsulatus* [63] are unable to form OL. Expression of *olsB* from *S. meliloti* in *Escherichia coli* causes the formation of lyso-ornithine lipid [61]. The OlsB of *S. meliloti* is predicted to be a water-soluble protein of 296 amino acids that encodes an *N*-acyltransferase converting ornithine

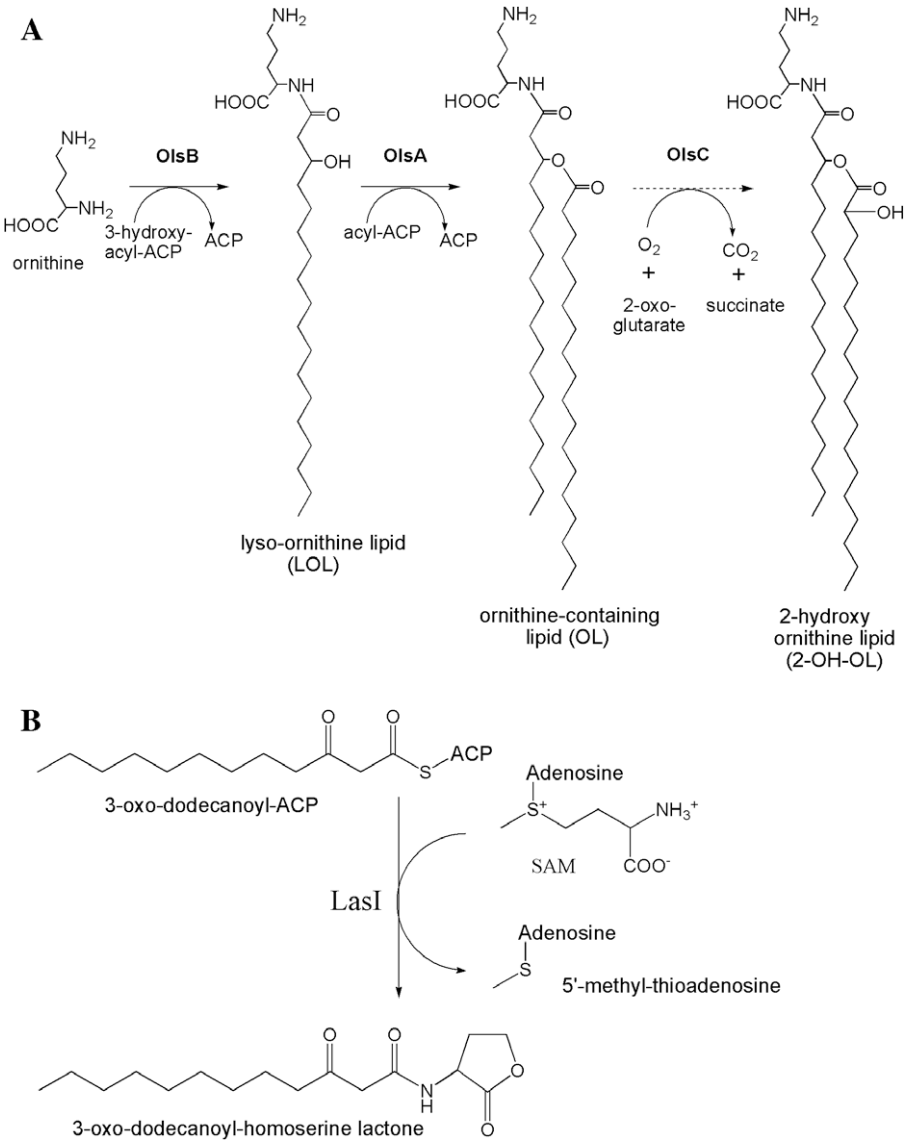


Fig. 5. Biosyntheses of ornithine-containing lipids (A) and of the *Pseudomonas aeruginosa* quorum sensing signal 3-oxo-dodecanoyl-homoserine lactone (B).

to lyso-ornithine lipid thereby catalyzing first step of OL biosynthesis. OlsB defines a concrete function for a whole cluster of orthologous group of proteins (COG3176) previously assigned as hypothetical or as putative hemolysins [61]. OlsB belongs to the acyl-CoA *N*-acyltransferase superfamily [64]. Heath and Rock [65] described a consensus peptide motif (H(X)4D) common to glycerolipid acyltransferases and demonstrated that an exchange of this conserved H for A eliminated the activity of *E. coli* PlsB. In OlsB, H87 and D92 might form such a motif that is conserved within OlsB homologues [61]. A search of PhyloFacts with OlsB identifies acyl-homoserine lactone synthases EsaI [66] and LasI [67] and the tertiary structure of OlsB is expected to be quite similar to the autoinducer synthase. Like OlsB, acyl-homoserine lactone synthases are *N*-acyl transferases that use acyl-ACPs and an amino acid derivative (SAM) as substrates (Fig. 5B). The *N*-acyl amino acid synthase FeeM from an uncultured soil microbe binds the acyl carrier protein FeeL, catalyzes the formation of *N*-acyl tyrosine, and its structure resembles that of acyl-homoserine lactone synthases EsaI and LasI [68].

Genome analyses indicate that like many bacterial groups, the order Rhodobacterales have an *olsBA* operon (containing *olsB1*) plus a gene coding for a second homologue of OlsB (*olsB2*) (Fig. 6). The *olsB2* gene is not located physically close to *olsBA*, but at another site in the genome. The OlsB2 homologues of different Rhodobacterales are more closely related to each other than to the OlsB1 homologues (Fig. 6). Interestingly, *R. sphaeroides* forms lipids that contain glutamine in addition to the well-known OL [69]. The initial step in the biosynthesis of glutamine-containing lipids might be catalyzed by the OlsB2 homologue found exclusively in the Rhodobacterales.

The only other examples where multiple *olsB* homologues are present within one genome are the α-proteobacteria *Magnetospirillum magneticum* and *M. magnetotacticum* (Fig. 6). In addition to OL, another unknown amino lipid has been described in the magnetosome membrane of *Magnetospirillum* which might be synthesized by the second homologue [70]. Remarkably, heterologous expression of microbial DNA extracted from environmental samples led to the identification of long-chain *N*-acyl derivatives of tyrosine, phenylalanine, tryptophan, and arginine, all of which have antibiotic activity [13,71,72]. It is possible that some of the long-chain *N*-acyl transferases involved in the formation of these compounds catalyze initial steps in the biosyntheses of other still unknown acyl–oxyacyl membrane lipids.

Genome sequencing data indicate, that in several organisms from the order *Alteromonadales* and in the ε-proteobacterium

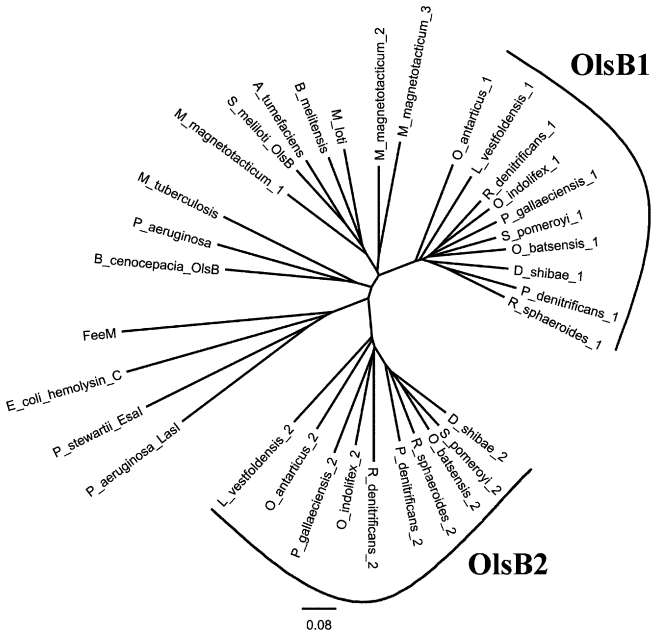


Fig. 6. Unrooted phylogenetic tree of *Sinorhizobium meliloti* OlsB, *Burkholderia cenocepacia* OlsB and OlsB-like ORFs. The tree was constructed using the program CLUSTALW (http://www.expasy.ch/). Distances between sequences are expressed as 0.08 changes per amino acid residue. Accession numbers are as follows: *Sinorhizobium meliloti* 1021 OlsB (NP_384499), *Burkholderia cenocepacia* J2315 OlsB (YP_002230419), *Escherichia coli* CFT073 HlyC (NP_755444), *Pantoea stewartii* subsp. *stewartii* EsaI (AAA82096), *Pseudomonas aeruginosa* PAO1 LasI (NP_250123), *N*-acyl transferase FeeM from an uncultured bacterium (AAM97306), *Agrobacterium tumefaciens* str. C58 (NP_353376), *Brucella melitensis* 16M (NP_540717), *Dinoroseobacter shibae* DFL 12 (YP_001532815 and YP_001533050), *Loktanella vestfoldensis* SKA53 (ZP_01003503 and ZP_01003690), *Magnetospirillum magnetotacticum* MS-1 (ZP_00050271, ZP_00055184, and ZP_00053729), *Mesorhizobium loti* MAFF303099 (NP_104372), *Mycobacterium tuberculosis* H37Rv (NP_217543), *Oceanicola batsensis* HTCC2597 (ZP_00999281 and ZP_00999194), *Oceanobulbus indolifex* HEL-45 (ZP_02153706 and ZP_02153839), *Octadecabacter antarticus* 307 (EDY79302 and EDY80135), *Paracoccus denitrificans* PD1222 (ZP_00631241 and ZP_00629627), *Phaeobacter gallaeciensis* 2.10 (ZP_02148428 and ZP_02148788), *Pseudomonas aeruginosa* PAO1 (NP_253040), *Rhodobacter sphaeroides* 2.4.1 (YP_354511 and YP_352676), *Roseobacter denitrificans* OCh 114 (YP_682889 and YP_683344), and *Silicibacter pomeroyi* DSS-3 (YP_167215 and YP_167705). OlsB1 and OlsB2 assign the two different subgroups of OlsB homologues present in bacteria from the order Rhodobacterales.

Arcobacter butzleri, the OlsB protein is *N*-terminally fused to a hypothetical protein domain of unknown function, suggesting that OL biosynthesis might turn out to be more complicated. The presence of OL has not been described in any of these species.

Also mutants of *S. meliloti* [62] or *R. capsulatus* [63] deficient in OlsA are unable to form OL. Overexpression of *olsB* in an *olsA*-deficient mutant of *S. meliloti* leads to the accumulation of lyso-ornithine lipid [61]. The OlsA of *S. meliloti* is a protein of 292 amino acids with a probable transmembrane helix close to the *N*-terminus. An alignment of OlsA with some prokaryotic enzymes displaying lysophosphatidic acid acyltransferase activities [62] demonstrates that there are two conserved regions (amino acids 67–83 and 139–154) where OlsA has the highest similarity to other members of this group. In lysophosphatidic acid acyltransferases, two motifs, NHQS and PEGTR, are conserved [73], and they are found in modified forms (NHVS, amino acids 72–75; PEGTT, amino acids 143–147) in the OlsA sequence [62]. Based on the consensus peptide motif (H(X)4D) [65] common to glycerolipid acyltransferases, in OlsA, H73 and D78 might form such a motif. It is striking, however, that sequences coding for enzymes with lysophosphatidic acid acyltransferase activities are overall quite dissimilar. For example, in the bacterium *Neisseria meningitidis* there are three enzymes (NlaA, NlaB, and a third activity detectable in *nlaA*-, *nlaB*-deficient double mutants) with lysophosphatidic acid acyltransferase activity in vitro [74]. Though NlaA and NlaB are from the same organism they are quite dissimilar and it is interesting to note that *nlaA*-deficient mutants and *nlaB*-deficient mutants show different phenotypes suggesting that NlaA and NlaB perform different biochemical functions in *N. meningitidis* in vivo. From its sequence, OlsA clearly groups within the present lysophosphatidic acid acyltransferases. Because *olsA*-deficient mutants are unable to form OL, but show no accumulation of lysophosphatidic acid and no impairment of glycerophospholipid biosynthesis, there must be a PlsC activity in *S. meliloti* responsible for these latter functions, presumably SMc00714 (http://sequence.toulouse.inra.fr/meliloti.html). OlsA is required for the enzymatic activity of a lyso-ornithine lipid- and acyl-AcpP-dependent *O*-acyltransferase that converts lyso-ornithine lipid into OL [62]. In addition to OlsA, *Pseudomonas fluorescens* possesses two more lysophosphatidic acid acyltransferase homologues, HdtS and PatB [75]. Either HdtS or PatB complement an *E. coli* PlsC-deficient mutant for growth, while the OlsA from *P. fluorescens* does not. Although HdtS or PatB can provide the PlsC function in vivo, they are not functionally identical. Mutants lacking PatB show reduced growth at elevated temperatures while HdtS-deficient mutants are affected in growth, motility and have reduced amounts of *cis*-vaccenic acid [75]. Also, *Rhodobacter capsulatus* possesses three lysophosphatidic acid acyltransferase homologues, OlsA, PlsC316, and PlsC3498 [76]. Either OlsA or PlsC316 from *R. capsulatus* complement an *E. coli* PlsC-deficient mutant for growth, while PlsC3498 does not. A PlsC316-deficient mutant has reduced amounts of C16 fatty acids [76]. Therefore, OlsA from *R. capsulatus* is able to acylate 1-acyl-*sn*-glycerol-3-phosphate in addition to lyso-ornithine lipid and therefore exhibits relaxed substrate specificity towards the acyl acceptor substrate [76]. It is expected that future studies of the present group of "lysophosphatidic acid acyltransferases" will reveal numerous subgroups with slightly different biochemical activities.

Little is known about the functions of OLs. *S. meliloti* mutants deficient in OL biosynthesis do not show any alteration of their macroscopic phenotype. The unability to form OL must be combined with deficient DGTS biosynthesis to obtain reduced cell yields when *S. meliloti* is grown under phosphorus-limiting conditions [54]. In *R. capsulatus*, OLs are required for optimal steady-state amounts of *c*-type cytochromes [63].

4.1.3. Hydroxylated ornithine-containing lipids

In bacteria like *Burkholderia cepacia*, *Flavobacterium* [12,77], *Thiobacillus* [58], *Gluconobacter* [78], *Streptomyces* [12], some *Ralstonia* spec. [79], and *R. tropici* [11] OL also have ester-linked fatty acyl groups with a hydroxyl group at the 2-position. The 2-hydroxyfatty acyl residues are not formed during standard fatty acid biosynthesis and specific enzymatic activities are required to introduce a hydroxyl group onto the 2-position of a fatty acyl residue. Similar *S*-2-hydoxyfatty acyl moieties are integral parts of *Salmonella typhimurium* lipid A and are thought to be of importance for pathogenesis of this organism. The *S*-2-hydroxylation is introduced after the fatty acyl group had been attached to the lipid A molecule and is catalyzed by the $Fe^{2+}/O_2/\alpha$-ketoglutarate-dependent LpxO-encoded dioxygenase [80,81]. It has been speculated that the hydroxyl groups might increase hydrogen bonding between adjacent lipid A molecules decreasing the outer membrane's permeability to lipophilic compounds under some growth conditions [81]. A similar dioxygenase (OlsC) might be responsible for the introduction of 2-hydroxy substitutions on the ester-linked fatty acyl group of OL [11] (Fig. 5). 2-Hydroxy substitutions on ester-linked fatty acyl groups occur also in bacterial sphingolipids and PE, other major components of the outer membrane in Gram-negative bacteria. PE is 2-hydroxylated on its *sn*-2-fatty acyl residue in *Burkholderia* [82,83]. Homologues of *S. typhimurium*

LpxO are found in the genome of *Burkholderia cenocepacia* J2315 (BCAM1214 and BCAM2401) and they might be candidates for introducing hydroxyl groups into the esterified fatty acyl residue of OL.

Rhizobium tropici CIAT899, an efficient symbiont of bean plants, is highly tolerant to acid, and produces four different classes of OL (termed S1, S2, P1, and P2) [11]. A mutant deficient in *olsC* is symbiotically defective and does not form P1 or P2. Overexpression of the *olsC* gene in the *olsC*-deficient mutant yielded P1 and P2 as major OLs, coupled with a near-complete lack of S1 and S2 and an acid-sensitive phenotype [11]. These results suggest that some classes of OL are important for acid tolerance (S1 and S2) and others for symbiotic effectiveness (P1 and P2), but in order to optimize both traits, an adequate balance of the four distinct classes of OLs is required [11]. The product encoded by *olsC* is a putative LpxO-like dioxygenase that might convert the two less polar forms of OLs (S1 and S2) to the two more polar forms (P1 and P2) [11] by hydroxylation at an unknown position. The OlsC of *R. tropici* is predicted to be a water-soluble protein of 281 amino acids [11].

4.1.4. Tauro-ornithine- and lysine-containing lipids

In *Gluconobacter cerinus*, ornithine-containing lipids hydroxylated in the 2-position of the ester-linked fatty acyl residue (2-OH-OL) are partially modified with a taurine residue that is amide-linked to the α-carboxy group of ornithine [78] (Fig. 7). The particulate fraction from *G. cerinus* requires ATP and Mn^{2+} to condense taurine to 2-OH-OL leading to the formation of tauro-ornithine lipid [84]. This tauro-ornithine lipid is also called cerilipin after the species of the bacterium from which it was isolated [78]. The gene encoding the taurine-condensing activity is unknown.

A lysine-containing lipid (LL) from an *Agrobacterium tumefaciens* strain [85] has the α-amino group of lysine *N*-acylated with a 3-hydroxypalmitoyl residue that is esterified with a fatty acid (Fig. 7). This LL is analogous to the OL with lysine instead of ornithine as a building block.

4.2. Glycine-containing lipids

The glycine-containing lipids (GLs) were identified in the gliding bacterium *Cytophaga johnsonae* C21 and the Gram-negative sea-water bacterium *Cyclobacterium marinus* WH [86,87]. GLs consist of the amino acid glycine and two fatty acyl residues, using the acyl-oxyacyl or piggyback structure. The structure of GL from *C. marinus* WH is principally a *N*-[3-D-(13-methyltetradecanoyloxy)-15-methylhexadecanoyl]glycine [87]. In this structure (Fig. 8), an iso-3-hydroxyfatty acyl group is amide-linked to glycine and its 3-hydroxy group is esterified to another iso-fatty acid. The absolute configuration of the hydroxy ester is 3-D [87]. This type of GL is called cytolipin because it was initially identified in the genus *Cytophaga* [86]. It constitutes about 6% and 5% of the total lipids in *C. johnsonae* C21 [86] and *C. marinus* WH [87], respectively. Based on chromatographic methods and specific stains it was assumed that lipoamino acids structurally similar to GL are presents in several gliding bacteria of the genus *Cytophaga* [88], and in some strains of Gram-negative fresh-water bacteria belonging to the genera *Arcocella* [89] and *Flectobacillus* [90] related to the genus *Cyclobacterium*. The three latter genera are systematically distant from the family *Cytophagaceae* and therefore it was suggested that GL might be widely distributed among Gram-negative aquatic bacteria [87]. However, an alternative explanation might be that the structural genes for GL formation were transferred horizontally.

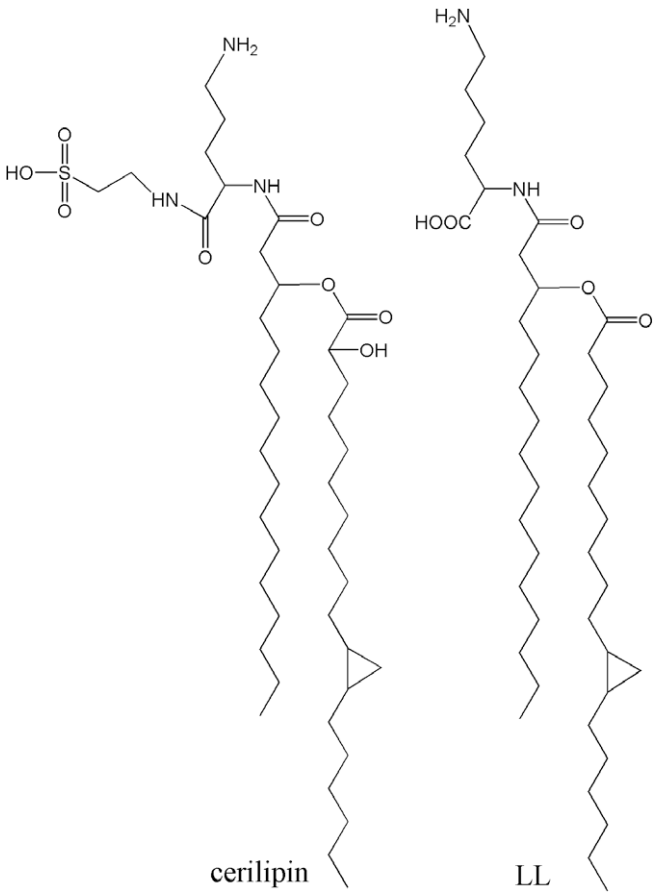


Fig. 7. Structures of tauro-ornithine-containing lipid (cerilipin) and of agrobacterial lysine-containing lipid (LL).

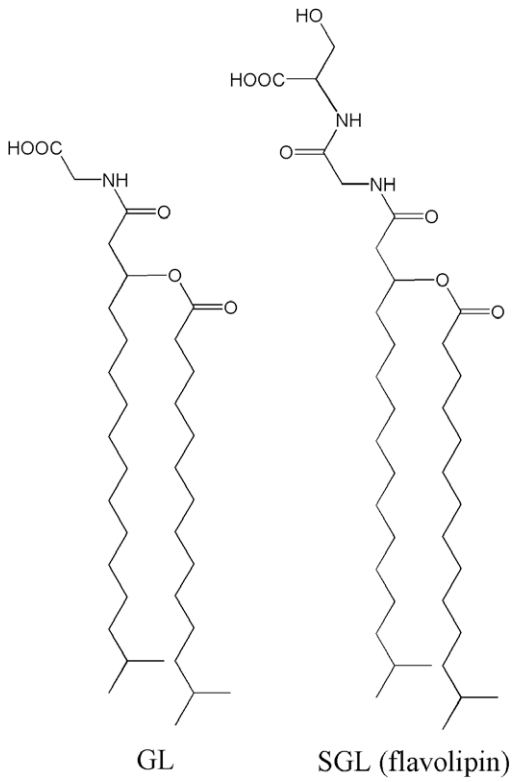


Fig. 8. Structures of glycine-containing lipid (GL) and of serineglycine-containing lipid (SGL; flavolipin).

The genes involved in the GL biosynthesis are not known although one might expect that they are similar to the genes involved in OL biosynthesis, due to the structural similarity between both molecules. Homologues to the above-described *olsB* fusions detected in species of the *Alteromonadales* are also found in genomes of several members of the genus *Cytophaga*. So far nothing is known about functions associated with GL.

4.3. Serineglycine-containing lipids

A serineglycine-containing lipid (SGL) was isolated from the opportunistic pathogen *Flavobacterium meningosepticum* [77]. This SGL was called "flavolipin" based on the genus name of the bacterium from which it was first isolated [77]. The initially proposed flavolipin structure of an *N*-(3-acyloxyacyl)serine, was incorrect [77,91] because it lacked a glycine residue. The synthesis of flavolipin to study its biological activities led to the correct structural assignment as a serineglycine-containing lipid (SGL) (Fig. 8) [92]. Flavolipin is not unique to *Flavobacterium* species, is also found in *C. marinus* WH [93], and therefore might be present in other sea-water bacteria as well. Flavolipin constitutes about 21% and 11% of the total lipids in *F. meningosepticum* [77] and *C. marinus* WH [93], respectively. Flavolipin shares the GL basic structure but has an additional serine residue (Fig. 8) which suggests that GL is a direct biosynthetic precursor for flavolipin formation in *C. marinus* WH [87]. In contrast to *C. marinus*, the seven species analyzed from the *Cytophaga* genus produce no flavolipin [88]. The genes involved in flavolipin biosynthesis are not known.

4.4. The innate immune response to amino acid-containing acyl-oxyacyl lipids

Bacterial lipids with an acyl-oxyacyl structure are recognized by toll-like receptors (TLRs) as pathogen-associated molecular patterns and trigger the innate immune response of mammals. The 3-acyl-oxyacylamide structure with (*R*)-configuration is present in OL, SGL, and lipid A. The best studied example is the bacterial endotoxin lipid A. Lipid A is the reactive part of LPS that stimulates Toll-like receptor 4 (TLR4) and the nuclear factor κB (NF-κB) to produce inflammatory cytokines. MD-2, a molecule that physically associates with TLR4 on the cell surface, confers the LPS responsiveness on the TLR4 receptor [94]. OL and SGL also induce inflammatory immune responses, measured by the formation of PGE_2, IL-1β, and tumor necrosis factor α by macrophages [95]. A recent study suggests that even the physical state of lipid A or OL affect their biological activities [96]. OL and SGL can be used as adjuvants [59,97–101], and when injected into mice before exposure to the endotoxin lipid A they prevent the lethal effects of the latter [102]. Because of the structural similarities between the two molecules, OL might function as an antagonistic blocker of lipid A-provoked events [102]. Like LPS, the inflammatory immune response-causing SGL signal is transduced via the TLR4–MD-2 complex [103].

5. Bacterial sphingolipids

Although sphingolipids are not amino acid-containing lipids in a strict sense, they are formed by condensing an amino acid (serine) to the fatty acyl-CoA forming the sphingolipid precursor 3-oxo-sphinganine, CoA and CO_2 (Fig. 9A). In eukaryotes, sphingolipids are ubiquitous and essential components of the plasma membrane and are crucial for signaling and organization of lipid rafts. In contrast, sphingolipids occur only in few bacteria, particularly some anaerobes, where they functionally replace other bacterial membrane lipids. Sphingolipids are found in the genera *Pedobacter* [104], *Bacteroides*, *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Sphingomonas*, *Sphingobacterium*, *Bdellovibrio*, *Cystobacter*, *Mycoplasma*, *Flectobacillus*, and possibly *Acetobacter* [4]. Their

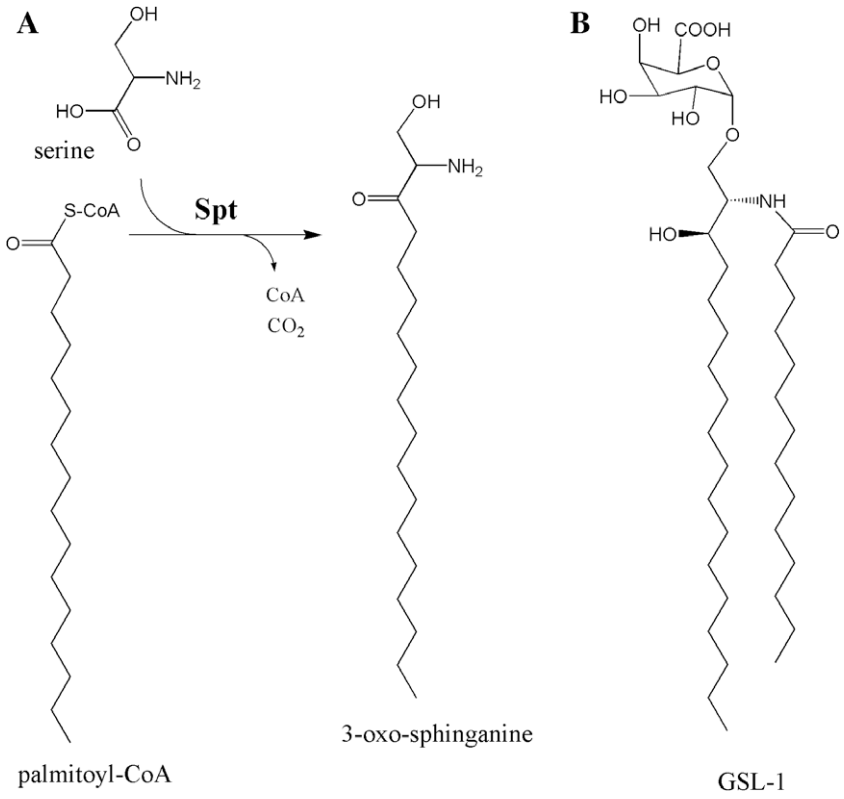


Fig. 9. Bacterial sphingolipids. The serine palmitoyl transferase (Spt)-catalyzed initial step of sphingolipid biosynthesis in bacteria (A) and the structure of glycosphingolipid GSL-1 from *Sphingomonas* (B).

occurrence in bacteria is thought so unusual that the genus name of the respective bacterium harbours the prefix "Sphingo", i.e. in *Sphingomonas* and *Sphingobacterium*. Some Gram-negative bacteria, such as *Sphingomonas capsulata*, lack lipopolysaccharide in their outer membrane and instead have glycosphingolipids as functional replacements. In *Sphingomonas paucomobilis*, two glycosphingolipids differing in their ceramide structures are substituted with the tetrasaccharide Man-Gal-GlcNAc-GlcA [105]. The chirality at carbon atoms C-2 and C-3 of the sphingoid base is D-erythro [4,106]. The variability of glycosphingolipids in the outer bacterial membrane of the *Sphingomonadaceae* is considerable [107], and only some of these glycosphingolipids, such as GSL-1 (Fig. 9B), are recognized by natural killer T cells which provide an innate-type immune response towards glycosphingolipid-containing bacteria [107,108]. Major molecular species of ceramides in sphingobacteria have been identified as 2-*N*-2′-hydroxy-13′-methyltetradecanoyl-15-methylhexadecasphinganine, 2-*N*-13′-methyltetradecanoyl-15-methylhexadecasphinganine, and 2-*N*-13′-methyltetradecanoyl-hexadecasphinganine [109–111]. Many *Bacteroides* species have two types of phosphosphingolipids, ceramide phosphorylethanolamine and ceramide phosphorylglycerol [112].

In eukaryotes, the biosynthesis of sphingolipids takes place in five stages. It begins with the condensation of serine and a fatty acyl-CoA to form 3-oxo-sphinganine (Stage 1), followed by its reduction to sphinganine (Stage 2), acylation to *N*-acylsphinganine (dihydroceramide) (Stage 3), and desaturation to ceramide (Stage 4) [106,113]. In Stage 5, ceramide is modified with different polar groups to form the great diversity of sphingolipids. Although the eukaryotic genes involved in the sphingolipid biosynthetis are known [114,115], little is known in bacteria. An exception is sphingolipid biosynthesis Step 1 catalyzed by serine palmitoyltransferase (EC 2.3.1.50) (Fig. 9A). Like other oxoamine synthases, the bacterial soluble serine palmitoyltransferase is pyridoxal 5′-phosphate-dependent and performs a Claisen condensation between serine and the acyl-CoA thioester with concomitant decarboxylation [116]. Although the serine palmitoyltransferase from *Sphingomonas* seems to be cytosolic, the serine palmitoyltransferases from *Sphingobacterium multivorum* and from *Bdellovibrio stolpii* are peripherically associated with the cytoplasmic side of the inner membrane [117,118]. The *S. paucimobilis* serine palmitoyltransferase crystal structure [119] at 1.3 Å resolution shows that the enzyme is a symmetrical homodimer with two active sites composed of monomers consisting of three domains. The pyridoxal 5′-phosphate cofactor is bound covalently to lysine 265 as an internal aldimine/Schiff base, and the active site is composed of residues from both subunits, located at the bottom of a deep cleft. Other bacterial α-oxoamine synthases are 8-amino-7-oxononanoate synthase (BioF; EC 2.3.1.47) which catalyzes the formation of 8-amino-7-oxononanoate from 6-carboxyhexanoyl-CoA and L-alanine during biotin biosynthesis, 5-aminolevulinate synthase (HemA; EC 2.3.1.37), which catalyzes the formation of 5-aminolevulinate from succinyl-CoA and glycine during tetrapyrrole and heme biosynthesis in α-proteobacteria [120], and 2-amino-3-oxobutyrate coenzyme A ligase (Kbl; EC 2.3.1.29), which cleaves 2-amino-3-oxobutyrate into acetyl-CoA and glycine during threonine degradation [116]. Phylogenetic analysis of bacterial α-oxoamine synthases (Fig. 10) suggests that distinct subgroups of serine palmitoyltransferases exist and that the encoding genes frequently form an operon with a putative acyl carrier protein gene. This finding suggests specialized acyl carrier proteins, instead of CoA, are used in some cases during the initial step of sphingolipid biosynthesis in bacteria. Based on our analysis (Fig. 10), the ability to form sphingolipids is more widespread in α-proteobacteria (*Gluconobacter, Granulibacter, Caulobacter*) than previously thought and might occur even in the β-proteobacterium *Nitrosomonas* and

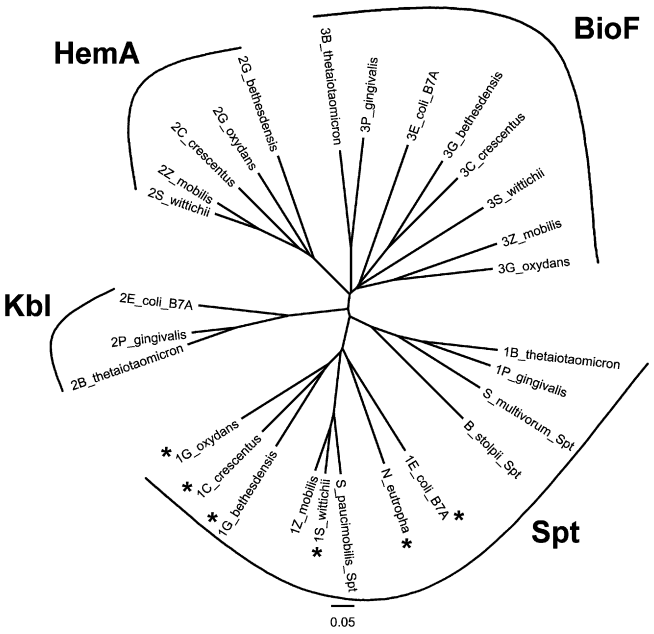


Fig. 10. Unrooted phylogenetic tree of selected bacterial serine palmitoyltransferases and other α-oxoamine synthases from bacteria. The tree was constructed using the program CLUSTALW (http://www.expasy.ch/). Distances between sequences are expressed as 0.05 changes per amino acid residue. The asterisks label species in which the serine palmitoyltransferase gene forms an operon with a putative acyl carrier protein. Accession numbers are as follows: *Bacteroides thetaiotaomicron* VPI-5482 (NP_809783, NP_810284, NP_810356), *Bacteriovorax stolpii* Spt (BAF73753), *Caulobacter crescentus* CB15 (NP_419978, NP_420168, NP_420387), *Escherichia coli* B7A (EDV60350, ZP_03029941, ZP_03030227), *Gluconobacter oxydans* 621H (AAW61792, YP_192033, YP_191153), *Granulibacter bethesdensis* CGDNIH1 (YP_744060, YP_744129, YP_744319), *Nitrosomonas eutropha* C91 (YP_746703), *Porphyromonas gingivalis* ATCC 33277 (YP_001929837, YP_001929605, YP_001929054), *Sphingobacterium multivorum* Spt (BAF73751), *Sphingomonas paucimobilis* Spt (BAB56013), *Sphingomonas wittichii* RW1 (YP_001264383, YP_001264306, YP_001261757), and *Zymomonas mobilis* subsp. *mobilis* ZM4 (YP_163005, YP_162933, YP_163652). Annotations of genes are: 8-amino-7-oxononanoate synthase (BioF), 2-amino-3-ketobutyrate coenzyme A ligase (Kbl), 5-aminolevulinate synthase (HemA), and serine palmitoyltransferase (Spt).

in several pathogenic *Escherichia coli* strains, i.e. in the enterotoxigenic *E. coli* (ETEC) B7A.

6. Sulfonolipids in the *Cytophaga* group

Gram-negative bacteria of the *Cytophaga* group move by gliding. Major lipids in the membranes of *Cytophaga johnsonae* are sulfonolipids, OL, and PE. Sulfonolipids and OL are predominantly localized to the outer membrane whereas PE is the predominant lipid of the inner membrane [121]. Sulfonolipids contain capnine that is formed by the condensation of cysteate with fatty acyl-CoA under the release of CO_2 [122,123] (Fig. 11A), in a reaction analogous to the one catalyzed by serine palmitoyltransferase [119]. Capnine is then converted to *N*-acyl-capnine, the membrane-forming sulfonolipid. The *N*-acylated residues are C14, C15, and C16 3-hydroxylated iso-fatty acids [124]. Mutants of *C. johnsonae*, deficient in gliding and sulfonolipid biosynthesis, were isolated and restoration of the sulfonolipid content by providing cysteate resulted in recovery of the ability to glide [123]. Therefore, sulfonolipids might be required for gliding motility. A structural variant of capnine exists in another member of the *Cytophaga* group, *Salinibacter ruber* [125]. The *Salinibacter* sulfonolipid contains an extra carboxylate at carbon 2 and an *O*-acyl group at carbon 3 (Fig. 11B) that is diagnostic for this extremely halophilic bacterial genus [125].

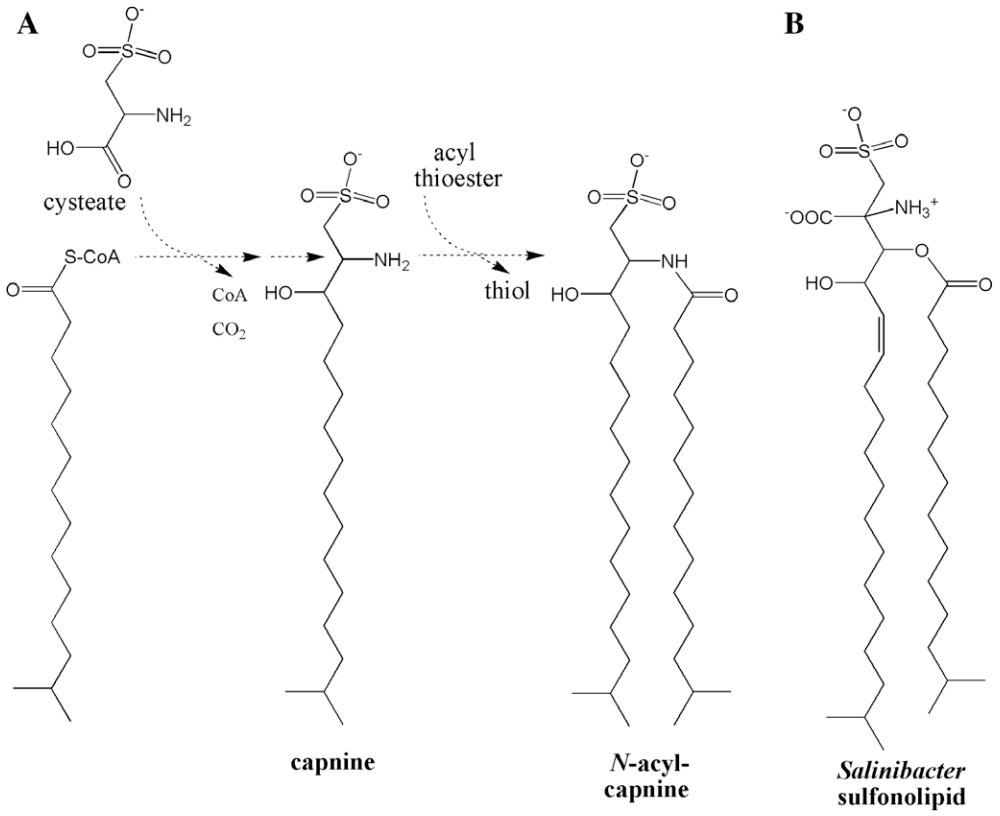


Fig. 11. Proposed pathway for sulfonolipid biosynthesis and an unusual sulfonolipid from *Salinibacter*.

7. Stress causes changes in bacterial membranes

Membrane lipid compositions of bacteria have usually been determined after the organisms had been grown on complex or defined culture media. Such determinations are reproducible and have led to the conviction that membrane lipid compositions are characteristic invariable traits of organisms. Unlike animal cells, however, plant and bacterial cells are not embedded in a controlled environment but are subject to many environmental changes and stresses. The bacterial membrane adapts to changing environments by altering the membrane lipid components by which it is formed.

It has long been known that reduced temperatures [1] or increased hydrostatic pressure [126] cause a reduction of membrane fluidity. In an attempt to maintain the fluidity of their membranes, bacteria include more unsaturated or branched fatty acyl chains into their membrane lipids thereby increasing packing disorder and fluidity of their membranes [127]. Acid stress in proteobacteria causes modifications of membrane lipids, such as formation of lysyl-PG [10], alanyl-PG [46] or the hydroxylation of OL [11]. Under phosphorus-limiting conditions, membrane phospholipids of some bacteria are partially replaced by lipids without phosphorus as demonstrated in *Bacillus subtilis* [128], *Pseudomonas diminuta* [129], *P. fluorescens* [130], and *Rhodobacter sphaeroides* [50]. In *S. meliloti*, these phosphorus-free lipids are sulfoquinovosyl diacylglycerol, OL, and DGTS [51]. In other bacteria, these phosphorus-free lipids include glycolipids as well. The ability to form OL or DGTS contributes to increased cell yields when *S. meliloti* is grown under the phosphorus-limiting condition [54]. In *S. meliloti*, the amounts of OL formed are strongly dependent on growth conditions [61]. However, in clinical isolates of *Flavobacterium* [77], *Burkholderia* isolates [82] and in pathogenic *Brucella* and *Bordetella* species [131], OL are normally major membrane lipids.

Lipid A-containing lipopolysaccharides (LPS) usually cover the outer surface of the outer membrane in Gram-negative bacteria and pose a major permeability barrier for hydrophilic and hydrophobic compounds. It is assumed that the hydrocarbon regions of the outer membrane are in a gel-like state of very low fluidity under physiological conditions [132]. Strong interactions between the lipid molecules forming the outer membrane are probably key to this gel-like behaviour and to its functions as a permeability barrier. Different environments/stresses require adjustments in the outer membrane that are accomplished by certain chemical modifications of LPS. The absence of divalent cations (Mg^{2+} and Ca^{2+}) will destabilize the outer membrane, and low Mg^{2+} concentrations activate the PhoPQ system to trigger a number of modifications of the LPS of *S. typhimurium* to stabilize the outer membrane [133]. Among these membrane-stabilizing modifications is the LpxO-catalyzed 2-hydroxylation of an esterified acyl residue of the lipid A of LPS [80,81]. Introduction of an additional hydroxyl group into the fatty acyl chain of a membrane lipid increases hydrogen bonding with neighboring molecules leading to membrane stabilization. In some bacterial groups, other lipids occur in the outer membrane either in addition to or in place of LPS. These include sphingolipids, sulfonolipids [132], and OLs [60]. Each of these outer membrane lipids have fatty acids with hydroxyl groups at the 2- and/or 3-position to stabilize the membrane. In addition, PE, which is enriched in the outer membrane, is 2-hydroxylated on its *sn*-2 fatty acid in *Burkholderia* [83].

8. Conclusions and perspectives

Membrane lipids act to form the lipid bilayer surrounding every cell and interact with other biomolecules based on their distinct chemical nature. Although phosphatidylserine has been

extensively studied in eukaryotes, other, less universal amino acid-containing membrane lipids are less well-known. Ornithine-containing lipids are formed in a direct two-step pathway whereas more steps are needed to form any of the glycerophospholipids. Therefore, making ornithine-containing lipids and other amino acid-containing acyl-oxyacyl lipids might be an easy way to build membranes in primitive biological systems. However, OL are certainly less resistant to extreme environmental conditions than archaeal ether lipids and probably would not have been of use when life originated on earth. Also, no OL-containing bacterium is known that is totally devoid of glycerophospholipids, leaving the question open whether a functional membrane can be formed by phosphorus-free membrane lipids only. Resolving the genetics and biochemistry of lysyl-phosphatidylglycerol, diacylglyceryl trimethylhomoserine, and ornithine-containing lipids in recent years has revealed the importance of these lipids in adapting to stress conditions and for the survival of bacteria. Nevertheless, we are only beginning to understand the functions of some of the amino acid-containing bacterial membrane lipids. The addition of amino acids into the structure of membrane lipids increases structural and chemical diversity, modifies net charge and polarity, and permits interaction with elements in the environment. For many minor amino acid-containing membrane lipids not much more than their structure and the producing bacterium are known. However, the immense information on bacterial genomes and improved bioinformatic tools will accelerate the detection of structural genes for many amino acid-containing bacterial membrane lipids. In addition to the more traditional approaches, another avenue for discovering new amino acid-containing bacterial membrane lipids will be the expression of metagenomic libraries and a subsequent screening for lipids. Finally, more biochemical studies are needed on the biosynthesis pathways as well as structural studies on the enzymes involved to provide feedback to impove bioinformatic predictions for ORFs involved in the biosynthesis of amino acid-containing bacterial membrane lipids.

Acknowledgments

This review is dedicated to Eugene P. Kennedy on the occasion of his 90th birthday. Work in our laboratory was supported by grants from DGAPA/UNAM (IN200806, IN217907, and IN218009) and the Consejo Nacional de Ciencia y Tecnología de México (CONACyT 46020-N, 49738-Q, and 82614).

References

[1] Rock CO. Fatty acid and phospholipid metabolism in prokaryotes. In: Vance DE, Vance JE, editors. Biochemistry of lipids lipoproteins and membranes. Amsterdam: Elsevier; 2008. p. 59–96.
[2] Raetz CR, Reynolds CM, Trent MS, Bishop RE. Lipid A modification systems in Gram-negative bacteria. Annu Rev Biochem 2007;76:295–329.
[3] Sohlenkamp C, López-Lara IM, Geiger O. Biosynthesis of phosphatidylcholine in bacteria. Prog Lipid Res 2003;42:115–62.
[4] Olsen I, Jantzen E. Sphingolipids in bacteria and fungi. Anaerobe 2001;7:103–12.
[5] Jorasch P, Wolter FP, Zähringer U, Heinz E. A UDP glucosyltransferase from *Bacillus subtilis* successively transfers up to four glucose residues to 1,2-diacylglycerol: expression of ypfP in *Escherichia coli* and structural analysis of its reaction products. Mol Microbiol 1998;29:419–30.
[6] Roy H, Ibba M. RNA-dependent lipid remodeling by bacterial multiple peptide resistance factors. Proc Natl Acad Sci USA 2008;105:4667–72.
[7] Jackson M, Crick DC, Brennan PJ. Phosphatidylinositol is an essential phospholipid in mycobacteria. J Biol Chem 2000;275:30092–9.
[8] Summons RE, Bradley AS, Jahnke LL, Waldbauer JR. Steroids, triterpenoids and molecular oxygen. Philos Trans Roy Soc B 2006;361:951–68.
[9] López-Lara IM, Sohlenkamp C, Geiger O. Membrane lipids in plant-associated bacteria: their biosyntheses and possible functions. Mol Plant Microbe Interact 2003;16:567–79.
[10] Sohlenkamp C, Galindo-Lagunas KA, Guan Z, Vinuesa P, Robinson S, Thomas Oates J, et al. The lipid lysyl-phosphatidylglycerol is present in membranes of *Rhizobium tropici* CIAT899 and confers increased resistance to polymyxin B under acidic growth conditions. Mol Plant Microbe Interact 2007;20:1421–30.
[11] Rojas-Jiménez K, Sohlenkamp C, Geiger O, Martínez-Romero E, Werner D, Vinuesa P. A ClC chloride channel homolog and ornithine-containing membrane lipids of *Rhizobium tropici* CIAT899 are involved in symbiotic efficiency and acid tolerance. Mol Plant Microbe Interact 2005;18:1175–85.
[12] Asselineau J. Bacterial lipids containing amino acids or peptides linked by amide bonds. Fortschr Chem Org Naturst 1991;56:1–85.
[13] Brady SF, Chao CJ, Clardy J. New natural product families from an environmental DNA (eDNA) gene cluster. J Am Chem Soc 2002;124:9968–9.
[14] Desai JD, Banat IM. Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 1997;61:47–64.
[15] Dowhan W, Bogdanov M, Mileykovskaya E. Functional roles of lipids in membranes. In: Vance DE, Vance JE, editors. Biochemistry of lipids, lipoproteins and membranes. Amsterdam, The Netherlands: Elsevier; 2008. p. 1–37.
[16] Sohlenkamp C, de Rudder KEE, Geiger O. Phosphatidylethanolamine is not essential for growth of *Sinorhizobium meliloti* on complex culture media. J Bacteriol 2004;186:1667–77.
[17] Koonin EV. A duplicated catalytic motif in a new superfamily of phosphohydrolases and phospholipid synthases that includes poxvirus envelope proteins. Trends Biochem Sci 1996;21:242–3.
[18] Vance DE, Vance JE. Phospholipid biosynthesis in eukaryotes. In: Vance DE, Vance JE, editors. Biochemistry of lipids, lipoproteins and membranes. Amsterdam, The Netherlands: Elsevier; 2008. p. 213–44.
[19] Langley KE, Yaffe MP, Kennedy EP. Biosynthesis of phospholipids in *Bacillus megaterium*. J Bacteriol 1979;140:996–1007.
[20] Nguyen NA, Sallans L, Kaneshiro ES. The major glycerophospholipids of the predatory and parasitic bacterium *Bdellovibrio bacteriovorus* HID5. Lipids 2008;43:1053–63.
[21] Hawrot E, Kennedy EP. Phospholipid composition and membrane function in phosphatidylserine decarboxylase mutants of *Escherichia coli*. J Biol Chem 1978;253:8213–20.
[22] Matsumoto K, Okada M, Horikoshi Y, Matsuzaki H, Kishi T, Itaya M, et al. Cloning, sequencing, and disruption of the *Bacillus subtilis psd* gene coding for phosphatidylserine synthase. J Bacteriol 1998;180:100–6.
[23] Vences-Guzmán MA, Geiger O, Sohlenkamp C. *Sinorhizobium meliloti* mutants deficient in phosphatidylserine decarboxylase accumulate phosphatidylserine and are strongly affected during symbiosis with alfalfa. J Bacteriol 2008;190:6846–56.
[24] Vance JE, Steenbergen R. Metabolism and functions of phosphatidylserine. Prog Lipid Res 2005;44:207–34.
[25] Rontein D, Wu WI, Voelker DR, Hanson AD. Mitochondrial phosphatidylserine decarboxylase from higher plants. Functional complementation in yeast, localization in plants, and overexpression in *Arabidopsis*. Plant Physiol 2003;132:1678–87.
[26] Langley KE, Hawrot E, Kennedy EP. Membrane assembly: movement of phosphatidylserine between the cytoplasmic and outer membranes of *Escherichia coli*. J Bacteriol 1982;152:1033–41.
[27] Lu Y-J, Zhang Y-M, Grimes KD, Qi J, Lee RE, Rock CO. Acyl-phosphates initiate membrane phospholipid synthesis in Gram-positive pathogens. Mol Cell 2006;23:765–72.
[28] O'Leary WM, Wilkinson SG. Gram-positive bacteria. In: Ratledge S, Wilkinson SG, editors. Microbial lipids. London: Academic Press; 1988. p. 117–201.
[29] Nahaie MR, Goodfellow M, Minnikin DE, Hajek V. Polar lipid and isoprenoid quinine composition in the classification of *Staphylococcus*. J Gen Microbiol 1984;130:2427–37.
[30] Op den Kamp JA, Redai I, van Deenen LL. Phospholipid composition of *Bacillus subtilis*. J Bacteriol 1969;99:298–303.
[31] Samant S, Hsu FF, Neyfakh AA, Lee H. The *Bacillus anthracis* protein MprF is required for synthesis of lysylphosphatidylglycerols and for resistance to cationic antimicrobial peptides. J Bacteriol 2009;191:1311–9.
[32] Fischer W, Leopold K. Polar lipids of four Listeria species containing L-lysylcardiolipin, a novel lipid structure, and other unique phospholipids. Int J Syst Bacteriol 1999;49:653–62.
[33] Houtsmuller UM, van Deenen L. Identification of a bacterial phospholipid as an O-ornithine ester of phosphatidyl glycerol. Biochim Biophys Acta 1963;70:211–3.
[34] Gould RM, Lennarz WJ. Biosynthesis of aminoacyl derivatives of phosphatidylglycerol. Biochem Biophys Res Commun 1967;26:512–4.
[35] Dos Santos Mota JM, Op den Kamp JAF, Verheij HM, van Deenen LLM. Phospholipids of *Streptococcus faecalis*. J Bacteriol 1970;104:611–9.
[36] Tocanne JF, Verheij HM, den Kamp JA, van Deenen LL. Chemical and physicochemical studies of lysylphosphatidylglycerol derivatives. Occurrence of a 2′ yields 3′ lysyl migration. Chem Phys Lipids 1974;13:389–403.
[37] Peschel A, Jack RW, Otto M, Collins VL, Staubitz P, Nicholson G, et al. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. J Exp Med 2001;193:1067–76.
[38] Oku Y, Kurokawa K, Ichihashi N, Sekimuzu K. Characterization of the *Staphylococcus aureus mprF* gene, involved in lysinylation of phosphatidylglycerol. Microbiology 2004;150:45–51.
[39] Staubitz P, Neumann H, Schneider T, Wiedemann L, Peschel A. MprF-mediated biosynthesis of lysylphosphatidylglycerol, an important

determinant in staphylococcal defensing resistance. FEMS Microbiol Lett 2004;231:67–71.
[40] Salzberg LI, Helmann JD. Phenotypic and transcriptomic characterization of *Bacillus subtilis* mutants with grossly altered membrane composition. J Bacteriol 2008;190:7797–807.
[41] Kenward MA, Brown MRW, Fryer JJ. The influence of calcium or manganese on the resistance to EDTA, polymyxin B or cold shock, and the composition of *Pseudomonas aeruginosa* grown in glucose- or magnesium-depleted batch cultures. J Appl Bacteriol 1979;47:489–503.
[42] Jones DE, Smith JD. Phospholipids of the differentiating bacterium *Caulobacter crescentus*. Can J Biochem 1979;57:424–8.
[43] Reeve WG, Tiwari RP, Worsley PS, Dilworth MJ, Glenn AR, Howieson JG. Constructs for insertional mutagenesis, transcriptional signal localisation and gene regulation studies in root nodule and other bacteria. Microbiology 1999;145:1307–16.
[44] Vinuesa P, Neumann-Silkow F, Pacios-Bras C, Spaink HP, Martinez-Romero E, Werner D. Genetic analysis of a pH-regulated operon from *Rhizobium tropici* CIAT899 involved in acid tolerant and nodulation competitiveness. Mol Plant Microbe Interact 2003;16:159–68.
[45] Reeve WG, Tiwari RP, Bräu l, Castelli J, Garau G, Dilworth MJ, et al. The *Sinorhizobium medicae* WSM419 *lpiA* gene is transcriptionally activated by low pH and regulated by FsrR. Microbiology 2006;152:3049–59.
[46] Klein S, Lorenzo C, Hoffmann S, Walther JM, Storbeck S, Piekarski T, et al. Adaptation of *Pseudomonas aeruginosa* to various conditions includes tRNA-dependent formation of alanyl-phosphatidylglycerol. Mol Microbiol 2009;71:551–65.
[47] Fischer W, Arneth-Seifert D. D-alanylcardiolipin, a major component of the unique lipid pattern of *Vagococcus fluvialis*. J Bacteriol 1998;180:2950–7.
[48] Peter-Katalinic J, Fischer W. A-D-Glucopyranosyl-, D-alanyl- and L-lysylcardiolipin from Gram-positive bacteria: analysis by fast atom bombardment mass spectrometry. J Lipid Res 1998;39:2286–92.
[49] Thedieck K, Hain T, Mohamed W, Tindall BJ, Nimtz M, Chakraborty T, et al. The MprF protein is required for lysinylation of phospholipids in listerial membranes and confers resistance to cationic antimicrobial peptides (CAMPs) on *Listeria monocytogenes*. Mol Microbiol 2006;62: 1325–39.
[50] Benning C, Huang ZH, Gage DA. Accumulation of a novel glycolipid and a betaine lipid in cells of *Rhodobacter sphaeroides* grown under phosphate limitation. Arch Biochem Biophys 1995;317:103–11.
[51] Geiger O, Röhrs V, Weissenmayer B, Finan TM, Thomas-Oates JE. The regulator gene phoB mediates phosphate stress-controlled synthesis of the membrane lipid diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine in *Rhizobium (Sinorhizobium) meliloti*. Mol Microbiol 1999;32:63–73.
[51a] Van Mooy BA, Fredricks HF, Pedler BE, Dyhrman ST, Karl DM, Koblízek M, et al. Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. Nature 2009;458:69–72.
[52] Klug RM, Benning C. Two enzymes of diacylglyceryl-O-4′-(*N*,*N*,*N*,-trimethyl) homoserine biosynthesis are encoded by btaA and btaB in the purple bacterium *Rhodobacter sphaeroides*. Proc Natl Acad Sci USA 2001;98: 5910–5915.
[53] Riekhof WR, Andre C, Benning C. Two enzymes, BtaA and BtaB, are sufficient for betaine lipid biosynthesis in bacteria. Arch Biochem Biophys 2005;441:96–105.
[54] López-Lara IM, Gao JL, Soto MJ, Solares-Pérez A, Weissenmayer B, Sohlenkamp C, et al. Phosphorus-free membrane lipids of *Sinorhizobium meliloti* are not required for the symbiosis with alfalfa but contribute to increased cell yields under phosphorus-limiting conditions of growth. Mol Plant Microbe Interact 2005;18:973–82.
[55] Riekhof WR, Sears BB, Benning C. Annotation of genes involved in glycerolipid biosynthesis in *Chlamydomonas reinhardtii*: discovery of the betaine lipid synthase $BTA1_{Cr}$. Eukaryot Cell 2005;4:242–52.
[56] Madigan MT, Martinko JM, Dunlap PV, Clark DP. Brock biology of microorganisms. 12th ed. San Francisco, CA: Pearson Benjamin Cummings; 2008.
[57] Lerouge P, Lebas MH, Agapakis-Caussé C, Promé JC. Isolation and structural characterization of a new non-phosphorylated lipoamino acid from *Mycobacterium phlei*. Chem Phys Lipids 1988;49:161–6.
[58] Knoche HW, Shively JM. The structure of an ornithine-containing lipid from *Thiobacillus thiooxidans*. J Biol Chem 1972;247:170–8.
[59] Kawai Y, Nakagawa Y, Matuyama T, Akagawa K, Itagawa K, Fukase K, et al. A typical bacterial ornithine-containing lipid *N*α-(D)-[3-(hexadecanoyloxy)hexadecanoyl]-ornithine is a strong stimulant for macrophages and a useful adjuvant. FEMS Immunol Med Microbiol 1999;23:67–73.
[60] Dees C, Shively JM. Localization and quantitation of the ornithine lipid of *Thiobacillus thiooxidans*. J Bacteriol 1982;149:798–9.
[61] Gao JL, Weissenmayer B, Taylor AM, Thomas-Oates J, López-Lara IM, Geiger O. Identification of a gene required for the formation of lyso-ornithine lipid, an intermediate in the biosynthesis of ornithine-containing lipids. Mol Microbiol 2004;53:1757–70.
[62] Weissenmayer B, Gao JL, López-Lara IM, Geiger O. Identification of a gene required for the biosynthesis of ornithine-derived lipids. Mol Microbiol 2002;45:721–33.
[63] Aygun-Sunar S, Mandaci S, Koch HG, Murria IVJ, Goldfine H, Daldal F. Ornithine lipid is required for optimal steady-state amounts of *c*-type cytochromes in *Rhodobacter capsulatus*. Mol Microbiol 2006;61:418–35.
[64] Gough J, Karplus K, Hughey R, Chothia C. Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure. J Mol Biol 2001;313:903–19.
[65] Heath RJ, Rock CO. A conserved histidine is essential for glycerolipid acyltransferase catalysis. J Bacteriol 1998;180:1425–30.
[66] Watson WT, Minogue TD, Val DL, Beck von Bodman S, Churchill MEA. Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. Mol Cell 2002;9:685–94.
[67] Gould TA, Schweizer HP, Churchill MEA. Structure of the *Pseudomonas aeruginosa* acylhomoserine lactone synthase LasI. Mol Microbiol 2004;53:1135–46.
[68] Van Wagoner RM, Clardy J. FeeM, an *N*-acyl amino acid synthase from an uncultured soil microbe: Structure, mechanism, and acyl carrier protein binding. Structure 2006;14:1425–35.
[69] Zhang X, Ferguson-Miller SM, Reid GE. Characterization of ornithine and glutamine lipids extracted from cell membranes of *Rhodobacter sphaeroides*. J Am Soc Mass Spectrom 2009;20:198–212.
[70] Schüler D. Molecular analysis of a subcellular compartment: the magnetosome membrane in *Magnetospirillum gryphiswaldense*. Arch Microbiol 2004;181:1–7.
[71] Brady SF, Clardy J. *N*-acyl derivatives of arginine and tryptophan isolated from environmental DNA expressed in *Escherichia coli*. Org Lett 2005;7:3613–6.
[72] Clardy J, Brady SF. Cyclic AMP directly activates NasP, an *N*-acyl amino acid antibiotic biosynthetic enzyme cloned from an uncultured β-proteobacterium. J Bacteriol 2007;189:6487–9.
[73] West J, Tompkins CK, Balantac N, Nudelman E, Meengs B, White T, et al. Cloning and expression of two human lysophosphatidic acid acyltransferase cDNAs that enhance cytokine-induced signaling responses in cells. DNA Cell Biol 1997;16:691–701.
[74] Shih GC, Kahler CM, Swartley JS, Rahman MM, Coleman J, Carlson RW, et al. Multiple lysophosphatidic acid acyltransferases in *Neisseria meningitidis*. Mol Microbiol 1999;32:942–52.
[75] Cullinane M, Baysse C, Morrissey JP, O'Gara F. Identification of two lysophosphatidic acid acyl transferase genes with overlapping function in *Pseudomonas fluorescens*. Microbiology 2005;151:3071–80.
[76] Aygun-Sunar S, Bilaloglu R, Goldfine H, Daldal F. *Rhodobacter capsulatus* OlsA is a bifunctional enyzme active in both ornithine lipid and phosphatidic acid biosynthesis. J Bacteriol 2007;189:8564–74.
[77] Kawai Y, Yano I, Kaneda K. Various kinds of lipoamino acids including a novel serine-containing lipid in an opportunistic pathogen *Flavobacterium*. Their structures and biological activities on erythrocytes. Eur J Biochem 1988;171:73–80.
[78] Tahara Y, Kameda M, Yamada Y, Kondo K. A new lipid; the ornithine and taurine-containing "cerilipin". Agric Biol Chem 1976;40:243–4.
[79] Galbraith L, Jonsson MH, Rudhe LC, Wilkinson SG. Lipids and fatty acids of *Burkholderia* and *Ralstonia* species. FEMS Microbiol Lett 1999;173:359–64.
[80] Gibbons HS, Lin S, Cotter RJ, Raetz CR. Oxygen requirement for the biosynthesis of the S-2-hydroxymyristate moiety in *Salmonella typhimurium* lipid A. Function of LpxO, a new Fe^{2+}/alpha-ketoglutarate-dependent dioxygenase homologue. J Biol Chem 2000;275:32940–9.
[81] Gibbons HS, Reynolds CM, Guan Z, Raetz CRH. An inner membrane dioxygenase that generates the 2-hydroxymyristate moiety of *Salmonella* lipid A. Biochemistry 2008;47:2814–25.
[82] Phung LV, Tran TB, Hotta H, Yabuuchi E, Yano I. Cellular lipid and fatty acid compositions of *Burkholderia pseudomallei* strains isolated from human and environment in Viet Nam. Microbiol Immunol 1995;39:105–16.
[83] Taylor CJ, Anderson AJ, Wilkinson SG. 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 1998;144:1737–45.
[84] Tahara Y, Shinmoto K, Yamada Y, Kondo K. Enzymatic synthesis of tauro-ornithine lipid in *Gluconobacter cerinus*. Agric Biol Chem 1978;42:205–6.
[85] Tahara Y, Yamada Y, Kondo K. A new lysine-containing lipid isolated from *Agrobacterium tumefaciens*. Agric Biol Chem 1976;40:1449–50.
[86] Kawazoe R, Okuyama H, Reichardt W, Sasaki S. Phospholipids and a novel glycine-containing lipoamino acid in *Cytophaga johnsonae* Stanier strain C21. J Bacteriol 1991;173:5470–5.
[87] Batrakov SG, Nikitin DI, Mosezhnyi AE, Ruzhitsky AO. A glycine-containing phosphorus-free lipoaminoacid from the Gram-negative marine bacterium *Cyclobacterium marinus* WH. Chem Phys Lipids 1999;99:139–43.
[88] Kawazoe R, Monde K, Reichardt W, Okuyama H. Lipoamino acids and sulfonolipids in *Cytophaga johnsonae* Stanier strain C21 and six related species of *Cytophaga*. Arch Microbiol 1992;158:171–5.
[89] Nikitin DI, Oranskaya MS, Pitryuk IA, Chernykh NA, Lysenko AM. A new ring-forming bacterium *Arcocella aquatica* gen. et sp. nov. Microbiology (Moscow) 1994;63:87–90 [Engl. Transl.].
[90] Raj HD, Maloy SR. Proposal of *Cyclobacterium marinus* gen. nov., comb. nov. for a marine bacterium previously assigned to the genus *Flectobacillus*. Int J Syst Bacteriol 1990;40:337–47.
[91] Shiozaki M, Deguchi N, Ishikawa T, Haruyama H, Kawai Y, Nishijima M. Revised structure of flavolipin and synthesis of its isomers. Tetrahedron Lett 1998;39:4497–500.
[92] Shiozaki M, Deguchi N, Mochizuki T, Wakabayashi T, Ishikawa T, Haruyama H, et al. Revised structure and synthesis of flavolipin. Tetrahedron 1998;54:11861–76.

[93] Batrakov SG, Nikitin DI, Sheichenko VI, Ruzhitsky AO. A novel sulfonic-acid analogue of ceramide is the major extractable lipid of the Gram-negative marine bacterium *Cyclobacterium marinus* WH. Biochim Biophys Acta 1998;1391:79–91.
[94] Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med 1999;189:1777–82.
[95] Kawai Y, Akagawa K. Macrophage activation by an ornithine-containing lipid or a serine-containing lipid. Infect Immun 1989;57:2086–91.
[96] Okemoto K, Hanada K, Nishijima M, Kawasaki K. The preparation of a lipidic endotoxin affects its biological activities. Biol Pharm Bull 2008;31:1952–4.
[97] Kato H, Goto N. Adjuvanticity of an ornithine-containing lipid of *Flavobacterium meningosepticum* as a candidate vaccine adjuvant. Microbiol Immunol 1997;41:101–6.
[98] Kawai Y, Kamoshita K, Akagawa K. B-lymphocyte mitogenicity and adjuvanticity of an ornithine-containing lipid or a serine-containing lipid. FEMS Microbiol Lett 1991;67:127–9.
[99] Kawai Y, Takasuka N, Inoue K, Akagawa K, Nishijima M. Ornithine-containing lipids stimulate CD14-dependent TNF-alpha production from murine macrophage-like J774.1 and RAW 264.7 cells. FEMS Immunol Med Microbiol 2000;28:197–203.
[100] Kawai Y, Okawarab AI, Okuyama H, Kura F, Suzuki K. Modulation of chemotaxis, O(2)(−) production and myeloperoxidase release from human polymorphonuclear leukocytes by the ornithine-containing lipid and the serineglycine-containing lipid of *Flavobacterium*. FEMS Immunol Med Microbiol 2000;28:205–9.
[101] Kawai Y, Watanabe M, Matsuura M, Nishijima M, Kawahara K. The partially degraded lipopolysaccharide of *Burkholderia cepacia* and ornithine-containing lipids derived from some Gram-negative bacteria are useful complex lipid adjuvants. FEMS Immunol Med Microbiol 2002;34:173–9.
[102] Kawai Y, Kaneda K, Morisawa Y, Akagawa K. Protection of mice from lethal endotoxemia by use of an ornithine-containing lipid or a serine-containing lipid. Infect Immun 1991;59:2560–6 [Erratum: Infect Immun 1992;60:320].
[103] Gomi K, Kawasaki K, Kawai Y, Shiozaki M, Nishijima M. Toll-like receptor 4-MD-2 complex mediates the signal transduction induced by flavolipin, an amino acid-containing lipid unique to *Flavobacterium meningosepticum*. J Immunol 2002;168:2939–43.
[104] Steyn PL, Segers P, Vancanneyt M, Sandra P, Kersters K, Joubert JJ. Classification of heparinolytic bacteria into a new genus, *Pedobacter*, comprising four species: *Pedobacter heparinus* comb. nov., *Pedobacter piscium* comb. nov., *Pedobacter africanus* sp. nov. and *Pedobacter saltans* sp. nov. proposal of the family *Sphingobacteriaceae* fam. nov.. Int J Syst Bacteriol 1998;48:165–77.
[105] Kawahara K, Seydel U, Matsuura M, Danbara H, Rietschel ET, Zähringer U. Chemical structure of glycosphingolipids isolated from *Sphingomonas paucimobilis*. FEBS Lett 1991;292:107–10.
[106] Hanada K. Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. Biochim Biophys Acta 2003;1632:16–30.
[107] Kinjo Y, Pei B, Bufali S, Raju R, Richardson SK, Imamura M, et al. Natural *Sphingomonas* glycolipids vary greatly in their ability to activate natural killer T cells. Chem Biol 2008;15:654–64.
[108] Wu D, Zajonc DM, Fujio M, Sullivan BA, Kinjo Y, Kronenberg M, et al. Design of natural killer T cell activators: Structure and function of a microbial glycosphingolipid bound to mouse CD1d. Proc Natl Acad Sci USA 2006;103:3972–7.
[109] Yano I, Tomiyasu I, Yabuuchi E. Long chain base composition of strains of three species of *Sphingobacterium* gen. nov.. FEMS Microbiol Lett 1982;15:303–7.
[110] Yano I, Imaizumi S, Tomiyasu I, Yabuuchi E. Separation and analysis of free ceramides containing 2-hydroxy fatty acids in *Sphingobacterium* species. FEMS Microbiol Lett 1983;20:449–53.
[111] Naka T, Fujiwara N, Yano I, Maeda S, Doe M, Minamino M, et al. Structural analysis of sphingophospholipids derived from *Sphingobacterium spiritivorum*, the type species of genus *Sphingobacterium*. Biochim Biophys Acta 2003;1635:83–92.
[112] Kato M, Muto Y, Tanaka-Bandoh K, Watanabe K, Ueno K. Sphingolipid composition in *Bacteroides* species. Anaerobe 1995;1:135–9.
[113] Nieto FL, Pescio LG, Favale NO, Adamo AM, Sterin-Speziale NB. Sphingolipid metabolism is a crucial determinant of cellular fate in non-stimulated proliferating Madin–Darby canine kidney (MDCK) cells. J Biol Chem 2008;283:25682–91.
[114] Kihara A, Mitsutake S, Mizutani Y, Igarashi Y. Metabolism and biological functions of two phosphorylated sphingolipids, sphingosine 1-phosphate and ceramide 1-phosphate. Prog Lipid Res 2007;46:126–44.
[115] Hirabayashi Y, Furuya S. Roles of L-serine and sphingolipid synthesis in brain development and neuronal survival. Prog Lipid Res 2008;47:188–203.
[116] Kerbarh O, Campopiano DJ, Baxter RL. Mechanism of α-oxoamine synthases: identification of the intermediate Claisen product in the 8-amino-7-oxononanoate synthase reaction. Chem Commun 2006:60–2.
[117] Ikushiro H, Hayashi H, Kagamiyama H. A water-soluble homodimeric serine palmitoyltransferase from *Sphingomonas paucimobilis* EY2395T strain. Purification, characterization, cloning, and overproduction. J Biol Chem 2001;276:18249–56.
[118] Ikushiro H, Islam MM, Tojo H, Hayashi H. Molecular characterization of membrane-associated soluble serine palmitoyltransferases from *Sphingobacterium multivorum* and *Bdellovibrio stolpii*. J Bacteriol 2007;198:5749–61.
[119] Yard BA, Carter LG, Johnson KA, Overton IM, Dorward M, Liu H, et al. The structure of serine palmitoyltransferase; gateway to sphingolipid biosynthesis. J Mol Biol 2007;370:870–86.
[120] Astner I, Schulze JO, van den Heuvel J, Jahn D, Schubert WD, Heinz DW. Crystal structure of 5-aminolevulinate synthase, the first enzyme of heme biosynthesis, and its link to XLSA in humans. EMBO J 2005;24:3166–77.
[121] Pitta TP, Leadbetter ER, Godchaux III W. Increase of ornithine amino lipid content in a sulfonolipid-deficient mutant of *Cytophaga johnsonae*. J Bacteriol 1989;171:952–7.
[122] White RH. Biosynthesis of the sulfonolipid 2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acid in the gliding bacterium *Cytophaga johnsonae*. J Bacteriol 1984;159:42–6.
[123] Abbanat DR, Leadbetter ER, Godchaux III W, Escher A. Sulphonolipids are molecular determinants of gliding motility. Nature 1986;324:367–9.
[124] Godchaux III W, Leadbetter ER. Sulfonolipids of gliding bacteria: structure of the *N*-acylaminosulfonates. J Biol Chem 1984;259:2982–90.
[125] Corcelli A, Lattanzio VMT, Mascolo G, Babudri F, Oren A, Kates M. Novel sulfonolipid in the extremely halophilic bacterium *Salinibacter ruber*. Appl Environ Microbiol 2004;70:6678–85.
[126] DeLong EF, Yayanos AA. Adaptation of the membrane lipids of a deep-sea bacterium to changes in hydrostatic pressure. Science 1985;228:1101–3.
[127] Zhang YM, Rock CO. Membrane lipid homeostasis in bacteria. Nat Rev Microbiol 2008;6:222–33.
[128] Minnikin DE, Abdolrahimzadeh H, Baddiley J. Variation of polar lipid composition of *Bacillus subtilis* Marburg with different growth conditions. FEBS Lett 1972;27:16–8.
[129] Minnikin DE, Abdolrahimzadeh H, Baddiley J. Replacement of acidic phospholipids by acidic glycolipids in *Pseudomonas diminuta*. Nature (London) 1974;249:268–9.
[130] Minnikin DE, Abdolrahimzadeh H. The replacement of phosphatidylethanolamine and acidic phospholipids by an ornithine-amide lipid and a minor phosphorus-free lipid in *Pseudomonas fluorescens* NCMB 129. FEBS Lett 1974;43:257–60.
[131] Thiehle OW, Schwinn G. The free lipids of *Brucella melitensis* and *Bordetella pertussis*. Eur J Biochem 1973;34:333–44.
[132] Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 2003;67:593–656.
[133] Murata T, Tseng W, Guina T, Millar SI, Nikaido H. PhoPQ-mediated regulation produces a more robust permeability barrier in the outer membrane of *Salmonella enterica* serovar *typhimurium*. J Bacteriol 2007;189:7213–22.

8.1.2. II Artículo

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 *Rhizobium tropici* CIAT899. *Mol. Microbiol. 79,* 1496-1514.

Los lípidos de ornitina (OLs) están ampliamente distribuidos en las bacterias Gram negativas. Los OLs estándar consisten de un residuo de ornitina y dos ácidos grasos. Un ácido graso está unido al grupo α-amino de la ornitina por enlace amida y el otro ácido graso está esterificado al C3 del ácido graso amidificado. Los OLs pueden ser hidroxilados en el ácido graso esterificado y esa modificación ha sido relacionada con un incremento en la tolerancia a estrés. *Rhizobium tropici* CIAT899 es resistente a estreses como pH bajo y altas temperaturas y forma nódulos en las raíces de plantas de frijol, en los cuales fija nitrógeno. *R. tropici* CIAT899 forma cuatro clases de OLs. Estudios de la función de estos OLs han sido obstaculizados debido a carencia de conocimiento sobre su biosíntesis. En este trabajo se describe que la biosíntesis de OLs se incrementa bajo estrés ácido y que OLs estan enriquecidos en la membrana externa. Usando un escrutinio de expresión funcional, se identificó la hidroxilasa OlsE de OLs, la cual en combinación con la hidroxilasa OlsC son responsables de la biosíntesis de los OLs modificados en *R. tropici* CIAT899. A diferencia de otras hidroxilaciones descritas, la hidroxilación catalizada por OlsE ocurre en el residuo de ornitina. Se caracterizaron Mutantes deficientes en OlsE u OlsC y la doble mutante OlsC/OlsE. Mutantes derivadas de *R. tropici* CIAT899 deficientes en la hidroxilación de OLs que se debe a OlsC son más susceptibles al estrés ácido y a la temperatura. Las tres mutantes que carecen de las hidroxilasas de OLs se ven afectadas durante la simbiosis.

Molecular Microbiology (2011) 79(6), 1496–1514 ■
doi:10.1111/j.1365-2958.2011.07535.x
First published online 23 January 2011

Hydroxylated ornithine lipids increase stress tolerance in *Rhizobium tropici* CIAT899

Miguel Á. Vences-Guzmán,[1] Ziqiang Guan,[2] Ernesto Ormeño-Orrillo,[1] Napoleón González-Silva,[1] Isabel M. López-Lara,[1] Esperanza Martínez-Romero,[1] Otto Geiger[1] and Christian Sohlenkamp[1]*

[1]*Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Av. Universidad s/n, Apdo. Postal 565-A, Cuernavaca, Morelos, CP62210, Mexico.*
[2]*Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, USA.*

Summary

Ornithine lipids (OLs) are widespread among Gram-negative bacteria. Their basic structure consists of a 3-hydroxy fatty acyl group attached in amide linkage to the α-amino group of ornithine and a second fatty acyl group ester-linked to the 3-hydroxy position of the first fatty acid. OLs can be hydroxylated within the secondary fatty acyl moiety and this modification has been related to increased stress tolerance. *Rhizobium tropici*, a nodule-forming α-proteobacterium known for its stress tolerance, forms four different OLs. Studies of the function of these OLs have been hampered due to lack of knowledge about their biosynthesis. Here we describe that OL biosynthesis increases under acid stress and that OLs are enriched in the outer membrane. Using a functional expression screen, the OL hydroxylase OlsE was identified, which in combination with the OL hydroxylase OlsC is responsible for the synthesis of modified OLs in *R. tropici*. Unlike described OL hydroxylations, the OlsE-catalysed hydroxylation occurs within the ornithine moiety. Mutants deficient in OlsE or OlsC and double mutants deficient in OlsC/OlsE were characterized. *R. tropici* mutants deficient in OlsC-mediated OL hydroxylation are more susceptible to acid and temperature stress. All three mutants lacking OL hydroxylases are affected during symbiosis.

Accepted 29 December, 2010. *For correspondence. E-mail chsohlen@ccg.unam.mx; Tel. (+52) 777 3131697; Fax (+52) 777 3175581.

© 2011 Blackwell Publishing Ltd

Introduction

Membranes of the Gram-negative model organism *Escherichia coli* only contain three major phospholipids, that is phosphatidylethanolamine, phosphatidylglycerol and cardiolipin (Heath *et al*., 2002). Some other bacteria also form the membrane lipids phosphatidylinositol or phosphatidylcholine (Jackson *et al*., 2000; Sohlenkamp *et al*., 2003). In addition to phospholipids, many bacteria also present phosphorus-free membrane lipids such as ornithine lipids (OLs), diacylglyceryl-*N*, *N*, *N*-trimethylhomoserine (DGTS) or sulpholipids (SLs) in their membranes (López-Lara *et al*., 2003; Geiger *et al*., 2010). In some cases, like for example *Rhodobacter sphaeroides* or *Sinorhizobium meliloti*, the formation of these phosphorus-free membrane lipids is induced by phosphate-limiting growth conditions (Benning *et al*., 1995; Geiger *et al*., 1999). Some bacteria such as *Brucella abortus* (Comerci *et al*., 2006; Bukata *et al*., 2008) or *Rhizobium tropici* (Rojas-Jiménez *et al*., 2005; Sohlenkamp *et al*., 2007) also form significant amounts of OLs during growth in standard laboratory media such as LB which contain phosphate in concentrations that are not growth-limiting.

Ornithine lipids are widespread among Gram-negative bacteria and have also been reported in some Gram-positive bacteria, like *Mycobacterium* and *Streptomyces* species, but seem to be absent from Archaea and Eukarya (López-Lara *et al*., 2003; Geiger *et al*., 2010). OLs contain a 3-hydroxy fatty acyl group that is attached in amide linkage to the α-amino group of ornithine. A second fatty acyl group is ester-linked to the 3-hydroxy position of the first fatty acid. It has been reported that in some bacteria the ester-linked fatty acid is hydroxylated at the 2 or 3 position (Asselineau, 1991). The genes *olsB* and *olsA* encoding the two enzymes essential for OL biosynthesis from ornithine and acyl-ACPs have been first described in *S. meliloti* (Weissenmayer *et al*., 2002; Gao *et al*., 2004). Although OLs are probably found in both membranes of Gram-negative bacteria, they seem to be enriched in the outer membrane (OM) as was shown in the acid-resistant species *Thiobacillus thiooxidans* (Dees and Shively, 1982). Therefore, Dees and Shively speculated about a role of OLs in acid resistance (Dees and Shively, 1982).

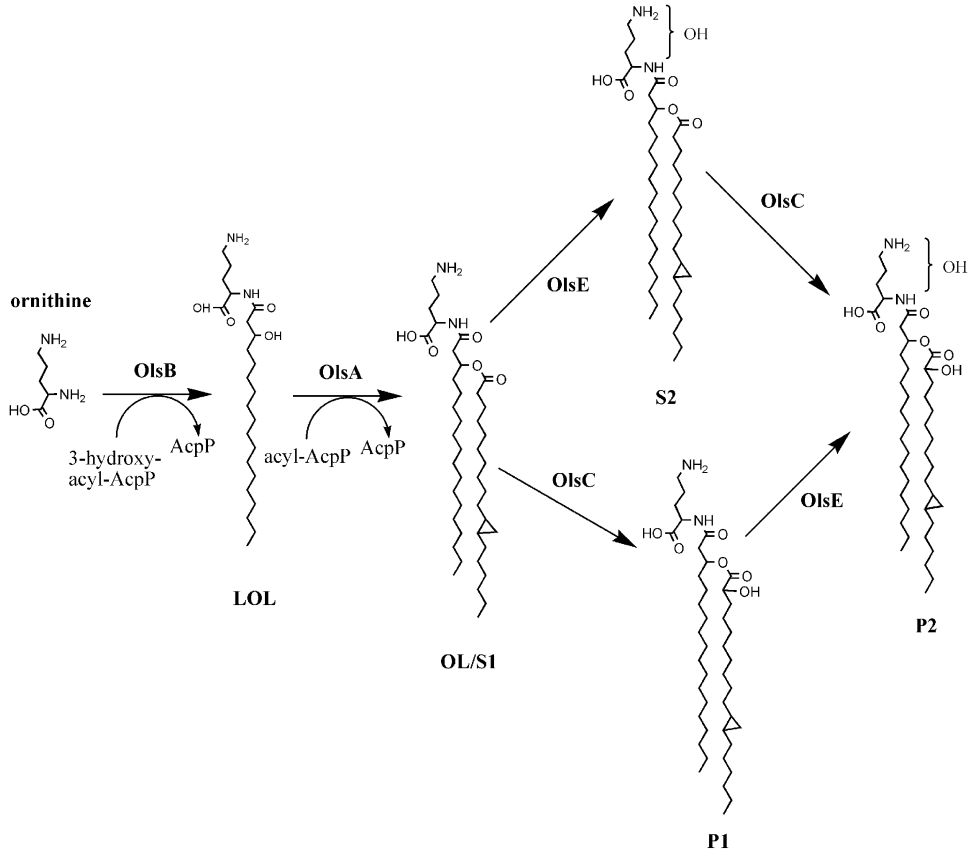


Fig. 1. Biosynthesis of ornithine lipids in *Rhizobium tropici* CIAT899. The genes coding for OlsB and OlsA have been first identified in *Sinorhizobium meliloti*, whereas the gene encoding the OL hydroxylase OlsC has been described first in *R. tropici*. Here we describe that the hydroxylation introduced by OlsC is in the 2 position of the secondary fatty acid. We also describe the identification of the gene encoding the OL hydroxylase OlsE introducing a hydroxyl group in the ornithine moiety of OL. Lyso-ornithine lipid (LOL), ornithine lipid (OL).

Rhizobium tropici CIAT899 is highly tolerant to many environmental stresses such as acidity or high temperatures. It can grow on acidified media down to pH 4.0, and it is a good competitor for nodule occupancy in *Phaseolus vulgaris* (common bean) and other hosts under acidic conditions (Martínez-Romero *et al*., 1991). A gene responsible for the hydroxylation of OL has been isolated in *R. tropici* using a transposon mutagenesis approach looking for mutants affected in their capacity to grow at pH 4.5 (Vinuesa *et al*., 2003; Rojas-Jiménez *et al*., 2005). Rojas-Jiménez *et al*. described the presence of four different species of OL in *R. tropici* membranes which were called S1, S2, P1 and P2. They showed that the putative hydroxylase OlsC is responsible for the formation of P1 and P2, presumably from OLs S1 and S2 functioning as substrates (Fig. 1), but they did not investigate on the position of the OlsC-dependent hydroxylation. No acid growth phenotype was observed for the *olsC*-deficient mutant, but constitutive expression of *olsC* was associated with the inability of the strain to grow at pH 4.5. Upon inoculation of the *olsC* mutant onto bean plants only poorly developed nodules were observed (Ndv^-) 21 days after inoculation of the plants (Rojas-Jiménez *et al*., 2005). In an earlier study, Taylor *et al*. (1998) had observed an increased formation of hydroxylated OLs at an elevated temperature in *Burkholderia cepacia*. These two previous results indicated a role of modified OLs in stress tolerance, and prompted us to investigate the synthesis of modified OLs and their role in stress tolerance in *R. tropici* in more detail. In this study we describe the isolation of the OL hydroxylase OlsE and the construction of *R. tropici* mutants deficient in the hydroxylation of OLs. We show that OlsC is introducing a hydroxyl group in the 2 position of the secondary fatty acid of OLs and that OlsE introduces a hydroxylation in the ornithine moiety of OLs. The characterization of these mutants shows that hydroxylated OLs are important for adaptation to stress conditions in *R. tropici*.

Results

Stress conditions alter the amount of modified OLs in R. tropici *indicating a role of OLs in stress adaptation*

Rhizobium tropici CIAT899 is a nodule-forming rhizobium well known for its ability to resist stress conditions such as acidic pH or high temperatures (Martínez-Romero *et al*., 1991). In an earlier study Rojas-Jiménez *et al*. (2005) had observed that *R. tropici* forms four different OLs. In addition to the unmodified OL which was named S1 (for substrate 1) three additional modified OLs probably derived from S1 are present. Taylor *et al*. (1998) had observed an increase in the relative amounts of hydroxylated OL when *B. cepacia* was grown at increased temperatures. To find out if the modification of OL also occurs as a stress response in *R. tropici* and if these modifications might have a role in stress adaptation, *R. tropici* CIAT899 was grown at 30°C, 37°C and 42°C and its lipid composition was analysed (Fig. 2A–C, Table 1). At the standard growth temperature of 30°C, all four OLs can be detected, with P1 being the most abundant OL. An increase in growth temperature to 37°C causes a decrease in the OLs S2 and P2 and a simultaneous increase in S1. When grown at 42°C the amounts of S1 and P1 decrease slightly. The OLs S2 and P2 cannot be detected in cells grown at 42°C. An unknown lipid which migrates similarly as the sulpholipid sulphoquinovosyl diacylglycerol is apparently formed at 42°C but not at lower growth temperatures. The decrease in OLs is accompanied by changes in the phospholipid composition: phosphatidylethanolamine (PE) decreases whereas phosphatidylcholine (PC), phosphatidylglycerol (PG) and cardiolipin (CL) increase. *R. tropici* CIAT899 was also grown in complex TY medium adjusted to different pH values (compare Fig. 2A, D and E, Table 2). In *R. tropici* cells grown at pH 4.5 the OLs S1 and S2 are not detectable, whereas P2 is

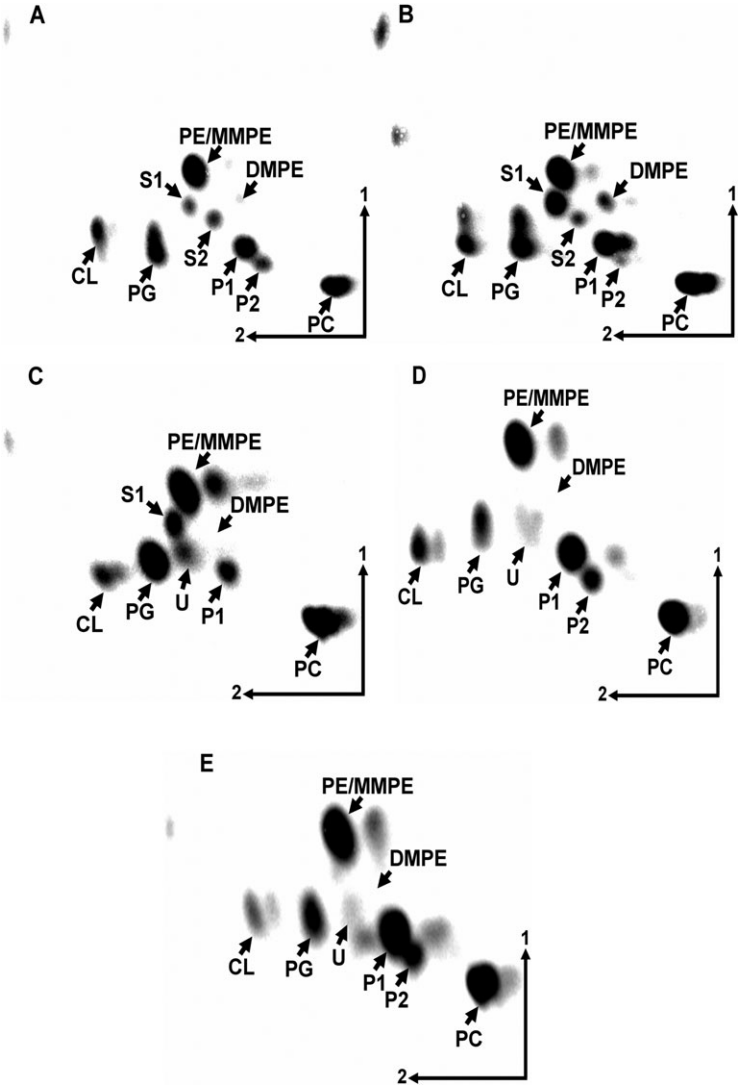


Fig. 2. Separation of [^{14}C]acetate-labelled lipids from *Rhizobium tropici* CIAT899 grown in complex TY medium at 30°C (A), at 37°C (B), at 42°C (C), at 30°C at pH 4.5 (D) or at 30°C at pH 4.0 (E) by two-dimensional thin-layer chromatography. The phospholipids phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL), monomethyl PE (MMPE), dimethyl PE (DMPE) and the ornithine lipids (OLs) S1, S2, P1 and P2 are indicated. U, unknown lipid.

© 2011 Blackwell Publishing Ltd, *Molecular Microbiology*, **79**, 1496–1514

Table 1. Membrane lipid composition of *Rhizobium tropici* wild-type CIAT899, *olsE*-deficient mutant MAV04, *olsC*-deficient mutant 899-*olsCΔ1* and *olsC/olsE*-deficient double mutant MAV05 after growth on complex TY medium at 30°C, 37°C or 42°C.

	Composition (% of total ^{14}C)											
	30°C				37°C				42°C			
Lipid	CIAT899	MAV04	899-*olsCΔ1*	MAV05	CIAT899	MAV04	899-*olsCΔ1*	MAV05	CIAT899	MAV04	899-*olsCΔ1*	MAV05
PC	22.6 ± 0.6	24.8 ± 2.9	20.7 ± 0.7	24.8 ± 0.1	28.9 ± 4.0	27.2 ± 4.8	23.5 ± 4.3	24.6 ± 1.2	26.8 ± 1.2	28.5 ± 1.6	27.0 ± 2.0	27.5 ± 2.1
PE	25.0 ± 0.1	24.6 ± 1.4	24.9 ± 2.1	25.6 ± 0.6	23.5 ± 5.0	23.2 ± 3.7	19.1 ± 1.0	20.4 ± 4.8	16.2 ± 1.3	17.9 ± 1.9	11.9 ± 1.3	11.8 ± 1.1
DMPE	1.1 ± 0.1	1.7 ± 0.2	1.6 ± 0.6	1.6 ± 0.4	1.4 ± 0.6	1.1 ± 0.2	1.4 ± 0.4	1.3 ± 0.2	0.4 ± 0.0	0.4 ± 0.1	0.6 ± 0.2	0.7 ± 0.1
PG	15.8 ± 0.8	13.9 ± 2.8	17.4 ± 1.5	16.0 ± 0.6	12.5 ± 0.9	11.8 ± 1.2	18.8 ± 1.7	16.9 ± 1.6	23.8 ± 0.3	22.1 ± 0.8	33.9 ± 1.0	29.2 ± 1.1
CL	4.0 ± 0.1	4.8 ± 0.1	3.0 ± 0.1	5.6 ± 0.3	5.3 ± 1.3	4.8 ± 1.0	8.0 ± 2.0	6.7 ± 2.4	7.5 ± 0.5	8.2 ± 0.3	11.1 ± 2.5	10.7 ± 1.3
S1	2.9 ± 0.2	6.8 ± 1.0	26.5 ± 1.7	26.4 ± 1.8	7.3 ± 1.0	9.0 ± 1.4	27.7 ± 0.9	30.1 ± 5.6	5.6 ± 0.2	4.7 ± 0.9	10.8 ± 0.8	16.0 ± 1.5
S2	3.5 ± 0.5	n.d.	5.9 ± 0.5	n.d.	1.6 ± 0.3	n.d.	1.5 ± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.
P1	20.6 ± 0.3	23.4 ± 1.8	n.d.	n.d.	18.2 ± 2.8	22.9 ± 0.1	n.d.	n.d.	15.2 ± 0.5	15.2 ± 0.7	n.d.	n.d.
P2	4.5 ± 0.3	n.d.	n.d.	n.d.	1.3 ± 0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
U	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.5 ± 0.5	3.0 ± 0.7	4.7 ± 0.7	4.1 ± 1.0

The values shown are mean values ± standard deviation derived from at least three independent experiments.
PC: phosphatidylcholine; PE: phosphatidylethanolamine; MMPE: monomethyl phosphatidylethanolamine; DMPE: dimethyl phosphatidylethanolamine; PG: phosphatidylglycerol; CL: cardiolipin; S1: substrate 1, unmodified ornithine lipid; S2, P1, P2: hydroxylated ornithine lipids; U: unidentified lipid; n.d.: not detected.

Table 2. Membrane lipid composition of *Rhizobium tropici* wild-type CIAT899, *olsE*-deficient mutant MAV04, *olsC*-deficient mutant 899-*olsCΔ1* and *olsC/olsE*-deficient double mutant MAV05 after growth on complex TY medium adjusted to pH 7.0, pH 4.5 or pH 4.0.

	Composition (% of total ^{14}C)											
	pH 7.0				pH 4.5				pH 4.0			
Lipid	CIAT899	MAV04	899-*olsCΔ1*	MAV05	CIAT899	MAV04	899-*olsCΔ1*	MAV05	CIAT899	MAV04	899-*olsCΔ1*	MAV05
PC	27.6 ± 0.7	25.0 ± 0.2	21.3 ± 0.3	24.6 ± 0.4	27.7 ± 0.2	28.1 ± 1.7	23.3 ± 2.2	23.8 ± 3.8	26.2 ± 0.3	26.6 ± 0.4	19.5 ± 0.5	18.1 ± 2.0
PE	26.7 ± 0.3	26.5 ± 0.1	21.7 ± 0.1	24.7 ± 0.5	27.4 ± 0.3	27.3 ± 2.1	12.8 ± 1.5	10.9 ± 0.4	22.3 ± 0.4	25.6 ± 0.3	14.9 ± 0.4	12.3 ± 2.0
DMPE	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.9 ± 0.0	0.8 ± 0.1	0.5 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	0.8 ± 0.2	0.2 ± 0.0	0.2 ± 0.1	0.6 ± 0.2
PG	12.2 ± 0.4	10.0 ± 0.1	13.1 ± 0.2	11.5 ± 0.1	8.2 ± 0.2	4.7 ± 0.4	10.1 ± 0.9	9.2 ± 0.9	6.8 ± 0.1	7.4 ± 1.3	12.4 ± 1.2	12.9 ± 1.1
CL	4.4 ± 0.2	5.7 ± 0.4	5.5 ± 0.3	3.7 ± 0.3	6.9 ± 0.3	2.4 ± 0.2	4.2 ± 0.7	4.3 ± 0.1	3.7 ± 0.5	4.2 ± 1.0	5.7 ± 0.4	4.7 ± 0.7
S1	2.9 ± 1.7	8.9 ± 0.2	32.5 ± 0.2	34.6 ± 0.2	n.d.	2.0 ± 0.5	40.6 ± 2.4	46.0 ± 2.9	n.d.	1.6 ± 0.3	40.7 ± 1.1	44.6 ± 3.6
S2	3.3 ± 0.3	n.d.	5.3 ± 0.5	n.d.	n.d.	n.d.	2.5 ± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.
P1	18.0 ± 0.4	23.3 ± 0.1	n.d.	n.d.	19.4 ± 0.3	32.0 ± 1.0	n.d.	n.d.	30.2 ± 0.1	31.9 ± 2.5	n.d.	n.d.
P2	4.5 ± 0.4	n.d.	n.d.	n.d.	8.3 ± 0.2	n.d.	n.d.	n.d.	8.1 ± 0.0	n.d.	n.d.	n.d.
U	n.d.	n.d.	n.d.	n.d.	1.3 ± 0.1	3.0 ± 0.3	5.5 ± 1.0	4.8 ± 0.5	1.9 ± 0.1	2.5 ± 0.4	6.6 ± 0.6	6.8 ± 0.5

The values shown are mean values ± standard deviation derived from at least three independent experiments. For abbreviations see Table 1.

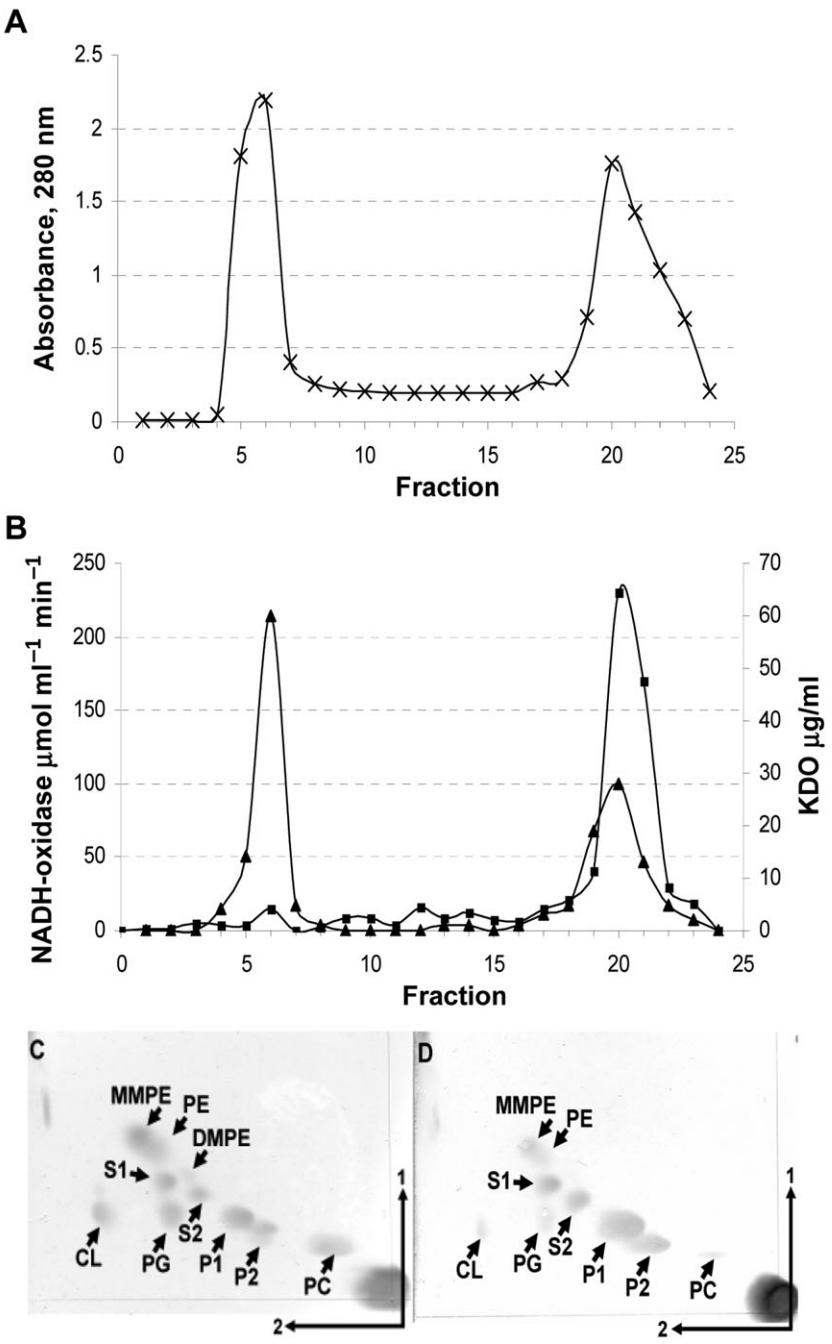


Fig. 3. Localization of OLs in membranes of wild-type *Rhizobium tropici* CIAT899.
A and B. Results of a sucrose density gradient centrifugation of cell membranes of *R. tropici* CIAT899. (A) A280 readings of the gradient fractions. (B) 2-Keto-3-oxyoctonate content (closed triangles) and NADH oxidase (closed squares) activity of the fractions.
C and D. Separation of membrane lipids extracted from the inner (C) and outer membrane (D). Fractions corresponding to the inner and outer membranes were pooled, lipids were extracted with 1-butanol and subsequently analysed using two-dimensional TLC. Lipids were visualized by spraying with ceric sulphate in sulphuric acid. The phospholipids phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL), monomethyl PE (MMPE), dimethyl PE (DMPE) and the ornithine lipids (OLs) S1, S2, P1 and P2 are indicated. A quantification of the lipids is shown in Table 3.

increased and no changes are detected for P1. When grown at pH 4.0 again OLs S1 and S2 cannot be detected, but P1 increases drastically and becomes the major membrane lipid (Fig. 2E).

OLs are enriched in the OM of R. tropici *CIAT899*

Dees and Shively (1982) had shown that in the acid-resistant species *T. thiooxidans* OL is present mainly in the OM and they had therefore speculated that it might play a role in conferring acid resistance to these bacteria. If such a hypothesis were true one would expect an accumulation of OLs also in the OM of the acid-tolerant bacterium *R. tropici*. Inner membrane (IM) and OM from *R. tropici* were separated and the lipids of both membranes were extracted and separated using two two-dimensional TLC (Fig. 3). The protein content of the fractions was estimated using absorption measurements at 280 nm. The protein-enriched fractions formed two peaks corresponding to the IM and OM (Fig. 3A). KDO (2-keto-3-deoxyoctanoate)

Table 3. Membrane lipid composition of the inner and outer membrane of *R. tropici* CIAT899.

Lipid	Inner membrane	Outer membrane
PC	23.9	9.1
PE	8.2	6.6
MMPE	5.4	5.9
DMPE	4.8	5.0
PG	17.2	7.7
CL	12.2	6.7
S1	6.2	11.4
S2	5.0	11.0
P1	11.1	25.3
P2	6.0	11.3

The data were obtained from the TLC plates shown in Fig. 3C and D using the program ImageQuant. Numbers present per cent of total lipids present in the TLC. For abbreviations see Table 1.

content and NADH oxidase activity that were used as markers for the OM and IM, respectively, indicated that the IM was contaminated to some extent by the OM, but that the OM was almost free of contamination by the IM (Fig. 3B). The TLC analysis of the Bligh-Dyer extracts showed that phospholipids are the major membrane lipids of the IM but are present in much smaller relative amounts in the OM (Fig. 3C and D, Table 3). A quantification of the lipids showed that phospholipids form more than 70% of the membrane lipids of the IM but only about 40% of the membrane lipids of the OM, excluding lipopolysaccharide (LPS). OLs form less than 30% of the membrane lipids of the IM but about 60% of the membrane lipids of the OM (again excluding LPS). Taking the contamination of the IM fractions with OM material into account the result overestimates the real concentration of OLs in the IM. Assuming that the outer leaflet of the OM is composed mainly of the lipid A moiety of LPS, this result indicates that the major proportion of the inner leaflet of the OM is composed of OLs.

Expression cloning of the OL-modifying enzyme OlsE from R. tropici

The experiments described earlier indicated a possible role for the different OLs in the *R. tropici* stress response. In *S. meliloti* only one type of OL is present. In contrast, four different types of OLs called S1, S2, P1 and P2 are present in *R. tropici* CIAT899 (Fig. 1). The gene *olsC* encoding the enzyme OlsC responsible for the synthesis of OLs P1 and P2 from the substrates S1 and S2 has been described earlier (Rojas-Jiménez *et al.*, 2005). It was not known, however, which gene encodes the hypothetical enzyme OlsE responsible for the synthesis of S2 and possibly also for the synthesis of P2 (Fig. 1). We suspected that S1, corresponding to the OL present in *S. meliloti*, was a substrate for the OlsE-catalysed reaction. The *S. meliloti* strain CS111.pNG25 lacking the ninhydrin-positive lipid PE and producing increased amounts of the OL S1 was constructed and transconjugants of CS111.pNG25 harbouring cosmids containing *R. tropici* CIAT899 genomic DNA were assayed for the presence of a second ninhydrin-positive lipid in addition to S1. In the transconjugant referred to as CS111.pNG25.pCos94, two ninhydrin-positive lipids with the expected R_f values for S1 and S2 were detected. A restriction analysis of pCos94 showed that it contains about 18–20 kb of inserted DNA. Restriction fragments of the pCos94 insert were subcloned into a broad-host-range vector and again conjugated into CS111.pNG25. The resulting transconjugants were analysed as described above for the cosmid bank (data not shown). A plasmid conferring the formation of the OL S2 was identified and its insert was sequenced. In addition to three predicted complete ORFs it contained two incomplete ORFs (GenBank Accession No. HM010770). BLAST searches using the NCBI database with the amino acid sequences of the three complete ORFs as query were made (Altschul *et al.*, 1997). The first ORF was annotated as a putative acetyltransferase, the second ORF as a putative aminoglycoside N(6′) acetyltransferase and the third ORF as a putative hydroxylase. The three candidate ORFs were cloned into a broad-host-range plasmid and the resulting plasmids were conjugated into CS111.pNG25. Labelling of the lipids of the three transconjugants with [^{14}C]acetate showed that ORF3 codes for the putative hydroxylase OlsE which is responsible for the formation of S2 (Fig. 4).

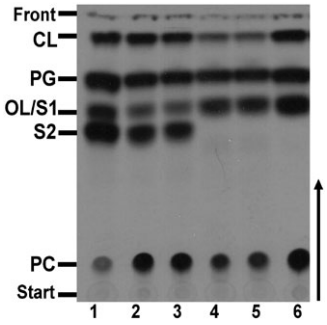


Fig. 4. Expression cloning of *olsE* from *R. tropici*. Lipids of *Sinorhizobium meliloti* CS111.pNG25 containing different plasmids or cosmids were radiolabelled with [^{14}C]acetate and separated by one-dimensional TLC. The following strains were analysed: CS111.pNG25.pCos94 (cosmid, lane 1), CS111.pNG25.pERMAV04 (ORF1 to 3/3.5 kb insert, lane 2), CS111.pNG25.pERMAV13 (ORF3, lane 3), CS111.pNG25.pERMAV12 (ORF2, lane 4), CS111.pNG25.pERMAV11 (ORF1, lane 5) and CS111.pNG25.pERMAV06 (negative control, lane 6). The phospholipids phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL) and the ornithine lipids (OLs) S1 and S2 are indicated.

OlsE belongs to the fatty acyl hydroxylase superfamily and is responsible for the hydroxylation of OL within the ornithine moiety

The gene *olsE* encodes a very hydrophobic protein of 330 amino acids predicted to form between four and six transmembrane helices. An analysis of the amino acid sequence shows that OlsE belongs to the fatty acyl hydroxylase superfamily (cl01132) which is characterized by the presence of two copies of the HXHH motif. This superfamily includes fatty acid and carotene hydroxylases, sterol desaturases (Mitchell and Martin, 1997), C-5 sterol desaturase (Arthington *et al*., 1991) and C-4 sterol methyl oxidase (Bard *et al*., 1996; Kennedy *et al*., 2000). A similar motif ($HX_{3-4}H$, $HX_{2-3}HH$, $HX_{2-3}H$) can be found in membrane-bound fatty acid desaturases such as OLE1 from *Saccharomyces cerevisiae* and is also present in bacterial alkane hydroxylase (Kok *et al*., 1989) and xylene monooxygenase (Suzuki *et al*., 1991). In these proteins the conserved histidine residues act to co-ordinate an oxo-bridged diiron cluster (Fe–O–Fe) that functions as part of the reaction centre (Fox *et al*., 1993; Shanklin *et al*., 1994).

The annotation of OlsE as a fatty acyl hydroxylase indicates that OlsE introduces a hydroxyl group into OL at an unknown position. To localize the hydroxyl group on the OL S2, lipids were extracted according to Bligh and Dyer (1959) from a 1 l culture of the *olsC*-deficient *R. tropici* mutant *899-olsCΔ1*. OLs S1 and S2 were purified from the total lipid extract and analysed by normal-phase LC-coupled electrospray ionization (ESI) mass spectrometry (MS) in the negative ion mode. Prior to fragmentation ions with *m/z* 691 and 707 corresponding to OLs S1 and S2 were detected. The molecular ion was shifted in case of S2 to an *m/z* 16 amu higher in comparison with S1 indicating the presence of an additional oxygen suggesting the presence of an additional hydroxyl group. Comparing the fragmentation patterns of S1 and S2 it was observed that the modification present in S2 is located within the ornithine moiety and not in the fatty acyl chains (Fig. 5). When assaying the two-dimensional TLC plates with *R. tropici* lipids with ninhydrin it was noticed that S2 and P2 react with delay in comparison with S1 and P1, and that the developed colour is different. While S1 and P1 upon reaction with ninhydrin develop a red to purple colour, the reaction of S2 and P2 causes the formation of an orange colour.

OlsC introduces a hydroxyl group at the 2 position of the secondary fatty acid of OL

OlsC is a homologue of the hydroxylase LpxO from *Salmonella typhimurium* that is responsible for the addition of a 2-hydroxy group to the myristate residue present at the 3′ position of lipid A. Rojas-Jiménez *et al*. (2005) had discovered the gene *olsC* and had shown that OlsC is a putative hydroxylase responsible for the formation of the OLs P1 and P2 from the OLs S1 and S2 in *R. tropici* (Fig. 1). However, it was not known in what part of the OL structure the OlsC-dependent hydroxylation occurs. To localize the hydroxyl group on the OL P1, OLs S1 and P1 were purified from the total lipid extracts and analysed by normal-phase LC-coupled ESI-MS in the negative ion mode. Prior to fragmentation ions with *m/z* 691 and 707 corresponding to OLs S1 and P1 were detected. The molecular ion of P1 was shifted to an *m/z* 16 amu higher in comparison with S1 indicating the presence of an additional oxygen suggesting the presence of an additional hydroxyl group. Comparing the fragmentation patterns of S1 and P1 it was observed that the modification present in P1 is located within the secondary fatty acyl chain (data not shown) which in case of S1 is mainly lactobacillic acid and in case of P1 hydroxy lactobacillic acid. In order to determine the position of the OlsC-dependent hydroxylation in P1 its fatty acids were transmethylated before the hydroxyl groups were derivatized to trimethylsilyl (TMS) ethers similar to the procedures described by Gibbons *et al*. (2008). Alpha- and beta-hydroxy fatty acid standards of 16 and 18 carbons were processed in parallel with the samples (Fig. S1A–D). GC/MS analysis of the derivatized fatty acids shows the presence of three peaks present in the samples derived from P1 that are not present in the samples derived from S1 (Fig. S1E and F). Their fragmentation pattern indicates that the OlsC-dependent hydroxylation occurs in the 2 position (Fig. S1G).

Lipid composition analysis of olsE *and* olsE/olsC *mutants*

To study the role of OLs in *R. tropici* in more detail, mutants deficient in *olsE* and double mutants deficient in *olsC* and *olsE* were constructed. Their lipid compositions were compared with the wild-type strain CIAT899 and the OlsC-deficient mutant 899-*olsCΔ1* (Fig. 6, Table 1). As expected the *olsE*-deficient mutant MAV04 lacked the OLs S2 and P2, the *olsC*-deficient mutant 899-*olsCΔ1* lacked P1 and P2 and in the double mutant MAV05 (Δ*olsC*Δ*olsE*) no S2, P1 or P2 were detectable. Apparently, the amount of OLs, being the sum of S1, S2, P1 and P2, is more or less stable between 20% and 35% when *R. tropici* is grown in complex TY medium at 30°C. No significant differences in the relative amounts of the phospholipids PE, PC, PG and CL were observed between the different strains. To show that the observed phenotypes were caused by the absence of the deleted genes, mutants MAV04 (Δ*olsE*) and MAV05 (Δ*olsC*Δ*olsE*) were also complemented. When *olsE* was present *in trans* in MAV04 again formation of S2 and P2 was detected and

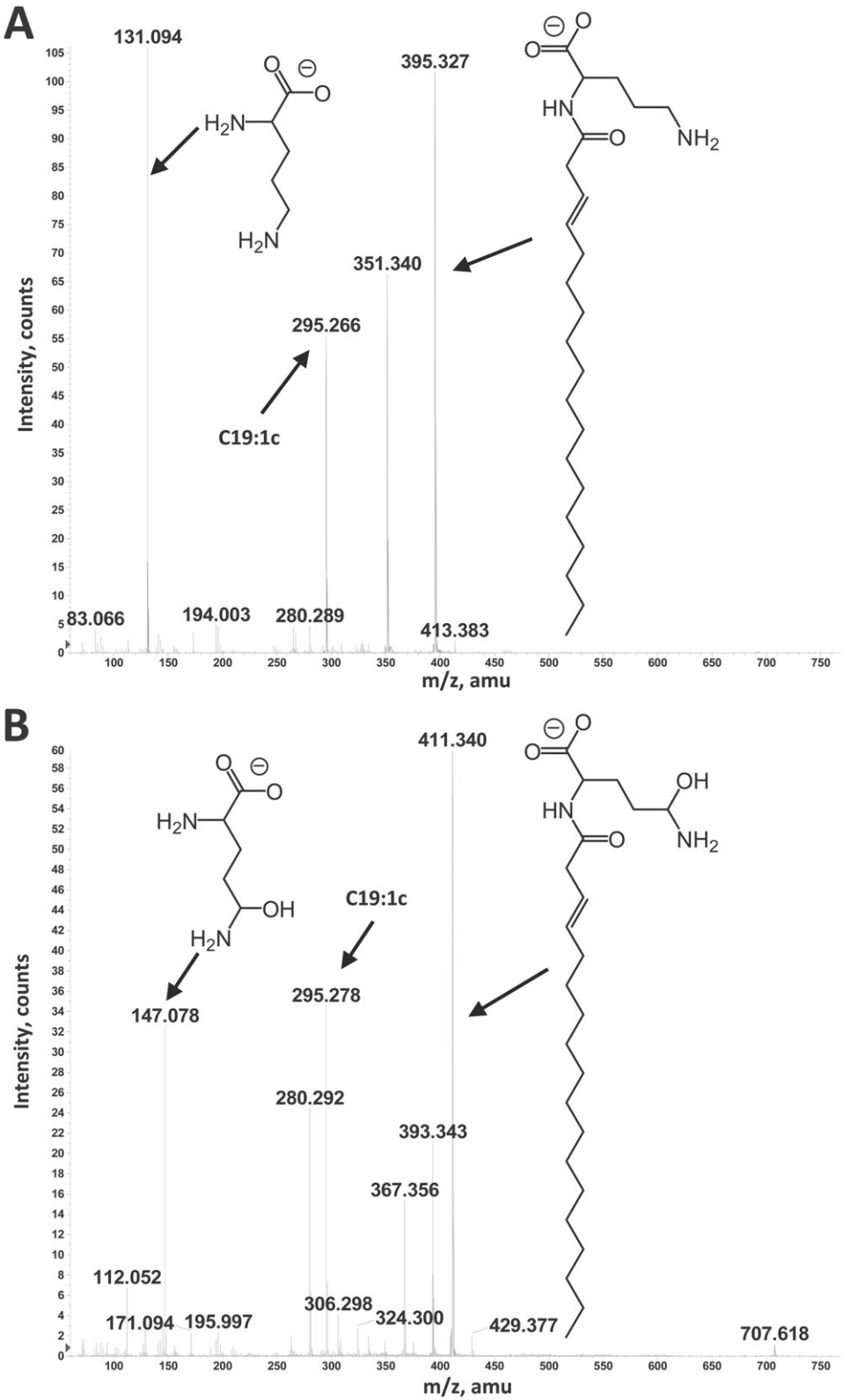


Fig. 5. Collision-induced dissociation mass spectra of ornithine lipids S1 and S2 detected in lipid extract of *R. tropici* mutant 899-*olsC*Δ*1*. Negative ion collision induced dissociation mass spectra of $[M\text{-}H]^-$ ions at *m/z* 671 (A) obtained from OL S1 and *m/z* 707 (B) obtained from OL S2. The structures of major fragment ions are indicated. The position of the hydroxyl group introduced in the ornithine moiety is assigned tentatively. Complete structures of the OLs are shown in Fig. 1.

© 2011 Blackwell Publishing Ltd,

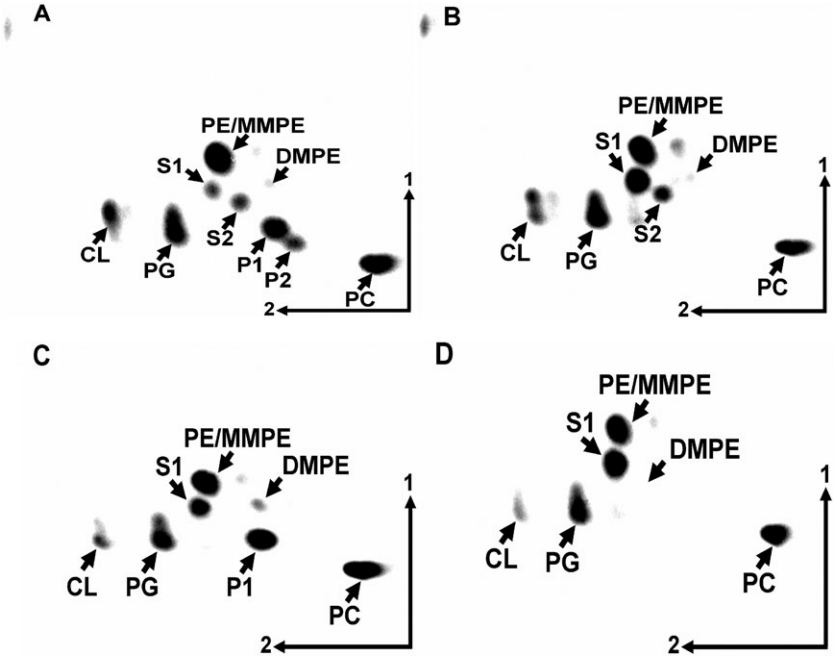


Fig. 6. Analysis of membrane lipid composition of *R. tropici* wild-type CIAT899 (A), *olsC*-deficient mutant 899-*olsC*Δ*1* (B), *olsE*-deficient mutant MAV04 (C) and *olsC/olsE*-deficient double mutant MAV05 (D). Lipids were labelled with [^{14}C]acetate during growth in complex TY medium at 30°C and separated using two-dimensional TLC. The phospholipids phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL), monomethyl PE (MMPE), dimethyl PE (DMPE) and the ornithine lipids (OLs) S1, S2, P1 and P2 are indicated.

when mutant MAV05 was complemented with *olsE* the OLs S2 and P2 could be detected, whereas S1 and P1 did not accumulate (data not shown). Constitutive expression of *olsC* and *olsE* together in MAV05 caused the accumulation of P2 while only trace amounts of the other OLs were observed (data not shown). Such an over-complementation leading to the accumulation of the reaction product(s) while almost completely consuming the substrate(s) had also been observed earlier for the complementation of the *olsC*-deficient mutant 899-*olsC*Δ*1* (Rojas-Jiménez *et al*., 2005).

*Growth and characterization of the lipid composition of mutants 899-*olsCΔ1*, MAV04 and MAV05: OlsC is important in conferring stress resistance*

An earlier study in *B. cepacia* (Taylor *et al*., 1998) had shown an increase in formation of hydroxylated OL when the bacteria were grown at higher temperature. In this study we have shown that the relative amounts of the different OLs shift in response to a change in growth temperature or pH of the medium. Rojas-Jiménez *et al*. (2005) had observed that a *R. tropici* strain constitutively expressing *olsC* was not able to grow at pH 4.5 any more. Therefore, it was expected that the *R. tropici* mutants deficient in OL hydroxylation would show a phenotype under conditions of acid or temperature stress. The wild-type *R. tropici* CIAT899 and the three mutants 899-*olsC*Δ*1*, MAV04 and MAV05 were cultivated in complex TY medium at pH 4.0, 4.5 and 7.0. At pH 7.0 all four strains divide at a similar rate (Fig. 7A). At pH 4.5 the wild-type CIAT899 and the mutant MAV04 (Δ*olsE*) grow at a similar rate compared with pH 7.0 whereas the other two mutants seem to present a longer generation time (Fig. 7B). At pH 4.0 the wild-type CIAT899 and the mutant MAV04 (Δ*olsE*) grow significantly slower than at pH 4.5 but still both cultures reach a final optical density larger than 1.0, whereas the mutants 899-*olsC*Δ*1* and MAV05 (Δ*olsC*Δ*olsE*) at most undergo one single division (Fig. 7C). To determine if the observed differences are related to changes in lipid composition, wild-type and mutant cells were grown and labelled in the corresponding media and analysed by TLC in two dimensions (Table 2). At pH 7.0 all four strains show similar concentrations of phospholipids and the distinct patterns of the different OLs typical for each mutant described above. At pH 4.5 both OlsC-deficient mutants (899-*olsC*Δ*1* and MAV05) show a drastic reduction in PE content and a strong increase in S1 to up to more than 40%. At pH 4.0 again, both OlsC-deficient mutants show a very similar lipid composition with S1 being the major membrane lipid and PE being drastically reduced. The wild-type CIAT899 apparently forms more P1 under these conditions. It seems that low-pH conditions cause the accumulation of OLs in all strains: in the wild-type and the mutant MAV04 (Δ*olsE*) the major lipid accumulated is P1, whereas in the mutants 899-*olsC*Δ*1* and MAV05 (Δ*olsC*Δ*olsE*) the major lipid is S1.

When the wild-type CIAT899 and the three mutants deficient in OL hydroxylation were cultivated in TY medium at 30°C, no differences in generation time can be observed between them (Fig. 7D). At 37°C, both strains lacking *olsC* (899-*olsC*Δ*1* and MAV05) seem to grow slightly slower than the other two strains (Fig. 7E). At 42°C

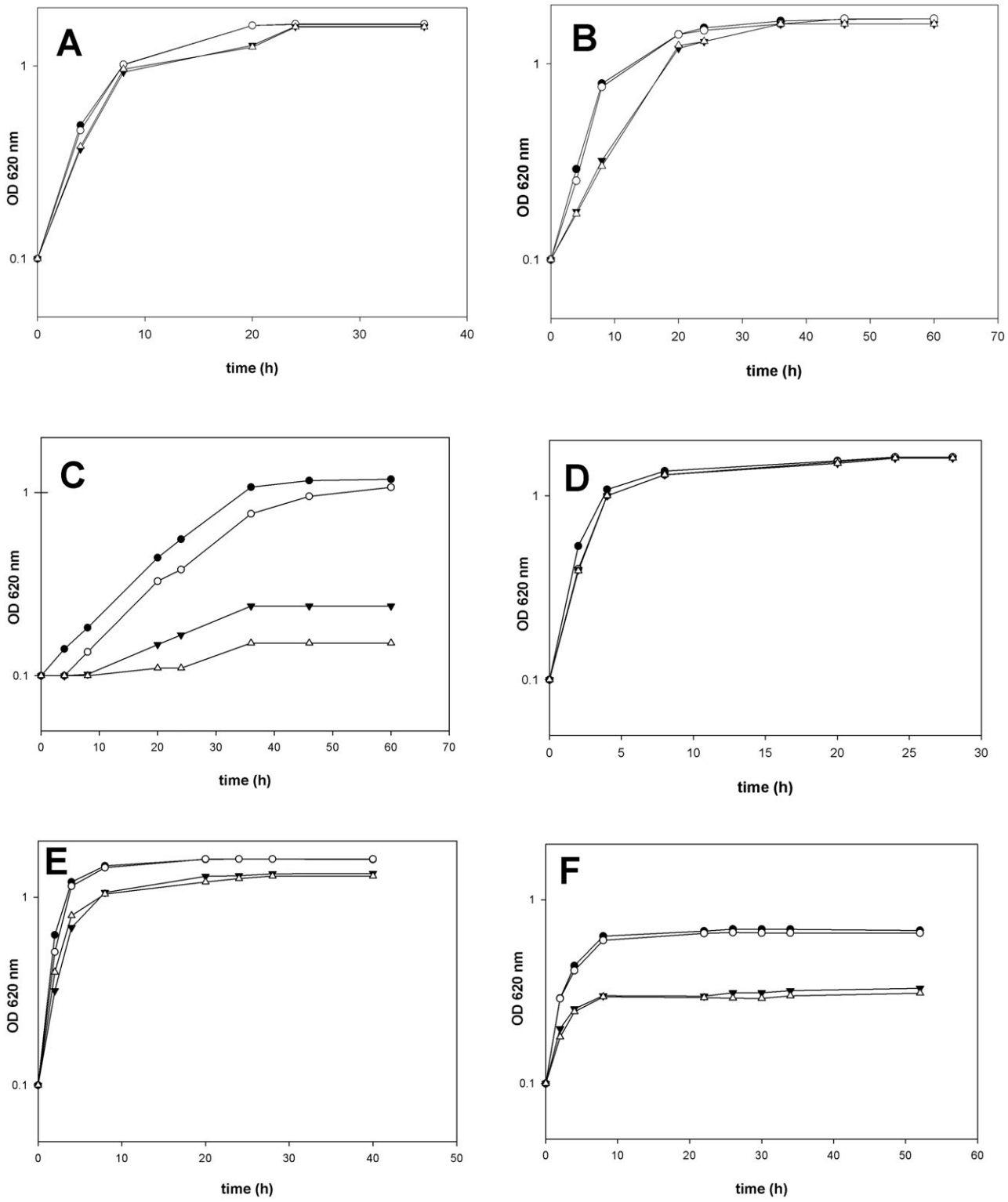


Fig. 7. Growth of *R. tropici* mutants lacking *olsC* is affected under stress conditions. *R. tropici* wild-type CIAT899 and mutants were grown in complex TY medium adjusted to pH 7.0 (A), pH 4.5 (B) or pH 4.0 (C) at 30°C or in complex TY medium at 30°C (D), 37°C (E) or 42°C (F). The result of a typical experiment is shown. CIAT899 – closed circles, MAV04 – open circles, MAV05 – open triangles, 899-*olsCΔ1* – closed triangles.

wild-type CIAT899 and mutant MAV04 (Δ*olsE*) grow slower than at the lower temperature and reach a final OD_{620} of only 0.65–0.68 (Fig. 7F). The mutants 899-*olsCΔ1* and MAV05 (Δ*olsC*Δ*olsE*) divide distinctly slower at 42°C than the two former strains and reach a final OD_{620} of only 0.3.

The lipid composition of the three mutants deficient in OL hydroxylation and the wild-type CIAT899 was also analysed at the different temperatures (Table 1). For each of the four strains the lipid compositions are very similar at 30°C and 37°C. At 42°C the amount of PG is increased by about 10–15% and also CL seems to be a bit more abundant at the higher temperature. The total of the four OLs is decreasing in all four strains. Whereas at 30°C and 37°C the sum of S1, S2, P1 and P2 is about 30%, at 42°C the strains contain only between 10% and 20% OLs.

R. tropici *mutants deficient in OlsC cause an increase in nodule number that is reverted by the deletion of* olsE

The *R. tropici* mutant deficient in OlsC (899-*olsCΔ1*) formed nodules on bean plants that were poorly developed 21 days after inoculation with the bacteria, lacked lenticels and presented a twofold reduction in nitrogen fixation (Rojas-Jiménez *et al.*, 2005). These results suggested that the *R. tropici* mutants MAV04 deficient in *olsE* and double mutants MAV05 deficient in *olsC* and *olsE* might also show nodulation phenotypes. Nodulation assays were performed in an agar-based medium in order to be able to observe the kinetics of nodule formation over time. While wild-type *R. tropici* CIAT899 and MAV04 (Δ*olsE*) produced reproducibly between 80 and 100 nodules per plant, the mutant 899-*olsCΔ1* caused the formation of more than 160 nodules per plant. When the *olsC*-deficient mutant was complemented with the *olsC* gene, it again formed nodules in numbers similar to the wild-type (Fig. 8A). Surprisingly, the double mutant MAV05 (Δ*olsC*Δ*olsE*) formed a similar number of nodules as the wild-type. The nodules were sectioned and while the wild-type caused almost exclusively the formation of nodules that were red inside indicating the formation of leghaemoglobin, while the mutants caused formation of many small nodules that were whitish on the inside indicating the absence of leghaemoglobin (Fig. S2). Roots from plants infected with the *olsC*-deficient mutant 899-*olsCΔ1* presented few red nodules and many whitish nodules and roots from plants infected with the *olsE*-deficient mutant MAV04 presented even less red and more white nodules. On roots infected with the *olsC/olsE*-deficient double mutant MAV05 almost no red nodules were formed (Fig. S2). Nitrogen fixation per hour and nodule fresh weight was affected in all three mutants in comparison with the wild-type (Fig. 8B). These results indicate that the absence of hydroxylated OLs strongly interferes with the development of functional nodules during *R. tropici*–bean symbiosis.

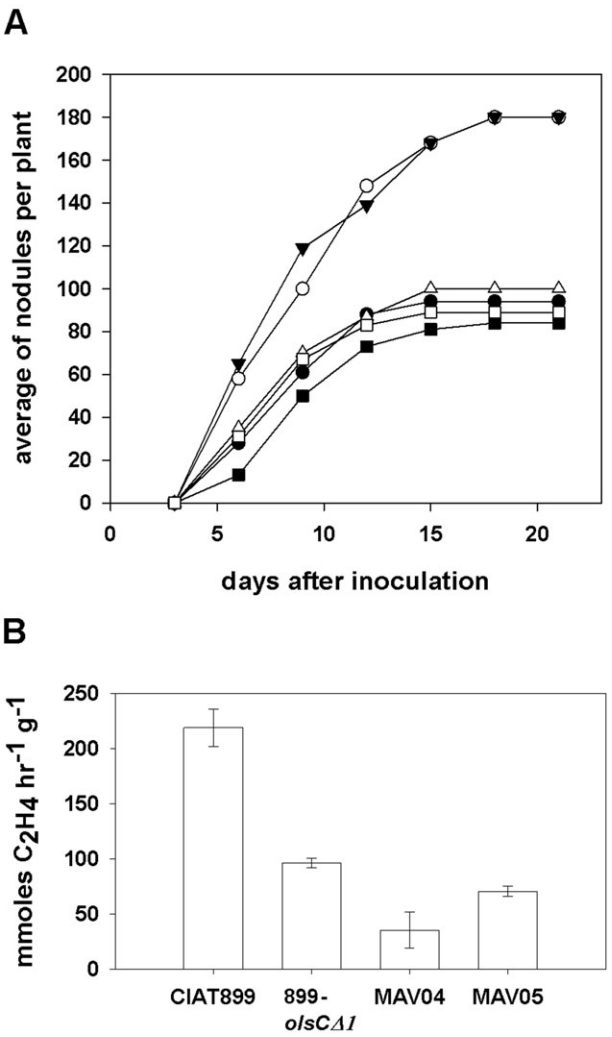


Fig. 8. Symbiotic phenotypes of *R. tropici* wild-type CIAT899 and strains deficient in OL modification on bean plants.
A. Nodulation assay. Nodules were counted every second or third day. Plants were harvested 21 days post inoculation; nodules were assayed for nitrogen fixation activity. The experiment was repeated three times with five plants for each strain. The result of a typical experiment is shown. CIAT899 (closed circles), 899-*olsCΔ1* (open circles), MAV04 (closed squares), MAV05 (open squares), 899-*olsCΔ1*.pERMAV05 (closed triangles), 899-*olsCΔ1*.pERMAV15 (open triangles). Uninoculated plants did not develop nodules.
B. Mean acetylene reduction of nodulated bean roots inoculated with wild-type *R. tropici* CIAT899 and mutants 899-*olsCΔ1*, MAV04 and MAV05. Values are the mean ± SD of three repetitions.

Discussion

Although OLs are widespread in eubacteria (López-Lara *et al.*, 2003; Geiger *et al.*, 2010) the genes *olsB* and *olsA* responsible for OL biosynthesis were only recently described in *S. meliloti* (Weissenmayer *et al.*, 2002; Gao

et al., 2004). In addition to the unmodified OL consisting of a 3-hydroxy fatty acid linked in an amide bond to the α-amino group of ornithine and a second fatty acid bound in an ester linkage to the first, several hydroxylated forms of OL have been described in organisms diverse as *B. cepacia* (Taylor *et al*., 1998), *R. tropici* (Rojas-Jiménez *et al*., 2005), *Flavobacterium* (Kawai *et al*., 1988), *Thiobacillus* (Knoche and Shively, 1972), *Streptomyces* (Asselineau, 1991) and some *Ralstonia* (Galbraith *et al*., 1999) species. The fact that the genes coding for the enzymes responsible for the hydroxylation of OLs have not been identified except for the case of *R. tropici* where the gene *olsC* was described (Rojas-Jiménez *et al*., 2005) has made it difficult to study the function of these hydroxylated forms of OL.

Apparently OL and especially their hydroxylated forms play a role in stress response as has been observed by Rojas-Jiménez *et al*. (2005) and Taylor *et al*. (1998). *R. tropici* mutants deficient in the formation of the hydroxylated OL P1 (899-*olsCΔ1* and MAV05) are affected in growth at low pH and at high temperature in comparison with the wild-type. It has to be mentioned that in an earlier study the mutant 899-*olsCΔ1* grew as well as the wild-type (Rojas-Jiménez *et al*., 2005). The explanation for this difference is unknown, but possibly slight differences in the pH of the medium cause drastic differences in the growth behaviour of the mutant. At pH 4.0 and 4.5 a drastic increase in the formation of OLs was observed when compared with growth at neutral pH. In the wild-type CIAT899 and in the mutant MAV04 (Δ*olsE*) especially P1 is increased, whereas in the *olsC*-deficient mutants unable to form P1 the substrate S1 is accumulating. This probably means that under acid growth conditions OL biosynthesis via OlsB and OlsA is induced. It is less clear what happens at the elevated growth temperature. Although the concentration of OLs is decreased during growth at 42°C in comparison with 30°C, again the presence of P1 seems to be of importance as *olsC*-deficient mutants show a growth phenotype under this condition. The elevated temperature also seems to interfere with OlsE activity as S2 and P2 cannot be detected.

Dees and Shively (1982) made the observation that in the extreme acid-tolerant bacterium *Thiobacillus oxidans* OLs are accumulated in the OM and therefore speculated about a role for OL in acid resistance in this organism (Dees and Shively, 1982). From the growth phenotype of the mutants unable to form P1 it is apparent that the hydroxylation at the 2 position of the secondary fatty acid is of importance under acid growth conditions. Our localization study confirms that although OLs seem to be present in both membranes, they show a higher relative abundance in the OM. Both studies therefore agree that OLs play a role in acid resistance, but it is not clear by which mechanism this effect of OLs is exerted. The hydroxyl group introduced by OlsC in the 2 position of the secondary fatty acid may increase hydrogen bonding between neighbouring OL molecules similarly as has been suggested for LpxO-hydroxylated lipid A in *Salmonella* and hydroxylated sphingolipids (Nikaido, 2003; Murata *et al*., 2007). These additional hydrogen bonds should result in bilayer stabilization and a decrease in membrane permeability which could explain the decrease in acid and temperature resistance of OlsC-deficient mutants.

In this study we identified the OL hydroxylase OlsE using a functional expression screening. OlsE belongs to the fatty acyl hydroxylase superfamily, unlike the other OL hydroxylase OlsC from *R. tropici* which belongs to the aspartyl-/asparaginyl β-hydroxylase protein family to which also the lipid A-myristate β-hydroxylase LpxO from *S. typhimurium* belongs (Gibbons *et al*., 2000; 2008). The closest homologues to OlsC from *R. tropici* are present in the α-proteobacteria *Agrobacterium radiobacter*, *Agrobacterium vitis*, *Ochrobactrum anthropi*, *Brucella* species, and in several cyanobacteria. Unlike other hydroxylations described in OL, the hydroxylation introduced by OlsE seems to be unique because it occurs in the ornithine moiety, but not in the fatty acid moieties as has been described for example in *T. thiooxidans, B. cepacia* or *R. tropici* (this study). Unrelated ornithine hydroxylases like for example PvdA from *Pseudomonas aeruginosa* have been described and studied in some detail (Visca *et al*., 1994; Meneely *et al*., 2009). PvdA is involved in pyoverdin biosynthesis and introduces a hydroxyl group in the δ-amino group of ornithine but is unrelated on sequence level to OlsE. It is not clear yet in which position the OlsE-catalysed hydroxylation occurs, but apparently the newly introduced hydroxyl group is close enough to the δ-amino group to change its reactivity with ninhydrin. As other members of the fatty acyl hydroxylase superfamily introduce hydroxyl groups at carbon atoms but not at nitrogen atoms OlsE possibly introduces a hydroxyl group at the δ-carbon. It is not clear how the OlsE-dependent hydroxylation might affect membrane characteristics. Possibly the OlsE-dependent hydroxylation enables the OLs S2 and P2 to form a lactone ring within the ornithine headgroup, the presence of which should change its biophysical properties drastically.

The closest OlsE homologues are present in some α-proteobacteria and more distant homologues are present in several actinobacteria, a few γ-proteobacteria and a few other α-proteobacteria. Possibly several of the closer homologues also function as OL hydroxylases. For the OlsE homologue Atu0318 from *Agrobacterium tumefaciens* we could show that is responsible for the formation of the OL S2 (data not shown). Distant OlsE homologues such as the one in *Bradyrhizobium japonicum* may use distinct substrates. One example for

bacterial lipids that are frequently hydroxylated is the hopanoids. In *B. japonicum*, an α-proteobacteria that forms hopanoids but no OL (Perzl *et al*., 1998; López-Lara *et al*., 2003) the OlsE homologue might be responsible for the hydroxylation of hopanoids.

The *R. tropici* mutants deficient in OL hydroxylation showed nodulation phenotypes, indicating that an adequate concentration of the correct OLs is required for the establishment of a successful symbiosis. It is possible that the nodulation phenotype is partly a consequence of the acid sensitivity phenotype, as during establishment of the symbiosis between rhizobia and legumes the bacteria are exposed to low-pH conditions in the rhizosphere and later again inside symbiosomes (Udvardi and Day, 1997). Other aspects, however, seem to be important as well as the OlsE-deficient mutant grows like the wild-type in media at pH 4.0, but still presents a severe nodulation phenotype. Modification of OL might be also of importance for the animal pathogen *Brucella* that has to survive acid pH conditions in the range of 4.0–4.5 inside phagosomes (Kohler *et al*., 2002). *Brucella* species form OL in a constitutive manner (Comerci *et al*., 2006; Bukata *et al*., 2008) and additionally have a close homologue to OlsC from *R. tropici* which makes it probable that they can form the hydroxylated OL P1. If hydroxylated OLs really play a role in conferring acid resistance then *Brucella* mutants deficient in their OlsC homologue might be affected in their survival inside phagosomes.

The exact function of OL S1 and its hydroxylated forms is still not known, although our data argue for an important role in stress resistance. The knowledge of the complete scheme of OL biosynthesis in *R. tropici* should facilitate future functional studies on the role of OLs. In addition, the phenomenon of over-complementation described above allows the construction of *R. tropici* strains principally accumulating one specific class of OL. Characterization of these strains should make it possible to assign roles to the different forms of OL.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in the present work and their relevant characteristics are shown in Table 4. *R. tropici* strains were grown in complex TY medium that contained 10 mM $CaCl_2$ (Beringer, 1974) at 30°C, 37°C or 42°C. Acidic media at pH 4.0 and 4.5 were buffered with 25 mM Homopipes (Research Organics, Cleveland, OH, USA) adjusted to the respective pH with NaOH, and media at pH 7.0 were buffered with 25 mM HEPES (Sigma). *E. coli* strains were grown in Luria–Bertani (LB) medium at 37°C (Sambrook and Russell, 2001). When needed, antibiotics were added at the following final concentrations (μg ml^{-1}): kanamycin (Km) 50; carbenicillin (Cb) 100; tetracycline (Tc) 10; nalidixic acid (Nal) 20; and chloramphenicol (Cm) 60.

DNA manipulations

Recombinant DNA techniques were performed according to standard protocols (Sambrook and Russell, 2001). The cosmid subclone containing *olsE* and PCR products were sequenced at Eurofins Medigenomix by the chain termination method. The DNA region containing *olsE* was analysed using the NCBI (National Center for Biotechnology Information) BLAST network server (Altschul *et al*., 1997). Oligonucleotide sequences are listed in Table S1.

Expression cloning of the R. tropici *OL hydroxylase gene* olsE

A cosmid library of *R. tropici* CIAT899 made in pVK102 using partially digested HindIII genomic DNA fragments (Vargas *et al*., 1990) was mobilized into *S. meliloti* CS111.pNG25 by triparental mating using pRK2013 as the helper plasmid (Figurski and Helinski, 1979). CS111.pNG25 was used to facilitate the screening: CS111 is a phosphatidylserine synthase-deficient mutant (Sohlenkamp *et al*., 2004) derived from the wild-type 1021 which is constitutively expressing the gene *olsB* from *Burkholderia cenocepacia*. CS111.pNG25 will form increased amounts of S1 which is one of the suspected substrates of OlsE while lacking the ninhydrin-positive membrane lipid phosphatidylethanolamine. Plasmid pNG25 was constructed as follows: the oligonucleotide primers oLOP111 and oLOP112, introducing NdeI and HindIII sites, respectively, were used in the PCR to amplify the gene *olsB* from *B. cenocepacia* J2315 using genomic DNA as template. After digestion of the PCR product the obtained fragment was cloned into the plasmid pET17b previously digested with the same enzymes to yield the plasmid pNG23. To obtain plasmid pNG25, the BglII/HindIII fragment containing *olsB* of *B. cenocepacia* together with the T7 promoter of pET17b was subcloned from pNG23 and cloned into BamHI/HindIII-digested pBBR1-MCS. Via diparental mating using *E. coli* S17-1 as a donor strain, pNG25 was introduced into *S. meliloti* CS111 to obtain CS111.pNG25 which was used as a receptor strain for the cosmid bank. Cosmid transconjugants were selected on TY containing the following antibiotics: tetracycline 10 μg ml^{-1}; nalidixic acid 20 μg ml^{-1}; chloramphenicol 60 μg ml^{-1}. Four hundred individual *S. meliloti* transconjugants harbouring random fragments of the library were picked and streaked for subsequent lipid analysis in small patches (1 cm by 1 cm) on fresh plates. After growth for 3 days, cells from each patch were collected with a toothpick and swirled in 60 μl of chloroform–methanol (1:1, v/v) as described previously (Benning and Somerville, 1992). After the addition of 20 μl of 1 M KCl-0.2 N H_3PO_4, the tubes were vortexed and centrifuged to separate the organic and aqueous phases. A 10 μl aliquot from the lipid-containing lower phase was spotted on a HPTLC silica gel 60 plate (Merck). The TLC was developed in one dimension using the solvent system chloroform–methanol–glacial acetic acid (130:50:20, v/v). Under these conditions unmodified OL was readily separated from the modified OL we were looking for and from other polar lipids such as PC, PG and CL. Lipids were detected first with iodine and subsequently primary amine containing lipids were visualized by spraying the plates with a solution of 0.2% ninhydrin in acetone and heating the

Table 4. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference
Rhizobium tropici strains		
CIAT899	Wild-type; acid-tolerant, Nal^r	Martínez-Romero *et al.* (1991)
899-*olsC*Δ*1*	CIAT899 carrying a 211 bp non-polar deletion in *olsC*	Rojas-Jiménez *et al.* (2005)
MAV04	CIAT899 carrying a deletion in *olsE*	This work
MAV05	CIAT899 carrying deletions in *olsC* and *olsE*	This work
Sinorhizobium meliloti strains		
CS111	*pssA*-deficient mutant of wild-type 1021	Sohlenkamp *et al.* (2004)
Burkholderia cenocepacia strains		
J2315	Wild-type	Holden *et al.* (2009)
Escherichia coli strains		
DH5α	*recA*1, Φ80 *lacZ*Δ*M15*; cloning strain	Hanahan (1983)
S17-1	*thi pro recA hsdR*$^-$ *hsdM*$^+$ RP4 integrated in the chromosome, 2-Tc::Mu, Km::Tn*7*(Tp^r/Sm^r)	Simon *et al.* (1983)
Plasmids		
pET17b	Expression vector, Cb^r	Studier (1991)
pET9a	Expression vector, Kan^r	Studier (1991)
pRK404	Broad-host-range vector, tetracycline-resistant	Ditta *et al.* (1985)
pBBR1MCS	Broad-host-range plasmid, chloramphenicol-resistant	Kovach *et al.* (1994)
pUC18	Cloning vector, ampicillin-resistant	Yanisch-Perron *et al.* (1985)
pRK2013	Helper plasmid; Km^r	Ditta *et al.* (1985)
pVK102	Cosmid vector	Vargas *et al.* (1990)
pK18mob*sacB*	Conjugative suicide vector, kanamycin-resistant	Schäfer *et al.* (1994)
pNG23	*olsB* of *B. cenocepacia* cloned in pET17b	This work
pNG25	*olsB* of *B. cenocepacia* subcloned as a BglII/HindIII fragment from pNG23 into BamHI/HindIII-digested pBBR1MCS	This work
pCCS98	*olsC* of *R. tropici* in pET9a	This work
pCos94	pVK102 derivative containing the *olsE* gene	This work
pEMAV01	1 kb fragment upstream of *olsE*, cloned as SmaI/BamHI fragment in pUC18	This work
pEMAV02	1 kb fragment downstream of *olsE*, cloned as BamHI/HindIII fragment in pUC18	This work
pEMAV03	1 kb upstream and 1 kb downstream sequences flanking *olsE*, cloned into pUC18	This work
pPMAV04	Suicide vector for construction of mutant MAV04 and MAV05	This work
pURMAV03	*olsE*-containing 3.5 kb fragment of pCos94 cloned as PstI/PstI fragment in pUC18	This work
pERMAV04	*olsE*-containing 3.5 kb fragment of pCos94 cloned as PstI/PstI fragment in pRK404	This work
pERMAV06	pET9a cloned as a BamHI fragment into pRK404	This work
pEMAV07	ORF1 in pET9a	This work
pEMAV08	ORF2 in pET9a	This work
pEMAV09	*olsE* in pET9a	This work
pERMAV11	pEMAV07 cloned as a BamHI fragment into pRK404	This work
pERMAV12	pEMAV08 cloned as a BamHI fragment into pRK404	This work
pERMAV13	pEMAV09 cloned as a BamHI fragment into pRK404	This work
pERMAV15	pCCS98 cloned as a BamHI fragment into pRK404	This work
pEMAV16	*olsC* cloned as a BamHI/BglII fragment into BamHI-digested pEMAV09	This work
pERMAV17	pEMAV16 cloned as a BamHI fragment into pRK404	This work

plates at 120°C. A transconjugant containing a gene modifying S1 should have two ninhydrin-positive lipids being either S2 and S1 similar to the lipid profile of the *R. tropici* mutant 899-*olsC*Δ*1* or S1 and P1. Once *S. meliloti* CS111.pNG25. pCos94 had been identified, cosmid pCos94 was isolated and re-introduced by conjugation into CS111.pNG25 to confirm that the lipid phenotype was caused by the presence of the cosmid and not by an independent mutation leading to the activation of an endogenous *S. meliloti* gene. In this independent transconjugant again the presence of S2 was observed. Next, the insert of pCos94 was digested with PstI. The resulting PstI/PstI fragments were subcloned into the broad host vector pRK404 and again mobilized into CS111. pNG25 repeating the lipid analysis described above. A pRK404-derived plasmid containing an approximately 3.5 kb insert was identified (pERMAV04) and its insert sequenced after subcloning into pUC18.

Expression of the three candidate ORFs from R. tropici *CIAT899*

The three candidate ORFs from plasmid pERMAV04 were separately amplified using genomic DNA from *R. tropici* CIAT899 as a template and XL-PCR polymerase (Applied Biosystems). Specific oligonucleotide primers incorporating

NdeI and BamHI sites into the final PCR products were used (oORF1-01 and oORF1-02 for ORF1; oORF2-01 and oORF2-02 for ORF2; oORF3-01 and oORF3-02 for ORF3). After digestion with the respective enzymes, the PCR products were cloned as NdeI/BamHI fragments into pET9a to yield the plasmids pEMAV07, pEMAV08 and pEMAV09 respectively. These three plasmids and pET9a were linearized with BamHI and were cloned into the BamHI site of pRK404, similarly to an earlier description (Gao *et al*., 2004) yielding the plasmids pERMAV11, pERMAV12, pERMAV13 and pERMAV06 respectively. Plasmids were mobilized into *S. meliloti* CS111.pNG25 and the lipids of the transconjugants were assayed as described above.

Deletion of the olsE *gene from* R. tropici *CIAT899*

Oligonucleotide primers oOlsX899ar1 and oOlsX899ar2 were used in a PCR (XL-PCR kit; Applied Biosystems) to amplify about 1.0 kb of genomic DNA upstream of the putative *olsE* gene from *R. tropici* CIAT899, introducing SmaI and BamHI sites into the PCR product. Similarly, primers oOlsX899ab1 and oOlsX899ab2 were used to amplify about 1.0 kb of genomic DNA downstream of the putative *olsE* gene from *R. tropici* CIAT899, introducing BamHI and HindIII sites into the PCR product. After digestion with the respective enzymes, PCR products were cloned as SmaI/BamHI or BamHI/HindIII fragments into pUC18 to yield the plasmids pUMAV01 and pUMAV02 respectively. Then, the BamHI/HindIII fragment from pUMAV02 was subcloned into pUMAV01 to yield pUMAV03. Plasmid pUMAV03 was digested with SmaI and HindIII to subclone the regions usually flanking the rhizobial *olsE* gene into the suicide vector pK18*mobsacB* (Schäfer *et al*., 1994) to yield pPMAV04. Via diparental mating using *E. coli* S17-1 (Simon *et al*., 1983) as a mobilizing strain, pPMAV04 was introduced into the wild-type strain *R. tropici* CIAT899. Transconjugants were selected on TY medium containing neomycin to select for single recombinants in a first step. The plasmid pK18*mobsacB* contains the *sacB* gene (Selbitschka *et al*., 1993), which confers sucrose sensitivity to many bacteria. Growth of the single recombinants on high sucrose will therefore select for double recombinants and the loss of the vector backbone of pK18*mobsacB* from the bacterial genome. Single recombinants were grown under non-selective conditions in complex medium for 1 day before being plated on TY medium containing 12% (w/v) sucrose. Several large and small colonies grew after 5 days, and the membrane lipids of eight candidates were analysed by *in vivo* labelling during growth on complex medium with [^{14}C]acetate and subsequent TLC (data not shown). Four clones lacking S2 and P2 were identified. Southern blot analysis confirmed that the S2- and P2-deficient strains were indeed double recombinants in which the gene *olsE* was deleted (data not shown).

Construction of a double mutant deficient in olsE *and* olsC

To construct a *R. tropici* double mutant deficient in *olsE* and *olsC*, the suicide plasmid pPMAV04 was conjugated into the *olsC*-deficient mutant 899-*olsCΔ1* (Rojas-Jiménez *et al*., 2005). The selection for double recombinants was performed in two steps as described above. Ten isolated colonies were chosen and their lipids were labelled with [^{14}C]acetate (see below). We used *R. tropici* CIAT899 and the mutant *899-olsCΔ1* as control strains. The lipids were analysed by TLC. One strain presented the expected phenotype which is the absence of the OLs S2, P1 and P2. Therefore this colony was called MAV05. Southern blot analysis confirmed that MAV05 was indeed a double recombinant in which the genes *olsC* and *olsE* were deleted (data not shown).

Complementation of the R. tropici *mutants MAV04, MAV05 and 899-*olsCΔ1

To show that the observed mutant phenotypes were caused by the introduced deletion and not by a secondary independent mutation, the mutants were complemented. The *olsE*-deficient mutant MAV04 was complemented with the plasmid pERMAV13. In this construct *olsE* is expressed under control of the T7 promoter. In earlier work we had observed constitutive expression from this promoter in different *Rhizobiaceae*. In the study published by Rojas-Jiménez *et al*. (2005) the mutant 899-*olsCΔ1* was complemented by *olsC* under its endogenous promoter, but in order to be able to compare the results from the complementation of the *olsC*-deficient mutant with the complementations of the mutants MAV04 and MAV05 a new plasmid was constructed.

The gene *olsC* was amplified using genomic DNA from *R. tropici* CIAT899 as a template and XL-PCR polymerase (Applied Biosystems). Specific oligonucleotide primers incorporating NdeI and BamHI sites into the final PCR product were used (o5B_olsC and o3_olsC). The digested PCR product was cloned into pET9a to yield the plasmid pCCS98. Plasmid pCCS98 was linearized with BamHI and cloned into BamHI-digested pRK404 to yield pERMAV15. To complement the double mutant MAV05 a plasmid containing both *olsC* and *olsE* under the control of the T7 promoter was constructed. A DNA fragment containing *olsC* under the control of the T7 promoter was subcloned from pCCS98 as BamHI/BglII fragment into the BamHI-digested pEMAV09 yielding plasmid pEMAV16. Plasmid pEMAV16 therefore contains the genes *olsC* and *olsE*, both under the control of separate T7 promoters. Subsequently, pEMAV16 was linearized with BamHI and cloned into BamHI-linearized pRK404 to yield pERMAV17.

In vivo *labelling of* S. meliloti *and* R. tropici *with [^{14}C]acetate and quantitative analysis of lipid extracts*

The lipid compositions of bacterial strains were determined following labelling with [1-^{14}C]acetate (Amersham Biosciences). Cultures (1 ml) of wild-type and mutant strains were inoculated from pre-cultures grown in the same medium. After addition of 0.5 μCi of [^{14}C]acetate (60 mCi mmol^{-1}) to each culture, the cultures were incubated for 4 h. The cells were harvested by centrifugation, washed with 500 μl of water and resuspended in 100 μl of water, and lipid extracts were obtained according to Bligh and Dyer (1959). Aliquots of the lipid extracts were spotted on high-performance TLC silica gel 60 (Merck, Poole, UK) plates and

were separated in two dimensions using chloroform/methanol/water (140:60:10, v/v) as a mobile phase for the first dimension and chloroform/methanol/glacial acetic acid (130:50:20, v/v) for the second dimension. Primary amine-containing lipids were visualized by spraying the plates with a solution of 0.2% ninhydrin in acetone and subsequent treatment at 120°C for 10 min. To visualize the membrane lipids, developed two-dimensional TLC plates were exposed to autoradiography film (Kodak) or to a PhosphorImager screen (Amersham Biosciences). The individual lipids were quantified using ImageQuant software (Amersham Biosciences).

Separation of IM and OM and determination of their respective lipid compositions

Membrane separation was performed as described previously (de Maagd and Lugtenberg, 1986; Klüsener *et al*., 2009), with minor modifications. A 400 ml culture *R. tropici* CIAT899 was grown in TY medium at 30°C overnight to an OD_{600} of 0.5–0.6. Cells were harvested by centrifugation at 10 000 *g*, 4°C, for 10 min. The cells were resuspended in 24 ml of lysis buffer [50 mM Tris-HCl, pH 7.5, 20% (w/v) sucrose, 0.2 M KCl, 0.2 mM dithiothreitol (DTT), 0.2 mg ml^{-1} DNase I, 0.2 mg ml^{-1} RNase A] and disrupted by two passages through a pre-chilled French pressure cell at 16 000 lb in^{-2}. The lysate was treated with 0.5 mg ml^{-1} lysozyme for 1 h on ice and centrifuged at 10 000 *g* for 20 min, 4°C, to remove the unbroken cells. The supernatant was centrifuged at 150 000 *g* (SW40Ti), 4°C, for 1 h to collect the membranes. The resulting membrane pellet was carefully resuspended in 2 ml of 20% (w/v) sucrose containing 5 mM EDTA, pH 7.5, and 0.2 mM DTT. Material that was not completely suspended was removed by centrifugation for 5 min at 16 000 *g*. The gradient was prepared by layering 7.5 ml of 53% (w/v) sucrose over a cushion of 2.5 ml of 70% (w/v) sucrose. Both sucrose solutions contained 5 mM EDTA, pH 7.5. The membrane suspension was layered on the top of the gradient, and sucrose density gradient ultracentrifugation was carried out at 100 000 *g* (SW40Ti), 4°C, for 16 h. After ultracentrifugation, the separated membranes were fractionated in 500 µl aliquots. For each fraction the protein concentration was estimated, and the density, the NADH activity and the 2-keto-3-deoxyoctonate (KDO) content were determined. The protein distribution was estimated using absorption measurements at 280 nm (Scopes, 1987). The NADH oxidase activity was determined by the method of Osborn *et al*. (1972) and the KDO content was determined as described earlier after the fractions had been precipitated twice with 10% (w/v) TCA (Karkhanis *et al*., 1978). NADH oxidase activity and KDO content were used as marker for the IM and OM respectively. Fractions corresponding to the IM and the OM were pooled and the lipids were extracted with 1-butanol (Bremer, 1963). Lipids were analysed using two-dimensional TLC as described above and the lipids were detected by oxidative charring using ceric sulphate in sulphuric acid (Villaescusa and Pettit, 1972). The lipid spots were quantified using the program ImageQuant (Applied Biosystems).

ESI-MS/MS analysis of lipids S1 and S2

In order to identify in which part of the OL S2 the modification is encountered, a 1 l culture of the mutant 899-*olsCΔ1* (Rojas-Jiménez *et al*., 2005) was grown to an optical density at 620 nm of 1.0 in TY medium, and lipids were extracted according to a modified Bligh-and-Dyer procedure (Bligh and Dyer, 1959). Lipids were fractionated using a silica column and chloroform/methanol/water (140:60:8, v/v) as a mobile phase. Fractions were analysed by one-dimensional TLC using chloroform/methanol/water (140:60:8, v/v) as a mobile phase. Fractions containing OLs were identified by iodine and ninhydrin staining as described above. OL-containing fractions were dried under N_2 stream and re-dissolved in methanol/chloroform (1:1, v/v). LC-ESI/MS of lipids was performed using an Agilent 1200 Quaternary LC system coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems, Foster City, CA). An Ascentis® Si HPLC column (5 µm, 25 cm × 2.1 mm) was used. Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v). Mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v). Mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v). The elution programme consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The total LC flow rate was 300 µl min^{-1}. The post-column splitter diverted ~10% of the LC flow to the ESI source of the Q-Star XL mass spectrometer, with MS settings as follows: IS = –4500 V, CUR = 20 psi, GS1 = 20 psi, DP = –55 V and FP = –150 V. Nitrogen was used as the collision gas. Data acquisition and analysis were performed using Analyst QS software version 1.1.

Determination of the position of the hydroxyl group introduced by OlsC into OLs

Large cultures (4 l) of the *R. tropici* mutant MAV05 and the strain MAV05.pERMAV15 were grown to an OD_{620} of 0.9 in TY medium. MAV05 only forms S1 and MAV05.pERMAV15 forms preferentially P1. Cells were harvested and lipids were extracted from the cell pellets according to a modified Bligh-and-Dyer method. OLs S1 and P1 were purified using preparative TLC using Si500F plates (Baker) in two steps. First chloroform/methanol/water (140:60:10, v/v) was used as a mobile phase and the OLs were purified from the silica. Enriched OLs were further purified by a second preparative TLC using chloroform/methanol/glacial acetic acid (130:50:20, v/v) as mobile phase. ESI-MS/MS analysis of S1 and P1 was performed as described above.

The derivatization of the lipids was performed essentially as described by Gibbons *et al*. (2008). Purified OLs S1 and P1 were hydrolysed in acidic methanol, and then converted to TMS ethers. Hydroxy fatty acid standards (α- and β-hydroxy palmitic acid, α- and β-hydroxy stearic acid) were processed and analysed in parallel with the samples. Typically, about 1 mg of sample was dried in a Reacti-vial and samples were hydrolysed by adding 300 µl of 1 M HCl in methanol and heated at 80°C for 16 h. The reactions were cooled and solvents were evaporated under a stream of nitrogen. Next, 200 µl Tri-Sil HTP reagent (Thermo) was added to the dried

samples. After incubation for 1 h at 25°C a 20 µl aliquot was diluted 1:6 in hexane and transferred to a new vial for GC/MS analysis.

GC/MS was performed using a Clarus 600T MS instrument coupled to a Clarus 600 gas chromatography system (Perkin Elmer). The column was a Elite-5 MS (0.32 mm internal diameter and 0.25 µm phase thickness) from Perkin Elmer. The temperature programme of the GC was as follows: the column oven temperature was initially held at 140°C for 6 min, increased to 250°C at a rate of 4°C min^{-1} and finally held at 250°C for 5 min. The total run time was 38.5 min. The injector was operated in the split mode, and the temperature of the injector was kept at 250°C. Helium was the carrier gas at a constant pressure of 7 psi. The instrument was operated in the electron impact (EI) mode with the electron energy set at 70 eV.

Plant tests

Phaseolus vulgaris seeds were surface-sterilized with 1.2% sodium hypochlorite and were germinated on 1% agar-water plates as described (Vinuesa *et al*., 1999). Seedlings were transferred to 250 ml flasks filled 220 ml of nitrogen-free nutrient solution (Fahraeus, 1957) containing agar at 0.7% and were inoculated with about 50 000 cfu ml^{-1} per plant. Plants were grown in a controlled growth chamber at 28°C with a 15 h day/9 h night cycle and harvested 21 days after inoculation. Nitrogenase activity of nodulated roots was determined by the acetylene reduction assay as described previously (Martínez *et al*., 1985). Nitrogen fixation activity per plant was normalized with respect to the nodule fresh weight per plant.

Acknowledgements

This research was supported by grants from CONACyT-Mexico (46020-N) and DGAPA/UNAM (IN217907 and IN201310). Z.G. and the mass spectrometry facility in the Department of Biochemistry, Duke University Medical Center, were supported by a LIPID MAPS glue grant (GM-069338) from the National Institutes of Health. M.Á.V.-G. is a PhD student from the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México and is a recipient of a scholarship from the Consejo Nacional de Ciencia y Tecnología, Mexico. We are grateful to Alfonso Leija Salas for his support in nodule analysis.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389–3402.

Arthington, B.A., Bennett, L.G., Skatrud, P.L., Guynn, C.J., Barbuch, R.J., Ulbright, C.E., and Bard, M. (1991) Cloning, disruption and sequence of the gene encoding yeast C-5 sterol desaturase. *Gene* **102:** 39–44.

Asselineau, J. (1991) Bacterial lipids containing amino acids or peptides linked by amide bonds. *Fortschr Chem Org Naturst* **56:** 1–85.

Bard, M., Bruner, D.A., Pierson, C.A., Lees, N.D., Biermann, B., Frye, L., *et al*. (1996) Cloning and characterization of ERG25, the *Saccharomyces cerevisiae* gene encoding C-4 sterol methyl oxidase. *Proc Natl Acad Sci USA* **93:** 186–190.

Benning, C., and Somerville, C.R. (1992) Isolation and genetic complementation of a sulfolipid-deficient mutant of *Rhodobacter sphaeroides*. *J Bacteriol* **174:** 2352–2360.

Benning, C., Huang, Z.H., and Gage, D.A. (1995) Accumulation of a novel glycolipid and a betaine lipid in cells of *Rhodobacter sphaeroides* grown under phosphate limitation. *Arch Biochem Biophys* **317:** 103–111.

Beringer, J.E. (1974) R factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol* **84:** 188–198.

Bligh, E.G., and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37:** 911–917.

Bremer, J. (1963) Carnitine in intermediary metabolism. The biosynthesis of palmitylcarnitine by cell subfractions. *J Biol Chem* **238:** 2774–2779.

Bukata, L., Altabe, S., de Mendoza, D., Ugalde, R.A., and Comerci, D.J. (2008) Phosphatidylethanolamine synthesis is required for optimal virulence of *Brucella abortus*. *J Bacteriol* **190:** 8197–8203.

Comerci, D.J., Altabe, S., de Mendoza, D., and Ugalde, R.A. (2006) *Brucella abortus* synthesizes phosphatidylcholine from choline provided by the host. *J Bacteriol* **188:** 1929–1934.

Dees, C., and Shively, J.M. (1982) Localization of quantitation of the ornithine lipid of *Thiobacillus thiooxidans*. *J Bacteriol* **149:** 798–799.

Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X.W., Finlay, D.R., *et al*. (1985) Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid* **13:** 149–153.

Fahraeus, G. (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *J Gen Microbiol* **16:** 374–381.

Figurski, D.H., and Helinski, D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci USA* **76:** 1648–1652.

Fox, B.G., Shanklin, J., Somerville, C., and Munck, E. (1993) Stearoyl-acyl carrier protein delta 9 desaturase from *Ricinus communis* is a diiron-oxo protein. *Proc Natl Acad Sci USA* **90:** 2486–2490.

Galbraith, L., Jonsson, M.H., Rudhe, L.C., and Wilkinson, S.G. (1999) Lipids and fatty acids of *Burkholderia* and *Ralstonia* species. *FEMS Microbiol Lett* **173:** 359–364.

Gao, J.L., Weissenmayer, B., Taylor, A.M., Thomas-Oates, J., López-Lara, I.M., and Geiger, O. (2004) Identification of a gene required for the formation of lyso-ornithine lipid, an intermediate in the biosynthesis of ornithine-containing lipids. *Mol Microbiol* **53:** 1757–1770.

Geiger, O., Röhrs, V., Weissenmayer, B., Finan, T.M., and Thomas-Oates, J.E. (1999) The regulator gene *phoB* mediates phosphate stress-controlled synthesis of the membrane lipid diacylglyceryl-*N,N,N*-trimethylhomoserine in *Rhizobium* (*Sinorhizobium*) *meliloti*. *Mol Microbiol* **32:** 63–73.

Geiger, O., González-Silva, N., López-Lara, I.M., and Sohlenkamp, C. (2010) Amino acid-containing membrane lipids in bacteria. *Prog Lipid Res* **49:** 46–60.

Gibbons, H.S., Lin, S., Cotter, R.J., and Raetz, C.R. (2000) Oxygen requirement for the biosynthesis of the *S*-2-hydroxymyristate moiety in *Salmonella typhimurium* lipid A. Function of LpxO, A new Fe2+/alpha-ketoglutarate-dependent dioxygenase homologue. *J Biol Chem* **275:** 32940–32949.

Gibbons, H.S., Reynolds, C.M., Guan, Z., and Raetz, C.R. (2008) An inner membrane dioxygenase that generates the 2-hydroxymyristate moiety of *Salmonella* lipid A. *Biochemistry* **47:** 2814–2825.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166:** 557–580.

Heath, R.J., Jackowski, S., and Rock, C.O. (2002) Fatty acid and phospholipid metabolism in prokaryotes. In *Biochemistry of Lipids, Lipoproteins and Membranes*. Vance, D.E., and Vance, J.E. (eds). Amsterdam: Elsevier, pp. 55–92.

Holden, M.T., Seth-Smith, H.M., Crossman, L.C., Sebaihia, M., Bentley, S.D., Cerdeno-Tarraga, A.M., *et al*. (2009) The genome of *Burkholderia cenocepacia* J2315, an epidemic pathogen of cystic fibrosis patients. *J Bacteriol* **191:** 261–277.

Jackson, M., Crick, D.C., and Brennan, P.J. (2000) Phosphatidylinositol is an essential phospholipid of mycobacteria. *J Biol Chem* **275:** 30092–30099.

Karkhanis, Y.D., Zeltner, J.Y., Jackson, J.J., and Carlo, D.J. (1978) A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of gram-negative bacteria. *Anal Biochem* **85:** 595–601.

Kawai, Y., Yano, I., and Kaneda, K. (1988) Various kinds of lipoamino acids including a novel serine-containing lipid in an opportunistic pathogen *Flavobacterium*. Their structures and biological activities on erythrocytes. *Eur J Biochem* **171:** 73–80.

Kennedy, M.A., Johnson, T.A., Lees, N.D., Barbuch, R., Eckstein, J.A., and Bard, M. (2000) Cloning and sequencing of the *Candida albicans* C-4 sterol methyl oxidase gene (ERG25) and expression of an ERG25 conditional lethal mutation in *Saccharomyces cerevisiae*. *Lipids* **35:** 257–262.

Klüsener, S., Aktas, M., Thormann, K.M., Wessel, M., and Narberhaus, F. (2009) Expression and physiological relevance of *Agrobacterium tumefaciens* phosphatidylcholine biosynthesis genes. *J Bacteriol* **191:** 365–374.

Knoche, H.W., and Shively, J.M. (1972) The structure of an ornithine-containing lipid from *Thiobacillus thiooxidans*. *J Biol Chem* **247:** 170–178.

Kohler, S., Porte, F., Jubier-Maurin, V., Ouahrani-Bettache, S., Teyssier, J., and Liautard, J.P. (2002) The intramacrophagic environment of *Brucella suis* and bacterial response. *Vet Microbiol* **90:** 299–309.

Kok, M., Oldenhuis, R., van der Linden, M.P., Raatjes, P., Kingma, J., van Lelyveld, P.H., and Witholt, B. (1989) The *Pseudomonas oleovorans* alkane hydroxylase gene. Sequence and expression. *J Biol Chem* **264:** 5435–5441.

Kovach, M.E., Phillips, R.W., Elzer, P.H., Roop, R.M., 2nd, and Peterson, K.M. (1994) pBBR1MCS: a broad-host-range cloning vector. *Biotechniques* **16:** 800–802.

López-Lara, I.M., Sohlenkamp, C., and Geiger, O. (2003) Membrane lipids in plant-associated bacteria: their biosyntheses and possible functions. *Mol Plant Microbe Interact* **16:** 567–579.

de Maagd, R.A., and Lugtenberg, B. (1986) Fractionation of *Rhizobium leguminosarum* cells into outer membrane, cytoplasmic membrane, periplasmic, and cytoplasmic components. *J Bacteriol* **167:** 1083–1085.

Martínez, E., Pardo, M.A., Palacios, R., and Cevallos, M.A. (1985) Reiteration of nitrogen fixation gene sequences and specificity of *Rhizobium* in nodulation and nitrogen fixation in *Phaseolus vulgaris*. *J Gen Microbiol* **131:** 1779–1786.

Martínez-Romero, E., Segovia, L., Mercante, F.M., Franco, A.A., Graham, P., and Pardo, M.A. (1991) *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int J Syst Bacteriol* **41:** 417–426.

Meneely, K.M., Barr, E.W., Bollinger, J.M., Jr, and Lamb, A.L. (2009) Kinetic mechanism of ornithine hydroxylase (PvdA) from *Pseudomonas aeruginosa*: substrate triggering of O2 addition but not flavin reduction. *Biochemistry* **48:** 4371–4376.

Mitchell, A.G., and Martin, C.E. (1997) Fah1p, a *Saccharomyces cerevisiae* cytochrome *b*5 fusion protein, and its *Arabidopsis thaliana* homolog that lacks the cytochrome *b*5 domain both function in the alpha-hydroxylation of sphingolipid-associated very long chain fatty acids. *J Biol Chem* **272:** 28281–28288.

Murata, T., Tseng, W., Guina, T., Miller, S.I., and Nikaido, H. (2007) PhoPQ-mediated regulation produces a more robust permeability barrier in the outer membrane of *Salmonella enterica* serovar *typhimurium*. *J Bacteriol* **189:** 7213–7222.

Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* **67:** 593–656.

Osborn, M.J., Gander, J.E., Parisi, E., and Carson, J. (1972) Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. *J Biol Chem* **247:** 3962–3972.

Perzl, M., Reipen, I.G., Schmitz, S., Poralla, K., Sahm, H., Sprenger, G.A., and Kannenberg, E.L. (1998) Cloning of conserved genes from *Zymomonas mobilis* and *Bradyrhizobium japonicum* that function in the biosynthesis of hopanoid lipids. *Biochim Biophys Acta* **1393:** 108–118.

Rojas-Jiménez, K., Sohlenkamp, C., Geiger, O., Martínez-Romero, E., Werner, D., and Vinuesa, P. (2005) A ClC chloride channel homolog and ornithine-containing membrane lipids of *Rhizobium tropici* CIAT899 are involved in symbiotic efficiency and acid tolerance. *Mol Plant Microbe Interact* **18:** 1175–1185.

Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schäfer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G., and Puhler, 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.

Scopes, R.K. (1987) Measurement of enzyme activity. In *Protein Purification – Principles and Practice*. Cantor, C.R. (ed.). New York: Springer, pp. 253–283.

Selbitschka, W., Niemann, S., and Pühler, A. (1993) Construction of gene replacement vectors for gram-negative bacteria using a genetically modified *sacRN* as a positive selection marker. *Appl Microbiol Biotechnol* **38:** 615–618.

Shanklin, J., Whittle, E., and Fox, B.G. (1994) Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. *Biochemistry* **33:** 12787–12794.

Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. *Biotechnology* **1:** 784–791.

Sohlenkamp, C., López-Lara, I.M., and Geiger, O. (2003) Biosynthesis of phosphatidylcholine in bacteria. *Prog Lipid Res* **42:** 115–162.

Sohlenkamp, C., de Rudder, K.E., and Geiger, O. (2004) Phosphatidylethanolamine is not essential for growth of *Sinorhizobium meliloti* on complex culture media. *J Bacteriol* **186:** 1667–1677.

Sohlenkamp, C., Galindo-Lagunas, K.A., Guan, Z., Vinuesa, P., Robinson, S., Thomas-Oates, J., *et al*. (2007) The lipid lysyl-phosphatidylglycerol is present in membranes of *Rhizobium tropici* CIAT899 and confers increased resistance to polymyxin B under acidic growth conditions. *Mol Plant Microbe Interact* **20:** 1421–1430.

Studier, F.W. (1991) Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J Mol Biol* **219:** 37–44.

Suzuki, M., Hayakawa, T., Shaw, J.P., Rekik, M., and Harayama, S. (1991) Primary structure of xylene monooxygenase: similarities to and differences from the alkane hydroxylation system. *J Bacteriol* **173:** 1690–1695.

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.

Udvardi, M.K., and Day, D.A. (1997) Metabolite transport across symbiotic membranes of legume nodules. *Annu Rev Plant Physiol Plant Mol Biol* **48:** 493–523.

Vargas, C., Martinez, L.J., Megias, M., and Quinto, C. (1990) Identification and cloning of nodulation genes and host specificity determinants of the broad host-range *Rhizobium leguminosarum* biovar *phaseoli* strain CIAT899. *Mol Microbiol* **4:** 1899–1910.

Villaescusa, F.W., and Pettit, G.R. (1972) Steroids and related natural products. 69. Synthesis of 20(22)-Dihydro-23-deoxodigitoxigenin. *J Org Chem* **37:** 569–572.

Vinuesa, P., Reuhs, B.L., Breton, C., and Werner, D. (1999) Identification of a plasmid-borne locus in *Rhizobium etli* KIM5s involved in lipopolysaccharide O-chain biosynthesis and nodulation of *Phaseolus vulgaris*. *J Bacteriol* **181:** 5606–5614.

Vinuesa, P., Neumann-Silkow, F., Pacios-Bras, C., Spaink, H.P., Martínez-Romero, E., and Werner, D. (2003) Genetic analysis of a pH-regulated operon from *Rhizobium tropici* CIAT899 involved in acid tolerance and nodulation competitiveness. *Mol Plant Microbe Interact* **16:** 159–168.

Visca, P., Ciervo, A., and Orsi, N. (1994) Cloning and nucleotide sequence of the *pvdA* gene encoding the pyoverdin biosynthetic enzyme L-ornithine N5-oxygenase in *Pseudomonas aeruginosa*. *J Bacteriol* **176:** 1128–1140.

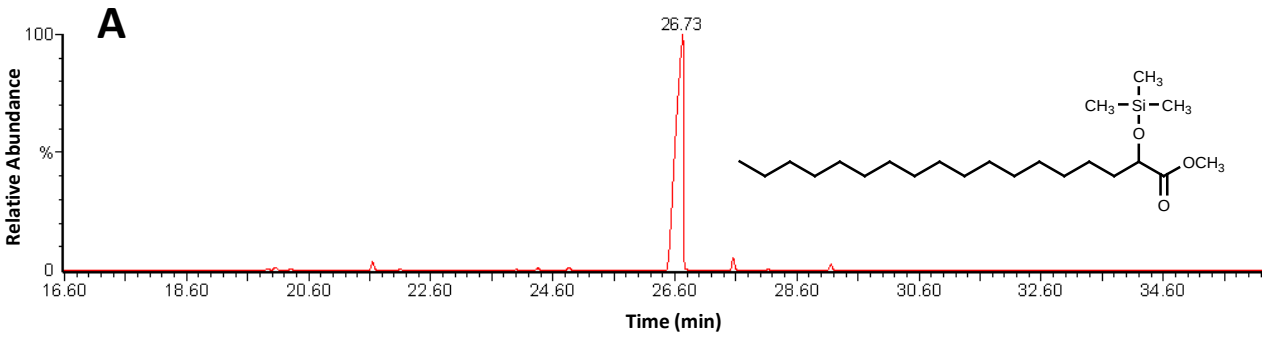
Weissenmayer, B., Gao, J.L., López-Lara, I.M., and Geiger, O. (2002) Identification of a gene required for the biosynthesis of ornithine-derived lipids. *Mol Microbiol* **45:** 721–733.

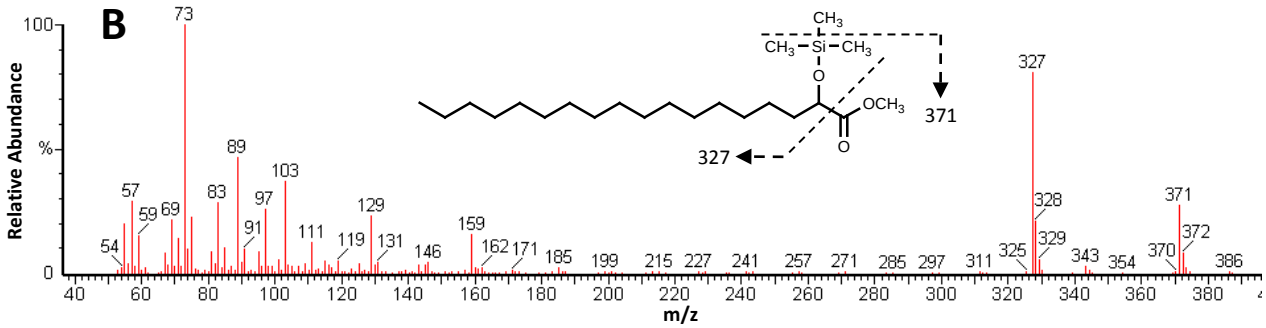
Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33:** 103–119.

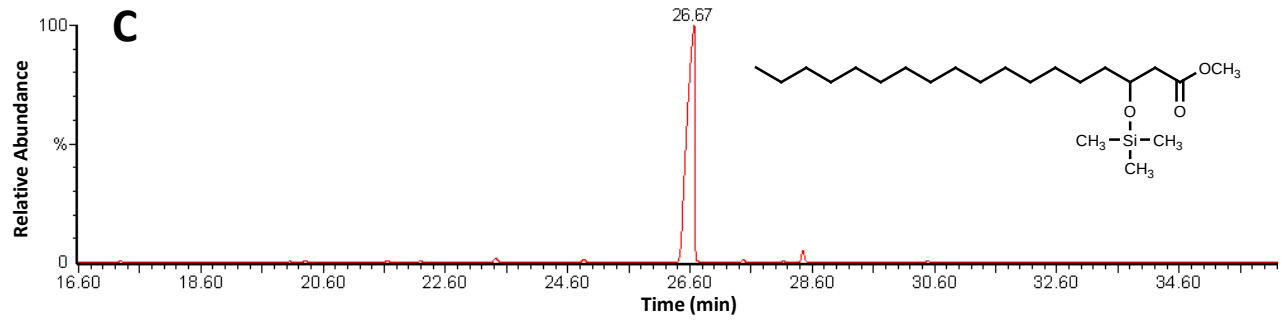
Supporting information

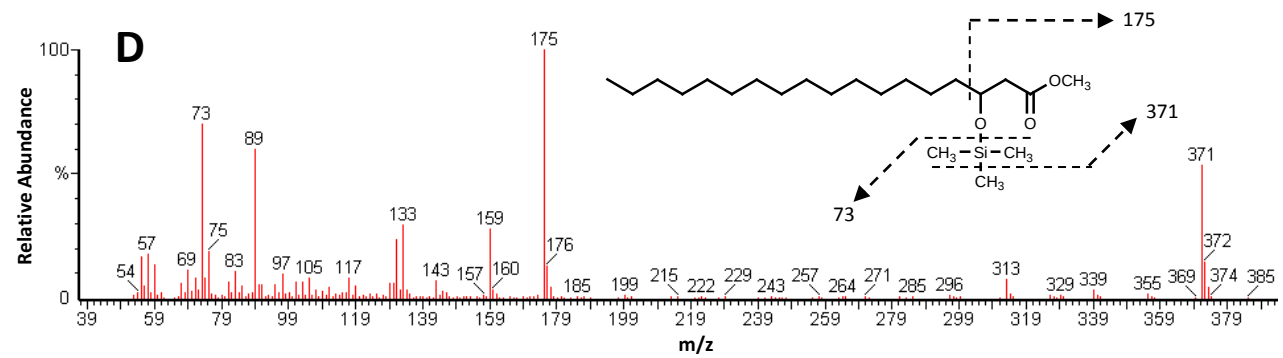
Additional supporting information may be found in the online version of this article.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.


A
26.73
Relative Abundance
Time (min)


B
73
89
103
57
83
97
129
59
69
91
111
119
131
146
159
162
171
185
199
215
227
241
257
271
285
297
311
325
327
328
329
343
354
370
371
372
386
371
327
Relative Abundance
m/z


C
26.67
Relative Abundance
Time (min)


D
175
73
89
57
75
69
83
97
105
117
133
143
157
159
160
176
185
199
215
222
229
243
257
264
271
285
296
313
329
339
355
369
371
372
374
385
175
371
73
Relative Abundance
m/z

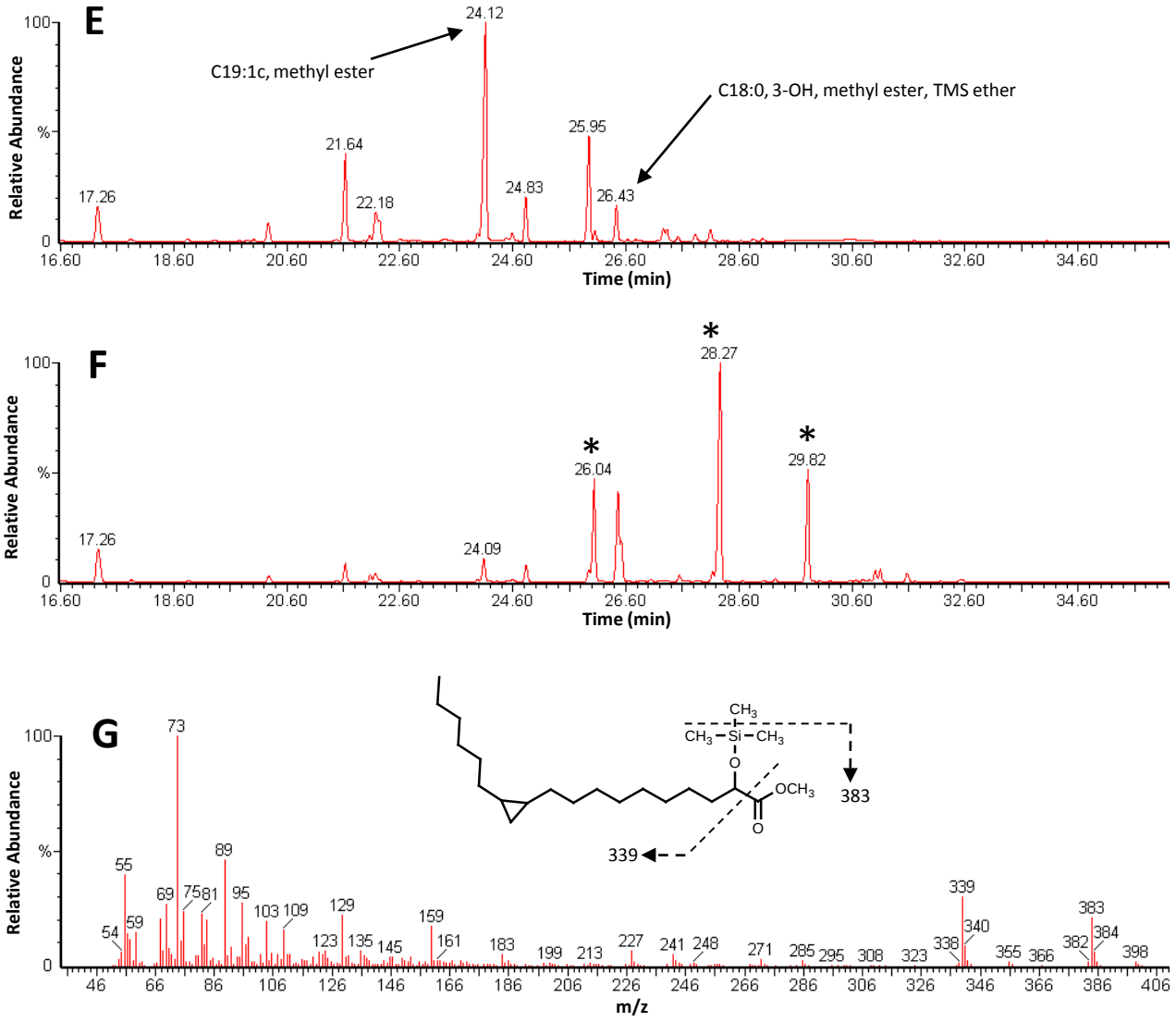


Figure S1. Determination of the position of the hydroxyl group introduced by OlsC into the esterified fatty acid of the ornithine lipid P1. 2-hydroxystearate and 3-hydroxystearate standards and ornithine lipids S1 and P1 were transmethylated, then converted to TMS derivatives and finally analyzed by GC/MS. (A) Total ion chromatogram (TIC) of the GC/MS analysis of derivatized 2-hydroxystearate. (B) EI/MS spectrum of the major peak in panel A showing a fragmentation pattern as expected for a TMS-2-hydroxystearoyl methyl ester. (C) TIC of the GC/MS analysis of derivatized 3-hydroxystearate. (D) EI/MS spectrum of the major peak in panel C showing a fragmentation pattern as expected for a TMS-3-hydroxystearoyl methylester. (E) TIC of the GC/MS analysis of the fatty acids derivatives obtained from the derivatization of ornithine lipid S1. (F) TIC of the GC/MS analysis of the fatty acid derivatives obtained from the derivatization of ornithine lipid P1. The asterics indicate peaks that are present in chromatogram F but absent in chromatogram E. (G) EI/MS spectrum of the major peak present in chromatogram F (P1) but absent from chromatogram E (S1) (retention time: 28.27 min). The observed fragmentation indicates that the spectrum is produced by TMS-2-hydroxy lactobacillic acid methyl ester. The proposed origins of the major fragments are indicated. The other two peaks labeled with asteriscs in panel F also fragment as expected for TMS-2-hydroxyl fatty acid methyl esters.

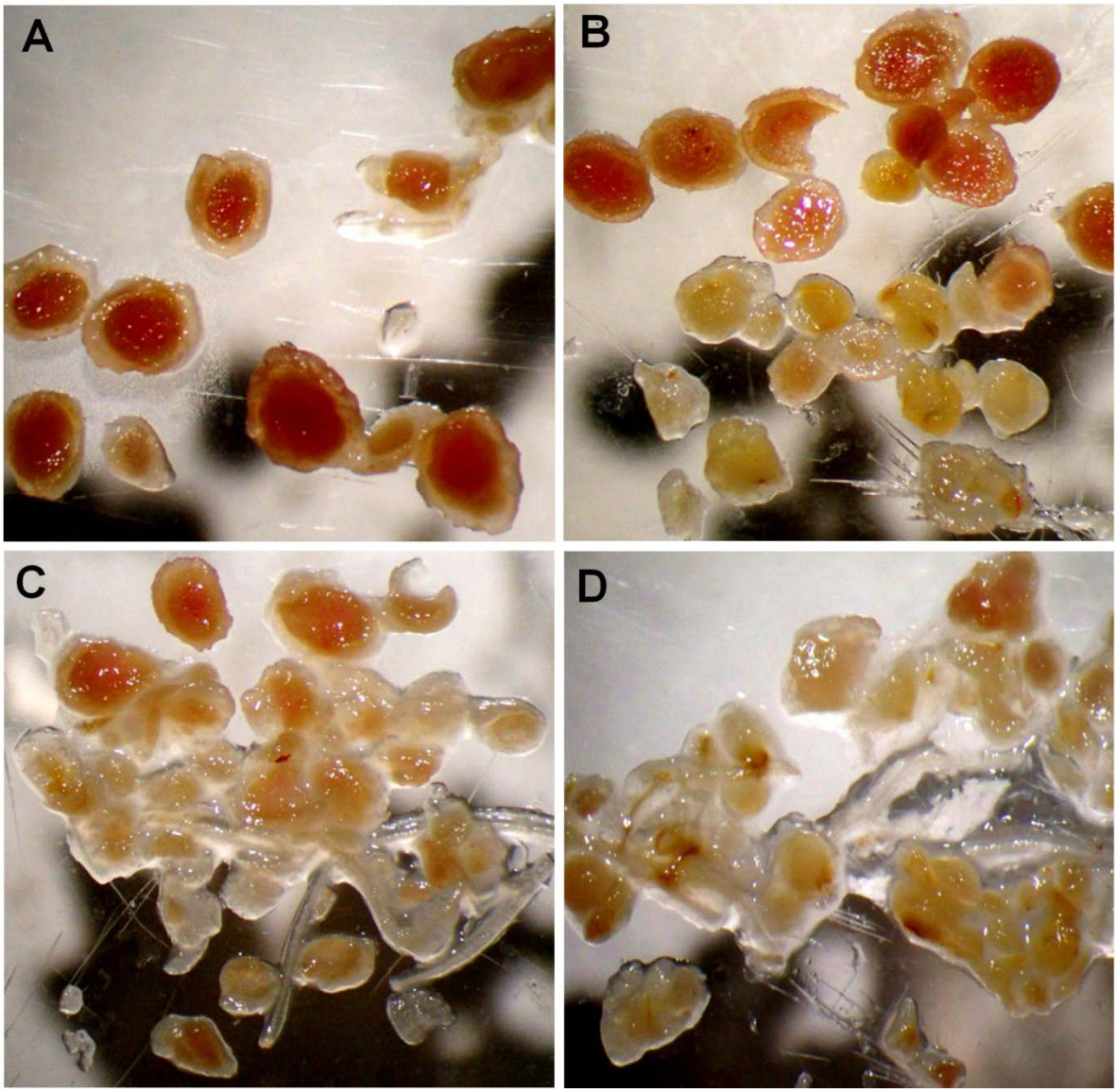


Figure S2. Bean plants were harvested 21 days after inoculation. A selection of nodules representing the different sizes and colors was harvested. Nodules were sectioned using a razorblade. Roots from bean plants were inoculated with the wild type *R. tropici* CIAT899 (A), OlsC-deficient mutant 899-*olsCΔ1*, OlsE-deficient mutant MAV04 (C), and OlsC/OlsE-deficient double mutant MAV05 (D).

Supplementary Table 1.
Oligonucleotides used in this study

Name	Sequence (5'-3')	Incorporated restriction site (underlined)
oLOP111	AggAATACATATgCgAgAACTgCCgACgCC	NdeI
oLOP112	ACCCAAgCTTTCAgCCCAggAAgTggCggg	HindIII
oORF1_01	AACTgCATATggATCTgACgAATTggAAgggCgTC	NdeI
oORF1_02	ACTgggATCCTAgCCgAATgTCAATTTCgTCTTC	BamHI
oORF2_01	AACTgCATATgCgCATAgAgCAggCggCAgACgAAC	NdeI
oORF2_02	ACTgggATCCATCAgTgCATCCgAggCAAACTCAgTg	BamHI
oORF3_01	ACTgACATATgATATTTCAAAgCAgTTCgCgTC	NdeI
oORF3_02	ACTgggATCCTAgCCTgATTTgCggATTTTAgC	BamHI
oOlsX899ar1	ACTgCCCgggATCgCTTTCgCTATCTTTTCgAgg	SmaI
oOlsX899ar2	ACTgggATCCATCgTCCTCAgaTTCTgATCggCg	BamHI
oOlsX899ab1	ACTgggATCCggCAAgAAgCCgAAggAATTgATC	BamHI
oOlsX899ab2	ACTgAAgCTTggCTATggCgCACggCCTTgTggAA	HindIII
o5B_olsC	ACgTCATATgACggAgAgTCCCTTgAgCgCACC	NdeI
o3_olsC	ACgTggATCCTCAgggCTTCgggCggTTTCTgAACg	BamHI

8.1.3. III Artículo

González-Silva, N., López-Lara, I. M., Reyes-Lamothe, R., Taylor, A. M., Sumpton, D., Thomas-Oates, J., and Geiger, O. (2011) The dioxygenase-encoding *olsD* gene from *Burkholderia cenocepacia* causes the hydroxylation of the amide-linked fatty acyl moiety of ornithine-containing membrane lipids. *Biochemistry 50,* 6396-6408.

Burkholderia cenocepacia es un patógeno oportunista importante, y una de sus características más sobresalientes es el repertorio de lípidos polares de su membrana, que incluye a fosfatidiletanolamina (PE) y lípidos de ornitina (OLs), así como sus derivados 2-hidroxilados de PE y OLs (2-OH-PE y 2-OH-OLs, respectivamente). Los últimos lípidos difieren de las versiones estándar por la presencia de un grupo hidroxilo en el C2 (2-OH) de un residuo de acilo esterificado. Similarmente, un grupo miristato esterificado al lípido A de *Salmonella typhimurium* puede tener una modificación 2-hidroxilo que se debe a la enzima LpxO. Nosotros postulamos que la 2-hidroxilación de los 2-OH-OLs podría ser catalizada por una nueva dioxigenasa homóloga a LpxO. En *B. cenocepacia*, identificamos dos marcos abiertos de lectura (BCAM1214 and BCAM2401) homólogos a LpxO de *S. typhimurium*. La introducción de *bcam2401* (nombrado *olsD*) en *Sinorhizobium meliloti* provocó la formación de un nuevo lípido y en *B. cenocepacia* de dos nuevos lípidos, Sorprendentemente, la modificación de los OLs debida a OlsD ocurre en la cadena de acilo unida a la amida. Este es el primer reporte de una modificación hidroxilo de los OLs en el residuo de acilo unido a la amida. La formación de los OLs hidroxilados ocurre solamente cuando la ruta de biosíntesis para OLs estándar no modificados está intacta. La modificación hidroxilo de los OLs en el residuo acilo unido a la amida ocurre solamente bajo condiciones de estrés ácido. Un ensayo ha sido desarrollado para la dioxigenasa OlsD, y una caracterización inicial de la enzima es presentada.

BIOCHEMISTRY
including biophysical chemistry & molecular biology

ARTICLE

pubs.acs.org/biochemistry

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

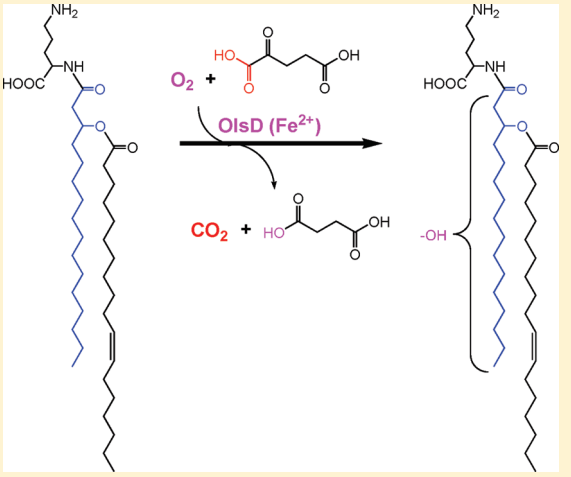
Napoleón González-Silva,[†] Isabel M. López-Lara,[†] Rodrigo Reyes-Lamothe,[†] Adrian M. Taylor,[‡] David Sumpton,[‡] Jane Thomas-Oates,[‡] and Otto Geiger[*,†]

[†]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

Received: May 6, 2011
Revised: June 24, 2011
Published: June 27, 2011

ACS Publications © 2011 American Chemical Society

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

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

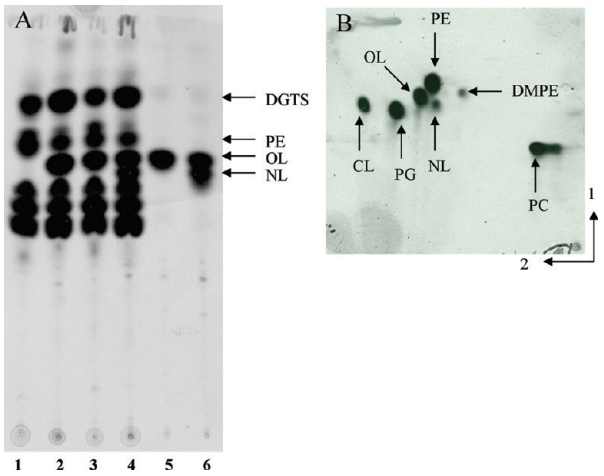


Figure 1. Expression of BCAM2401 (OlsD) in *S. meliloti* causes the formation of a new ornithine-containing lipid (NL). (A) Lipid analysis of *S. meliloti* strains carrying different plasmids after growth on Sherwood minimal medium containing a low phosphate concentration and grown either in the presence 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

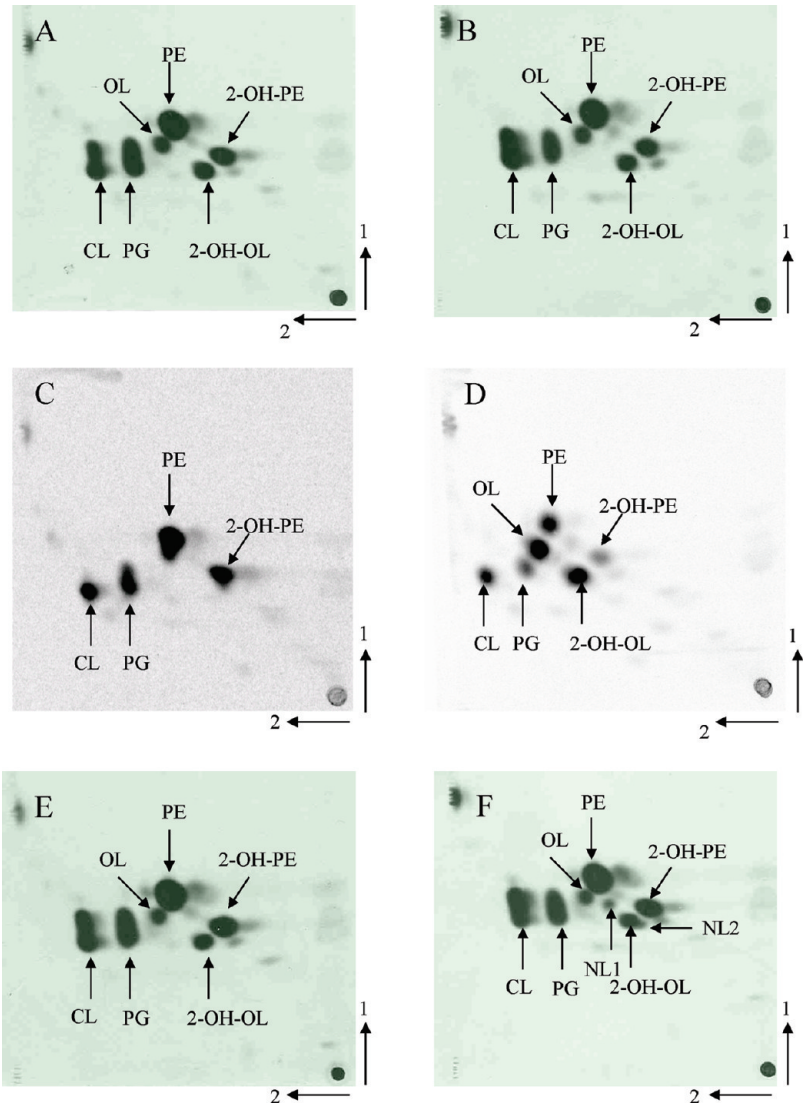


Figure 2. Membrane lipid profiles of *B. cenocepacia* strains defective with respect to OL biosynthesis genes (*olsB* or *olsD*) or overexpressing them. Separation of [^{14}C]acetate-labeled lipids by two-dimensional TLC from wild-type *B. cenocepacia* J2315 (A), wild-type *B. cenocepacia* J2315 containing 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

dx.doi.org/10.1021/bi200706v |*Biochemistry* 2011, 50, 6396–6408

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 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.

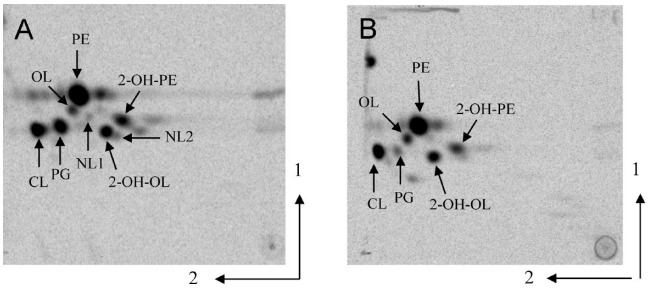


Figure 3. OlsD-dependent modification of OLs in *B. cenocepacia* occurs under acidic growth conditions. Separation of [^{14}C]acetate-labeled lipids by 2D-TLC from wild-type *B. cenocepacia* J2315 (A) or *olsD*-deficient knockout mutant *B. cenocepacia* AQ3 (B) after growth on acidic (pH 4) Homopipes/Na-buffered 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.

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

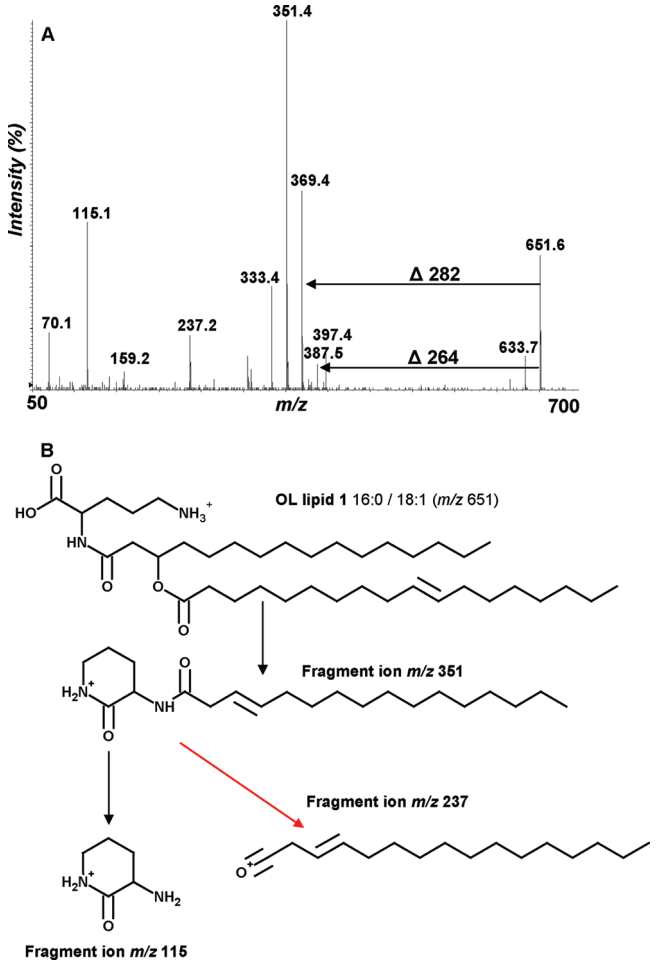


Figure 4. Positive ion mode product ion mass spectrum of OL lipid 1 (m/z 651) (A) and proposed fragmentation scheme for OL (B). The step shown with the red arrow shows the loss of the newly formed unsaturated internal fatty acyl residue from ornithine as an acylium cation, producing the proposed diagnostic 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

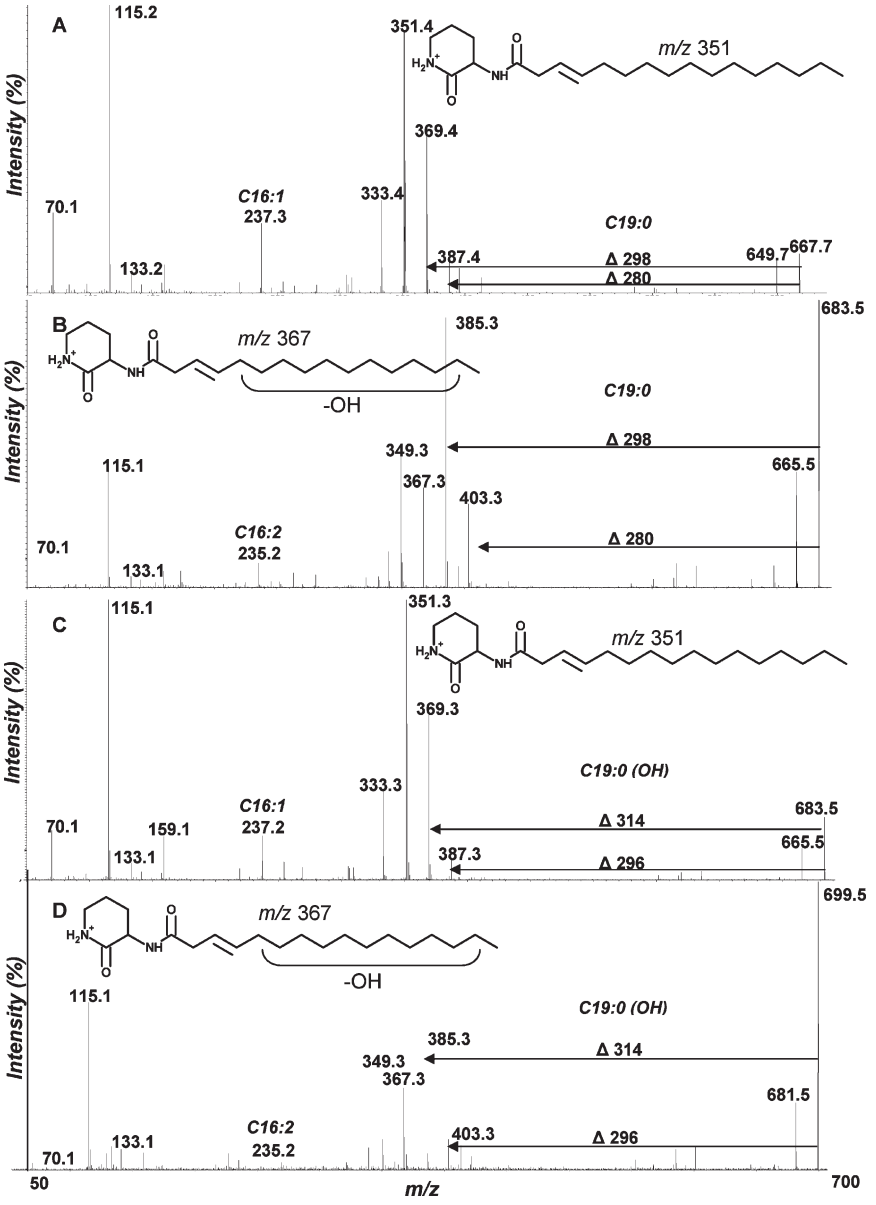


Figure 5. Positive mode product ion mass spectra of the ions at *m*/*z* 667 from OL spot extract (A), *m*/*z* 683 from NL1 spot extract (B), *m*/*z* 683 from 2-OH/NL2 spot extract (C), and *m*/*z* 699 from 2-OH-OL/NL2 spot extract (D). Peak intensities are normalized to the most intense fragment ion.

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.

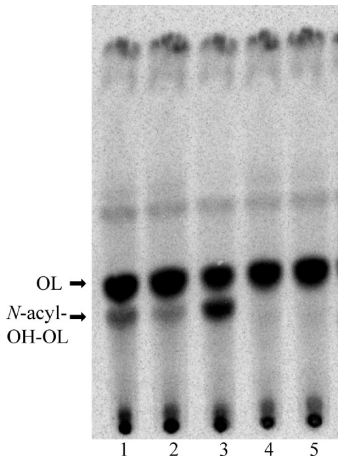


Figure 6. Membrane-associated OlsD dioxygenase converts OL to OL that is hydroxylated in the amidified acyl residue. Cell-free extracts of *E. coli* BL21(DE3) pLysS pNG40 expressing OlsD (lane 1), BL21(DE3) pLysS containing the empty pET16b vector (lane 4), the soluble protein fraction from *E. coli* 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

dx.doi.org/10.1021/bi200706v |*Biochemistry* 2011, 50, 6396–6408

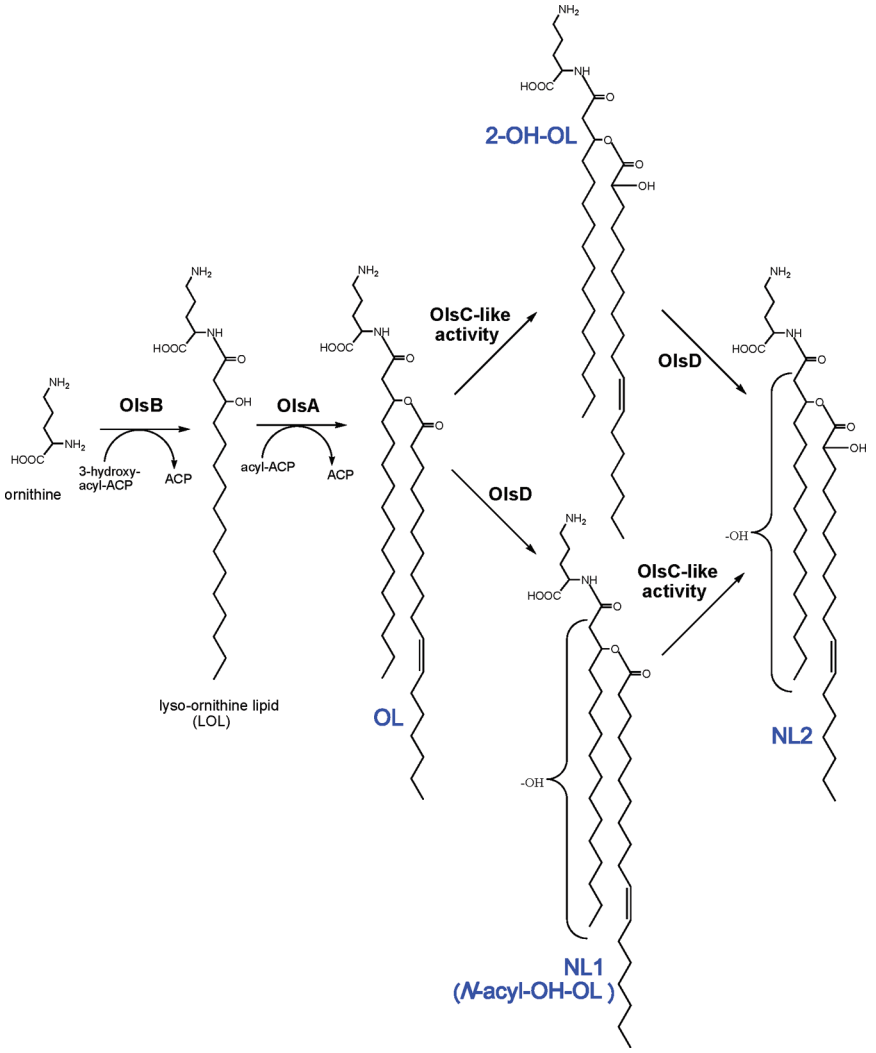


Figure 7. Biosynthesis of OLs in *B. cenocepacia* J2315. The genes encoding OlsB and OlsA have first been identified in *S. meliloti*, whereas the gene encoding the OL 2-hydroxylase OlsC has been described in *R. tropici*. We show that *bcal1281* from *B. cenocepacia* encodes the OlsB *N*-acyltransferase 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

dx.doi.org/10.1021/bi200706v |*Biochemistry* 2011, 50, 6396–6408

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.

Funding Sources

N.G.-S. was supported during his Ph.D. studies (Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México) by a scholarship from the Consejo Nacional de Ciencia y Tecnología (Mexico). This work was supported by grants from DGAPA/UNAM (200806 and 218009) and the Consejo Nacional de Ciencia y Tecnología de México (CONACyT 82614). The York group gratefully acknowledges A.M.T.'s studentship provided by the Royal Society of Chemistry (RSC) and the Engineering and Physical Sciences Research Council (EPSRC) and D.S.'s studentship supported by the EPSRC and the European Union (ICA4-CT-2001-10056), and J.T.-O. gratefully acknowledges funding from the Analytical Chemistry Trust Fund, the RSC Analytical Division, and EPSRC.

■ ACKNOWLEDGMENT

This article is dedicated to Franz Lingens on the occasion of his 86th birthday. We thank Alhondra Solares-Pérez for help in the construction of OlsD-deficient mutant AQ3.

■ 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.

■ REFERENCES

(1) Mahenthiralingam, E., Urban, T. A., and Goldberg, J. B. (2005) The multifarious, multireplicon *Burkholderia cepacia* complex. *Nat. Rev. Microbiol. 3*, 144–156.

(2) 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.

(3) 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.

(4) Knoche, H. W., and Shively, J. M. (1972) The structure of an ornithine-containing lipid from *Thiobacillus thiooxidans*. *J. Biol. Chem. 247*, 170–178.

(5) Geiger, O., Röhrs, V., Weissenmayer, B., Finan, T. M., and Thomas-Oates, J. E. (1999) The regulator gene *phoB* mediates phosphate stress-controlled synthesis of the membrane lipid diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine in *Rhizobium (Sinorhizobium) meliloti*. *Mol. Microbiol. 32*, 63–73.

(6) Gao, J.-L., Weissenmayer, B., Taylor, A. M., Thomas-Oates, J., López-Lara, I. M., and Geiger, O. (2004) Identification of a gene required for the formation of lyso-ornithine lipid, an intermediate in the biosynthesis of ornithine-containing lipids. *Mol. Microbiol. 53*, 1757–1770.

(7) Weissenmayer, B., Gao, J.-L., López-Lara, I. M., and Geiger, O. (2002) Identification of a gene required for the biosynthesis of ornithine-derived lipids. *Mol. Microbiol. 45*, 721–733.

(8) Gibbons, H. S., Lin, S., Cotter, R. J., and Raetz, C. R. (2000) Oxygen requirement for the biosynthesis of the *S*-2-hydroxymyristate moiety in *Salmonella typhimurium* lipid A. Function of LpxO, a new Fe^{2+}/α-ketoglutarate-dependent dioxygenase homologue. *J. Biol. Chem. 275*, 32940–32949.

(9) Gibbons, H. S., Reynolds, C. M., Guan, Z., and Raetz, C. R. H. (2008) An inner membrane dioxygenase that generates the 2-hydroxymyristate moiety of *Salmonella* lipid A. *Biochemistry 47*, 2814–2825.

(10) Rojas-Jiménez, K., Sohlenkamp, C., Geiger, O., Martínez-Romero, E., Werner, D., and Vinuesa, P. (2005) A CIC chloride channel homolog and ornithine-containing membrane lipids of *Rhizobium tropici* CIAT899 are involved in symbiotic efficiency and acid tolerance. *Mol. Plant-Microbe Interact. 18*, 1175–1185.

(11) 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.

(12) Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E., and Ausubel, F. M. (1982) Physical and genetic characterization of symbiotic

and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *J. Bacteriol. 149*, 114–122.

(13) Sohlenkamp, C., de Rudder, K. E. E., and Geiger, O. (2004) Phosphatidylethanolamine is not essential for growth of *Sinorhizobium meliloti* in complex culture media. *J. Bacteriol. 186*, 1667–1677.

(14) Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol. 166*, 557–580.

(15) Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in Gram-negative bacteria. *Nat. Biotechnol. 1*, 784–791.

(16) Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene 33*, 103–119.

(17) Ditta, G., Schmidhauser, T., Yacobson, E., Lu, P., Liang, X.-W., Finlay, D. R., Guiney, D., and Helinski, D. R. (1985) Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring expression. *Plasmid 13*, 149–153.

(18) Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M., II, and Peterson, K. M. (1994) pBBR1MCS: A broad-host-range cloning vector. *BioTechniques 16*, 800–802.

(19) Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II, and Peterson, K. M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene 166*, 175–176.

(20) Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol. 185*, 60–89.

(21) Seed, B. (1987) An LFA-3 cDNA encodes a phospholipids-linked membrane protein homologous to its receptor CD2. *Nature 329*, 840–842.

(22) 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.

(23) Beringer, J. E. (1974) R factor transfer in *Rhizobium leguminosarum*. *J. Gen. Microbiol. 84*, 188–198.

(24) Sherwood, M. T. (1970) Improved synthetic medium for the growth of *Rhizobium*. *J. Appl. Bacteriol. 33*, 708–713.

(25) Miller, J. H. (1972) *Experiments in molecular genetics*, Cold Spring Harbor Laboratory Press, Plainview, NY.

(26) Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.

(27) Bligh, E. G., and Dyer, J. W. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol. 37*, 911–917.

(28) de Rudder, K. E. E., Thomas-Oates, J. E., and Geiger, O. (1997) *Rhizobium meliloti* mutants deficient in phospholipid *N*-methyltransferase still contain phosphatidylcholine. *J. Bacteriol. 179*, 6921–6928.

(29) Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem. 150*, 76–85.

(30) Holden, M. T. G., Seth-Smith, H. M. B., Crossman, L. C., Sebaihia, M., Bentley, S. D., Cerdeno-Tarraga, A. M., Thomson, N. R., Bason, N., Quail, M. A., Sharp, S., Cherevach, I., Churcher, C., Goodhead, I., Hauser, H., Holroyd, N., Mungall, K., Scott, P., Walker, D., White, B., Rose, H., Iversen, P., Mil-Homens, D., Rocha, E. P. C., Fialho, A. M., Baldwin, A., Dowson, C., Barrell, B. G., Govan, J. R., Vandamme, P., Hart, C. A., Mahenthiralingam, E., and Parkhill, J. (2009) The genome of *Burkholderia cenocepacia* J2315, an epidemic pathogen of cystic fibrosis patients. *J. Bacteriol. 191*, 261–277.

(31) Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev. 67*, 593–656.

(32) Tomer, K. B., Crow, F. W., Knoche, H. W., and Gross, M. L. (1983) Fast atom bombardment and mass spectrometry/mass spectrometry for analysis of a mixture of ornithine-containing lipids from *Thiobacillus thiooxidans*. *Anal. Chem. 55*, 1033–1036.

(33) Zhang, X., Ferguson-Miller, S. M., and Reid, G. E. (2009) Characterization of ornithine and glutamine lipids extracted from cell membranes of *Rhodobacter sphaeroides*. *J. Am. Soc. Mass Spectrom. 20*, 198–212.

(34) Dees, C., and Shively, J. M. (1982) Localization and quantitation of the ornithine lipid of *Thiobacillus thiooxidans*. *J. Bacteriol. 149*, 798–799.

(35) Geiger, O., González-Silva, N., López-Lara, I. M., and Sohlenkamp, C. (2010) Amino acid-containing membrane lipids in bacteria. *Prog. Lipid Res. 49*, 46–60.

(36) López-Lara, I. M., Gao, J. L., Soto, M. J., Solares-Pérez, A., Weissenmayer, B., Sohlenkamp, C., Verroios, G. P., Thomas-Oates, J., and Geiger, O. (2005) Phosphorus-free membrane lipids of *Sinorhizobium meliloti* are not required for the symbiosis with alfalfa but contribute to increased cell yields under phosphorus-limiting conditions of growth. *Mol. Plant-Microbe Interact. 18*, 973–982.

(37) Aygun-Sunar, S., Mandaci, S., Koch, H.-G., Murria, I. V. J., Goldfine, H., and Daldal, F. (2006) Ornithine lipid is required for optimal steady-state amounts of *c*-type cytochromes in *Rhodobacter capsulatus*. *Mol. Microbiol. 61*, 418–435.

(38) Drevinek, P., Holden, M. T. G., Ge, Z., Jones, A. M., Ketchell, I., Gill, R. T., and Mahenthiralingam, E. (2008) Gene expression changes linked to antimicrobial resistance, oxidative stress, iron depletion and retained mobility are observed when *Burkholderia cenocepacia* grows in cystic fibrosis sputum. *BMC Infect. Dis. 8*, 121.

(39) Peeters, E., Sass, A., Mahenthiralingam, E., Nelis, H., and Coenye, T. (2010) Transcriptional response of *Burkholderia cenocepacia* J2315 sessile cells to treatments with high doses of hydrogen peroxide and sodium hypochlorite. *BMC Genomics 11*, 90.

(40) Yoder-Himes, D. R., Konstantinidis, K. T., and Tiedje, J. M. (2010) Identification of potential therapeutic targets for *Burkholderia cenocepacia* by comparative transcriptomics. *PLoS One 5*, e8724.

(41) Kawai, Y., Yano, I., and Kaneda, K. (1988) Various kinds of lipoamino acids including a novel serine-containing lipid in an opportunistic pathogen *Flavobacterium*. Their structures and biological activities on erythrocytes. *Eur. J. Biochem. 171*, 73–80.

(42) Asselineau, J. (1991) Bacterial lipids containing amino acids or peptides linked by amide bonds. *Fortschr. Chem. Org. Naturst. 56*, 1–85.

(43) Tahara, Y., Kameda, M., Yamada, Y., and Kondo, K. (1976) An ornithine-containing lipid isolated from *Gluconobacter cerinus*. *Biochim. Biophys. Acta 450*, 225–230.

(44) Galbraith, L., Jonsson, M. H., Rudhe, L. C., and Wilkinson, S. G. (1999) Lipids and fatty acids of *Burkholderia* and *Ralstonia* species. *FEMS Microbiol. Lett. 173*, 359–364.

(45) Silipo, A., Molinaro, A., Ierano, T., de Soyza, A., Sturiale, L., Garozzo, D., Aldridge, C., Corris, P. A., Khan, C. M. A., Lanzetta, R., and Parrilli, M. (2007) The complete structure and pro-inflammatory activity of the lipooligosaccharide of the highly epidemic and virulent Gram-negative bacterium *Burkholderia cenocepacia* ET-12 (strain J2315). *Chem.—Eur. J. 13*, 3501–3511.

(46) Chung, H. S., and Raetz, C. R. H. (2010) Dioxygenases in *Burkholderia ambifaria* and *Yersinina pestis* that hydroxylate the outer Kdo unit of lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A. 108*, 510–515.

(47) Ernst, R. K., Yi, E. C., Guo, L., Lim, K. B., Burns, J. K., Hackett, M., and Miller, S. I. (1999) Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science 286*, 1561–1565.

(48) Aygun-Sunar, S., Bilaloglu, R., Goldfine, H., and Daldal, F. (2007) *Rhodobacter capsulatus* OlsA is a bifunctional enzyme active in both ornithine lipid and phosphatidic acid biosynthesis. *J. Bacteriol. 189*, 8564–8574.

(49) Wyckoff, T. J., Lin, S., Cotter, R. J., Dotson, G. D., and Raetz, C. R. (1999) Hydrocarbon rulers in UDP-*N*-acetylglucosamine acyltransferases. *J. Biol. Chem. 273*, 32369–32372.

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

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

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).

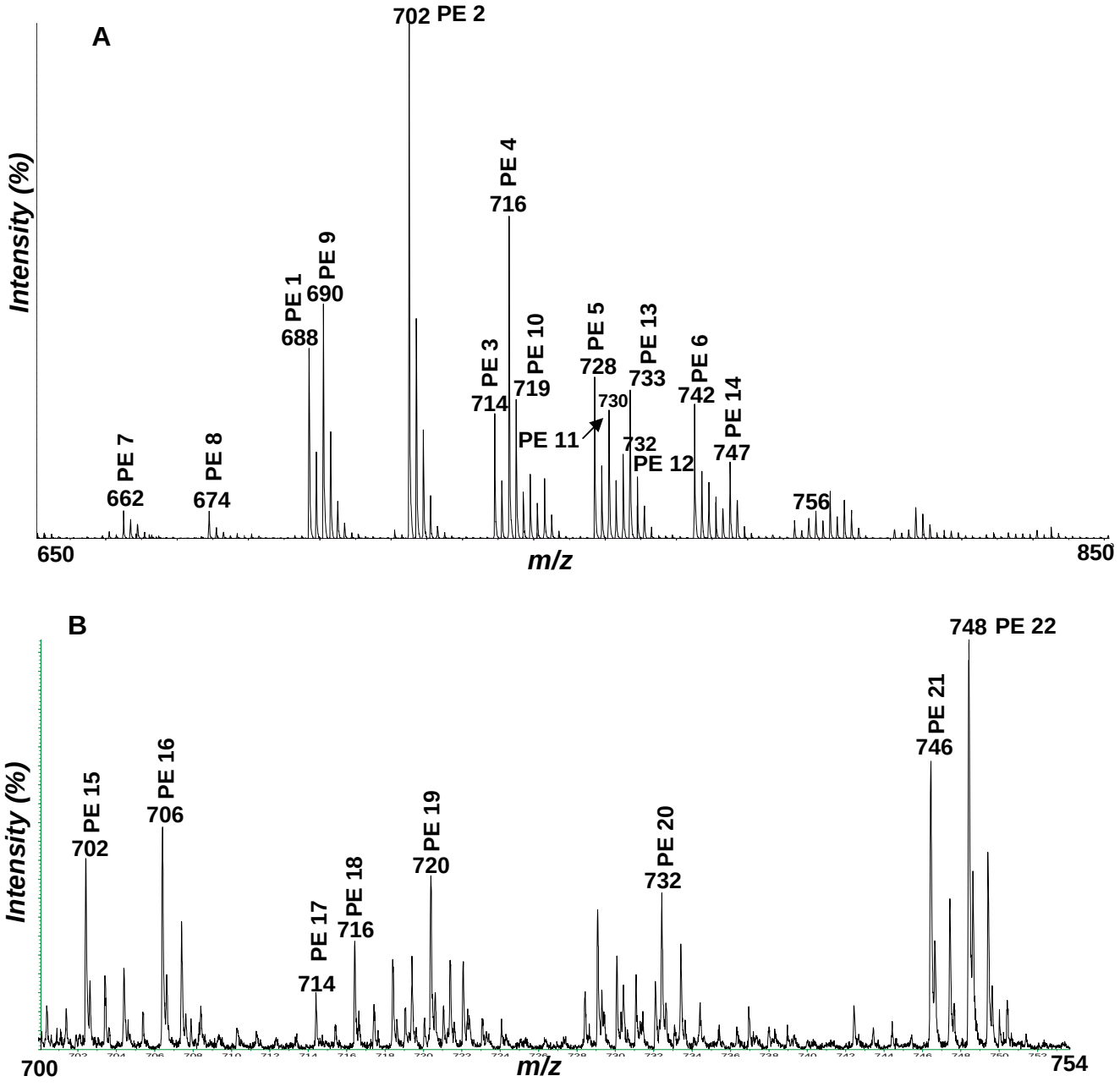


Figure S1 continued:

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.

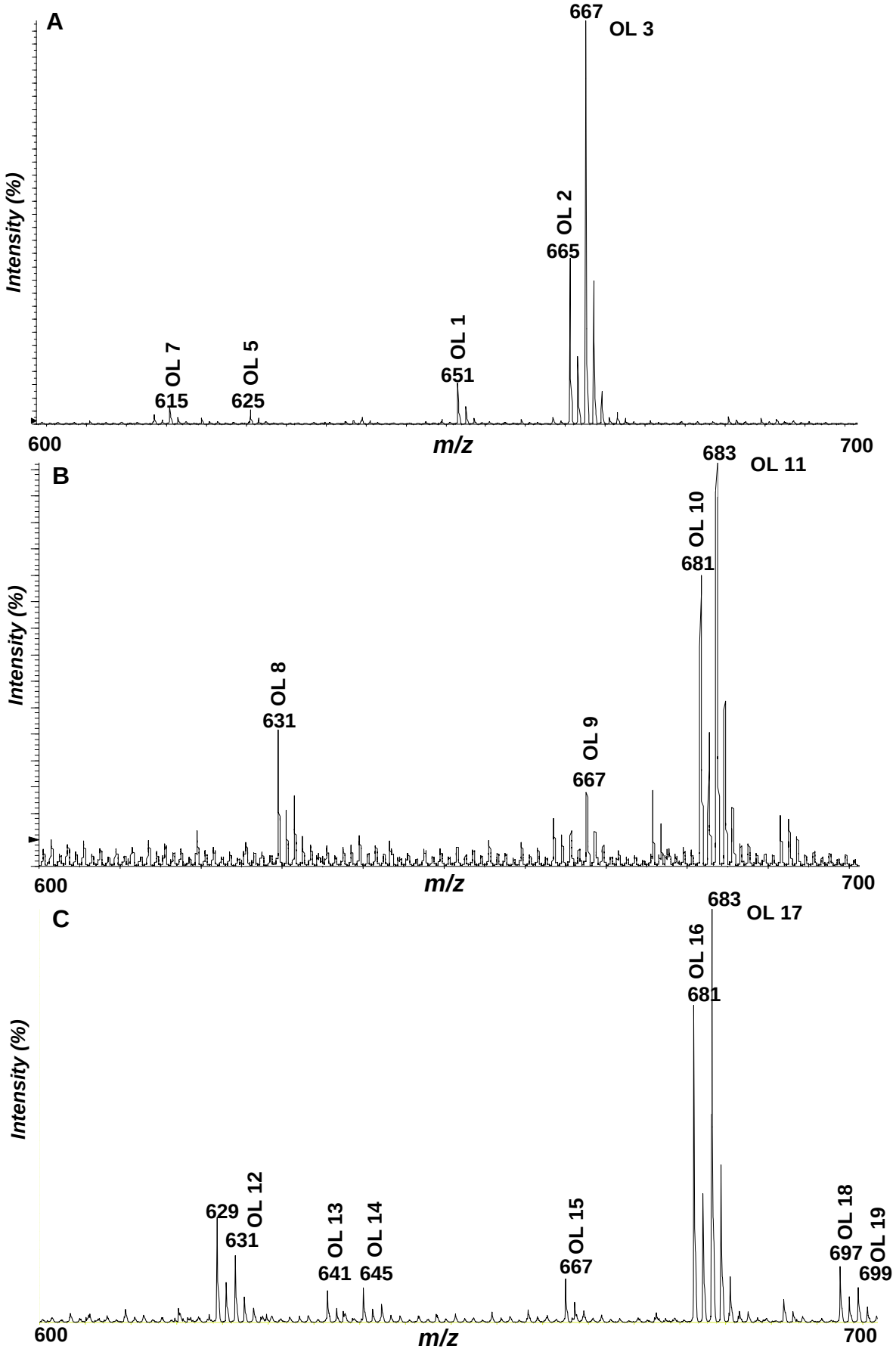


Figure S3. (A) Electrospray mass spectrum of the total lipids from *Sinorhizobium meliloti* x pSphx04 that contains the *olsD* gene from *Burkholderia cenocepacia* J2315. Peak intensity is normalized to the most intense signal in the region displayed. (B) Positive ion mode CID spectrum of the hydroxylated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

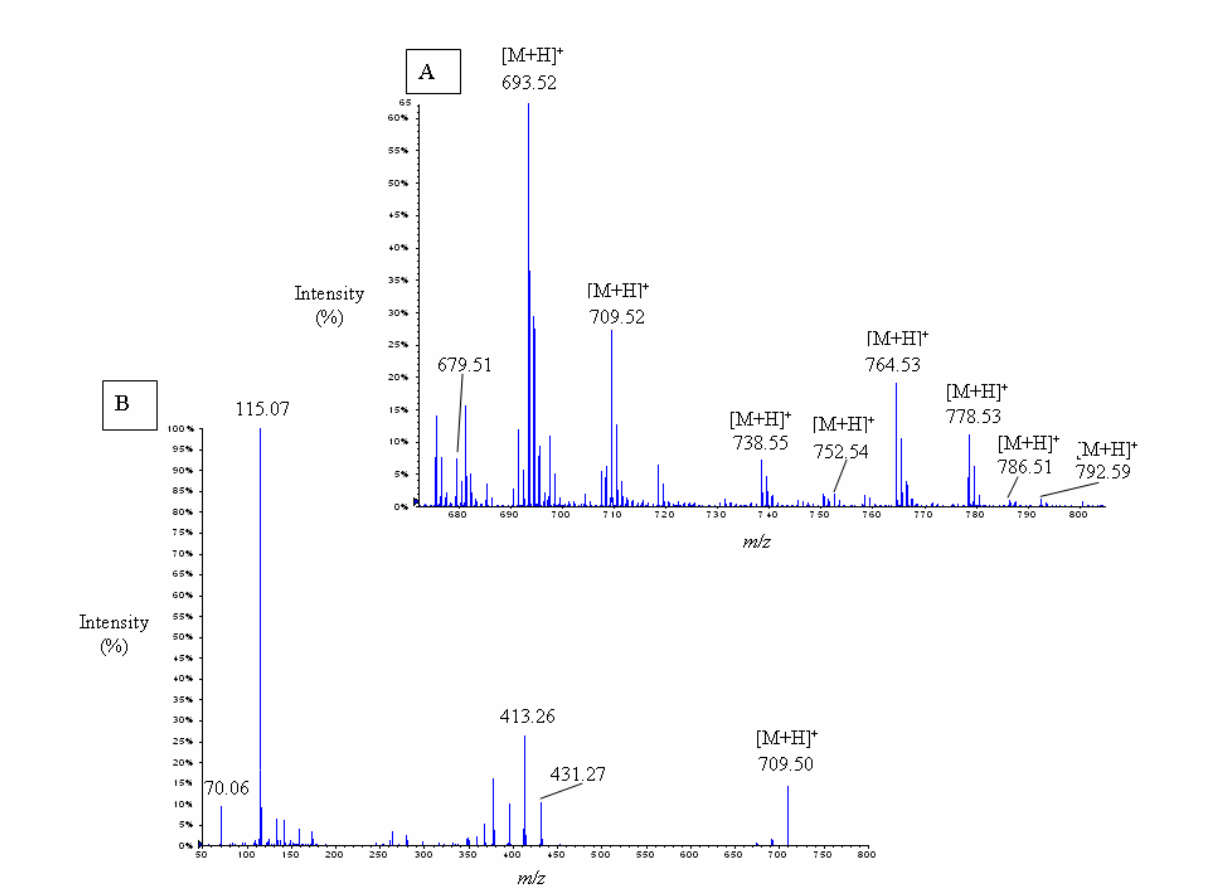
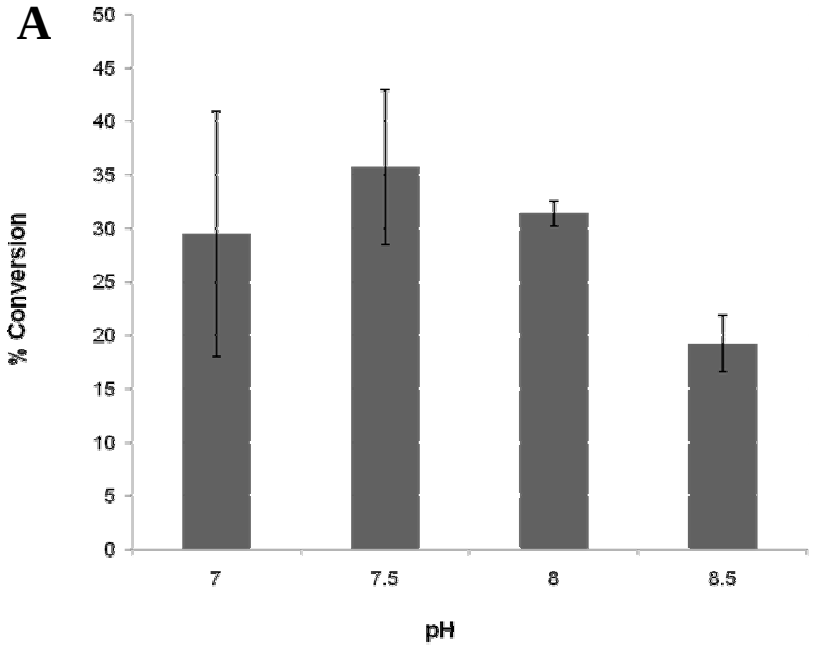


Figure S4. pH and detergent dependence of OlsD activity. (A) OlsD-catalyzed conversion of OL to its hydroxylated derivative *N*-acyl-OH-OL was monitored after incubation in 50 mM Hepes/KOH buffer at the pH values 7.0, 7.5, 8.0, and 8.5. (B) OlsD-catalyzed conversion of OL to its hydroxylated derivative *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.



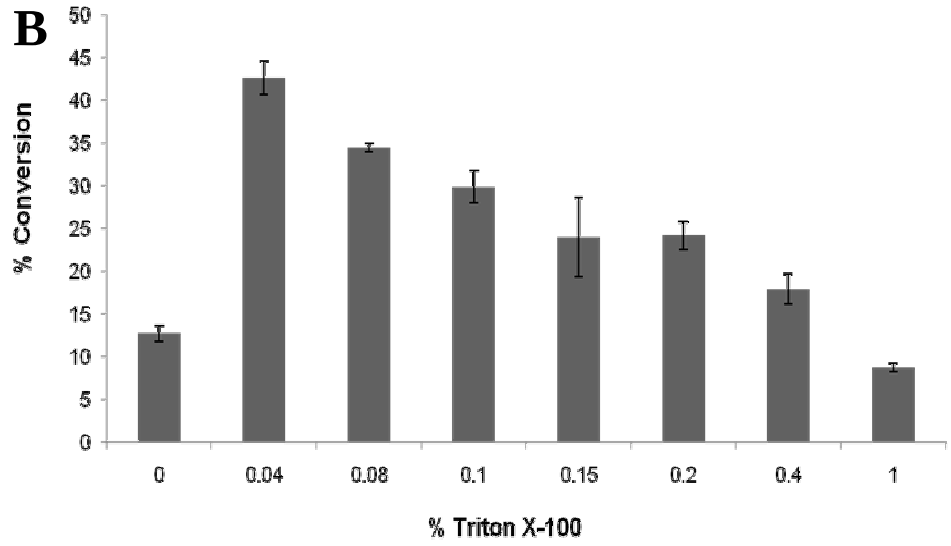
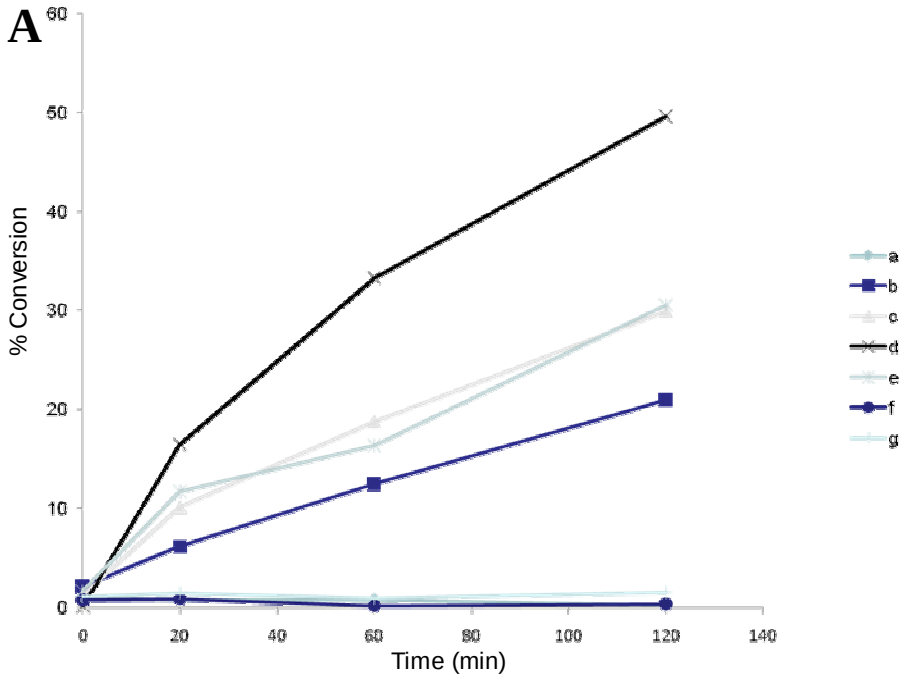


Figure S5. Dependence of OlsD activity on soluble cofactors. (A) A standard OlsD assay mixture containing 1 mg/mL of protein was prepared in which either α-ketoglutarate (a), ascorbate (b), iron (c), or dithiothreitol (d) was omitted. The complete assay (e) is described in Experimental Procedures and 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.



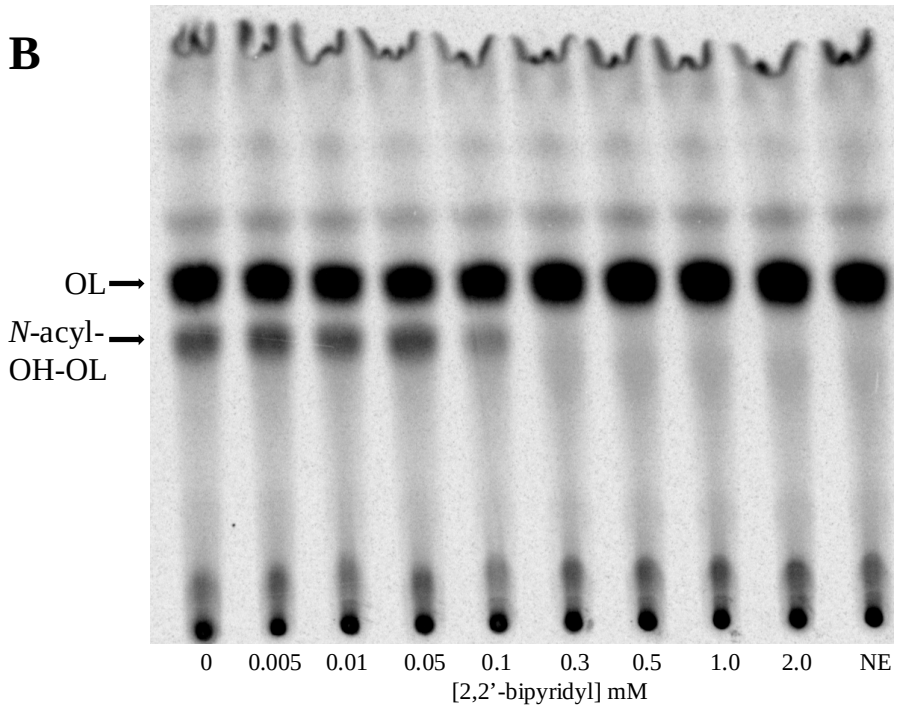
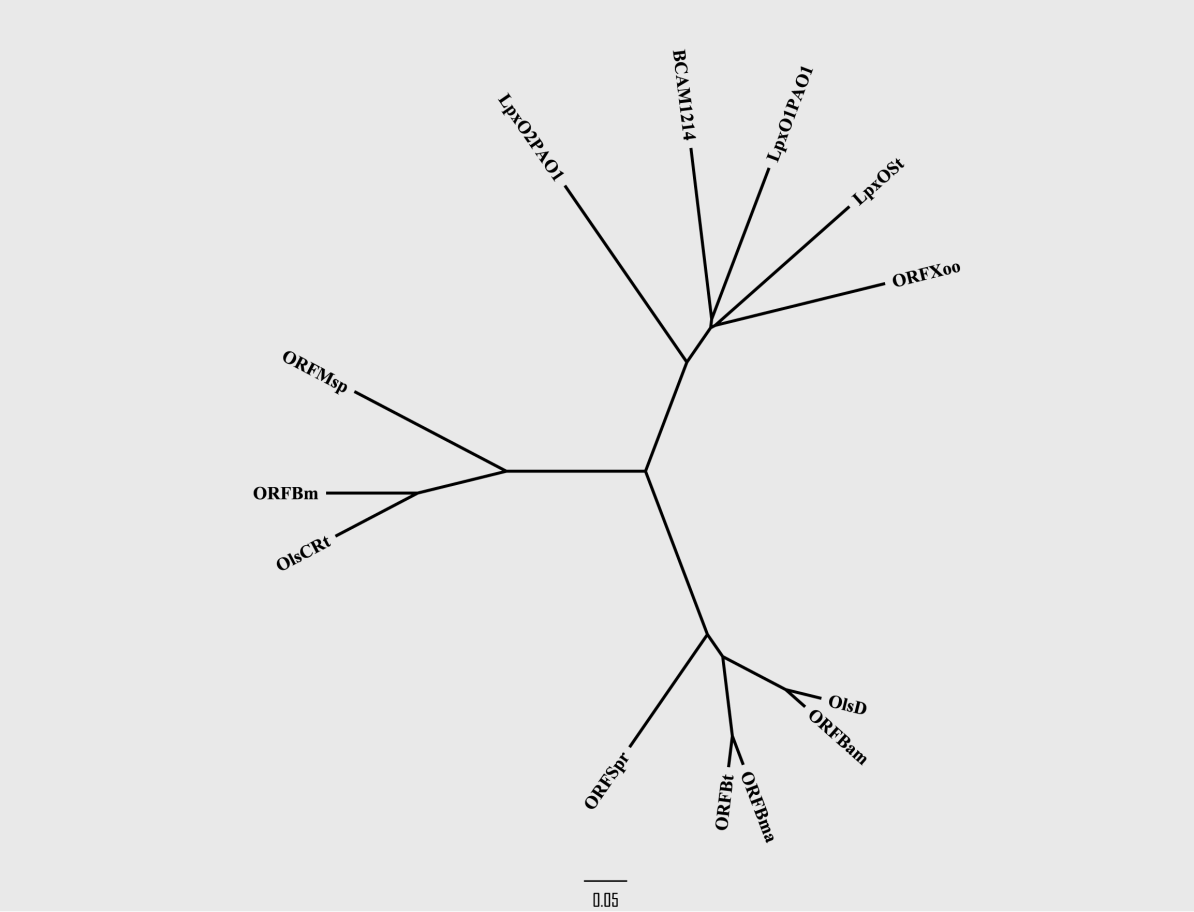


Figure S6. Unrooted phylogenetic tree of LpxO-like bacterial dioxygenases. The tree was constructed using the program CLUSTAL W (http://www.expasy.ch/). Distances between the sequences are expressed as 10 changes per amino acid residue. Accession numbers and locus tags are as follows: ORFBm (*Brucella 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).



8.2. Resultados adicionales

Tabla 2. Cepas y plásmidos, y sus características relevantes. En la sección de procedimientos experimentales de resultados adicionales se describe detalladamente su construcción.

Capas	**Características relevantes**	**Fuente o referencia**
S. meliloti 1021	str-21	Meade et. al., 1982
S. meliloti AAK1	*olsB*::*kan*	Gao et. al., 2004
S. meliloti CS111	*pssA*::*gm*	Sohlenkamp et. al., 2004
S. meliloti NG8	*Smc02490*::*cat*	Este trabajo
Rhizobium tropici 899-*olsCΔ1*	Tiene una deleción no polar de 211 bp en el gen *olsC*	Rojas-Jiménez et al., 2005
B. cenocepacia J2315	Wild type	LMG Bacteria Collection
B. cenocepacia AQ3	*olsD*::*cat*	González-Silva et al., 2011
B. cenocepacia NG1	*olsB*::*cat*	González-Silva et al., 2011
B. cenocepacia NG4	*bcam1214*::*cat*	Este trabajo
E. coli		
JM110	*rpsL* (*Str*^{r}) *thr leu thi-l lacY galK galT ara tonA tsx dam dmc supE44* Δ(*lac-proAB*) *F'*[*traD36proAB+ lacI*^{q} *lacZiΔM15*], cepa deficinete en las metilasas de DNA Dam y Dcm	Yanisch-Perron et al., 1985
DH5α	*rec*A1, Φ80 *lac*ZΔ*M15*, cepa usada para clonar	Hanahan, 1983
S17-1	Contiene el plásmido RP4 modificado integrado en su genoma	Simon et al., 1983
BL21 (DE3)	F^{-} *dcm ompT hsdS*(r_B^{-} m_B^{-}) *gal* λ(DE3), cepa de expresión	Studier et al., 1990
pLysS	Contiene un gen que codifica para la lisozima T7, inhibidor de ARN polimerasa T7, Cm^{R}	Studier et al., 1990
Plásmidos		
pET9a	Vector de expresión, Kn^{R}	Studier et al., 1990
pET16b	Vector de expresión, Ap^{R}	Studier et al., 1990
pET17b	Vector de expresión, Ap^{R}	Seed, 1987
pET3d	Vector de expresión, Ap^{R}	Novagen

pUC18	Vector de clonación, Ap^R	Yanisch-Perron et al., 1985
pBluescriptSK+	Vector de clonación, Ap^R	Stratagene
pCR®2.1-TOPO	Vector de clonación, Ap^R Kn^R	Invitrogen
pBBR1MCS	Vector de amplio rango de hospedero, Cm^R	Kovach et al., 1994
pBBR1MCS-5	Plásmido de amplio rango de hospedero, Gm^R	Kovach et al., 1995
pRK404	Vector de amplio rango de hospedero, Tc^R	Ditta et. al., 1985
pK18mob*sacB*	Vector suicida, Kn^R	Schäfer et. al., 1994
pIML21	*acpP* de *S. meliloti* clonado en pRK404 como *Bam*HI-*Bgl*II, Tc^R	Isabel M. López Lara
pJG21	*olsB* de *S. meliloti* clonado en pRK404, Kn^R Tc^R	Gao et al,.2004
pILAS03	*olsB* de *S. meliloti* clonado en pBBR1MCS-5	González-Silva et al., 2011
pERMAV1	*olsB* de *R. tropici* clonado en pET9a como *Nde*I-*Bam*HI, Kn^R	Miguel A. Vences Guzmán
pERMAV32	pERMAV1 clonado en pRK404 como *Bam*HI, Kn^R Tc^R	Miguel A. Vences Guzmán
pCCS98	*olsC* corto de *R. tropici* clonado en pET9a como *Nde*I-*Bam*HI, Kn^R	Christian Sohlenkamp
pCCS82	*olsC* largo de *R. tropici* clonado en pET9a como *Nde*I-*Bam*HI, Kn^R	Christian Sohlenkamp
pNG12	F1 de *bcam1214* clonado en pBluescriptSK+ como *Eco*RV-*Bam*HI, Ap^R	Este trabajo
pNG18	*bcam2775* clonado en pET9a como *Nde*I-*Bgl*II, Kn^R	Este trabajo
pNG20	pNG18 clonado en pRK404 como *Bgl*II, Kn^R Tc^R	Este trabajo
pNG23	$olsB_{Bc}$ clonado en pET17b, Ap^R	Vences-Guzmán et al., 2011
pNG24	pNG23 clonado en pRK404 como *Bgl*II, Ap^R Tc^R	González-Silva et al., 2011
pNG25	$olsB_{Bc}$ clonado en pBBR1MCS, Cm^R	Vences-Guzmán et al., 2011
pNG26	*bcal0511* clonado en pET9a, Kn^R	Este trabajo
pNG27	pNG26 clonado en pRK404 como *Bam*HI, Kn^R Tc^R	Este trabajo
pNG28	pET17b clonado en pRK404 como *Bam*HI, Ap^R Tc^R	González-Silva et al., 2011
pNG29	$olsC_{corto}$ clonado en pNG23 como *Bam*HI-*Bgl*II, Ap^R	Este trabajo
pNG30	$olsC_{largo}$ clonado en pNG23 como *Bam*HI-*Bgl*II, Ap^R	Este trabajo
pNG31	pNG29 clonado en pRK404 como *Bam*HI, Ap^R Tc^R	Este trabajo
pNG32	pNG30 clonado en pRK404 como *Bam*HI, Ap^R Tc^R	Este trabajo
pNG33	$olsB_{Sm}$ clonado en pIML21 como *Bam*HI-*Bgl*II, Tc^R	Este trabajo

pNG39	pET3d clonado en pRK404 como *Bam*HI, Ap^R Tc^R	Este trabajo
pNG40	*olsD* clonado en pET16b, Ap^R	González-Silva et al., 2011
pNG42	$olsC_{Rt\ corto}$ clonado en pET16b como *Nde*I-*Bam*HI, Ap^R	Este trabajo
pNG43	pNG42 clonado en pRK404 como *Bam*HI, Ap^R Tc^R	Este trabajo
pNG45	F2 de *smc02490* clonado en pBluescriptSK+ como *Bam*HI-*Xba*I, Ap^R	Este trabajo
pNG49	F2 de *bcam1214* clonado en pUC18 como *Bam*HI-*Xba*I, Ap^R	Este trabajo
pNG50	F1 de *smc02490* clonado en pCR®2.1-TOPO®, Ap^R Kn^R	Este trabajo
pNG51	F2 de *bcam1214* clonado en pNG12 como *Bam*HI-*Xba*I, Ap^R	Este trabajo
pNG52	F1 de *smc02490* clonado en pNG45 como *Eco*RV-*Bam*HI, Ap^R	Este trabajo
pNG53	*cat* clonado en pNG51 como *Bam*HI, Ap^R Cm^R	Este trabajo
pNG54	*cat* clonado en pNG52 como *Bam*HI, Ap^R Cm^R	Este trabajo
pNG55	F1 de *bcam1214*+ *cat* + F2 de *bcam1214* clonado en pK18mob*sacB* como *Eco*RV-*Xba*I, Kn^R Cm^R	Este trabajo
pNG56	F1 de *Smc02490* + *cat* + F2 de *Smc02490* clonado en pK18mob*sacB* como *Eco*RV-*Xba*I, Kn^R Cm^R	Este trabajo

a: Kn^R resistencia a kanamicina, Tc^R resistencia a tetraciclina, Cm^R resistencia a cloranfenicol, Ap^R resistencia a ampicilina, Gm^R resistencia a gentamicina, F1 y F2, región rio arriba y rio abajo de un gen, respectivamente.

8.2.1. Los lisolípidos (LOLs) derivados de los OLs modificados por OlsD tienen menor movilidad en TLC con respecto a los LOLs derivados de OLs no modificados

De acuerdo a los datos de espectrometría de masas, OlsD modifica con un grupo hidroxilo el ácido graso amidificado a la ornitina de los OLs. Para confirmar este resultado se analizó la movilidad en TLC de los OLs no modificados y modificados por OlsD, así como la de sus respectivos LOLs generados después de deacilarlos químicamente por un tratamiento alcalino suave. Se observó que los OLs modificados tienen menor movilidad en TLC posiblemente debido a que son más polares porque presumiblemente poseen un grupo hidroxilo adicional respecto a los OLs no modificados (fig. 11). También se observó que los LOLs derivados de los OLs modificados por OlsD tienen una menor movilidad en la TLC respecto a los LOLs derivados de los OLs no modificados (fig. 11). El resultado anterior sugiere que el grupo hidroxilo adicional que supuestamente poseen los OLs modificados se

localiza en el ácido graso amidificado o en el residuo de ornitina. Por lo tanto, este resultado es consistente con los datos obtenidos por el análisis de espectrometría de masas de los OLs no modificados y modificados que sugieren que OlsD modifica con un grupo hidroxilo el ácido graso amidificado a la ornitina de los OLs.

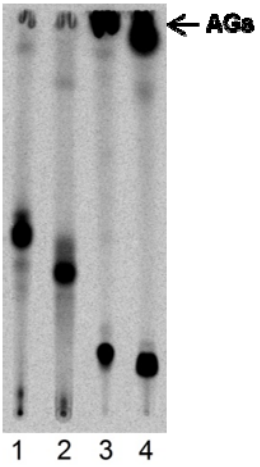


Figura. 11. Separación por TLC de lípidos de ornitina no modificados y modificados por OlsD, así como sus respectivos lisolípidos (LOLs). OLs no modificados (línea 1), OLs modificados por OlsD (línea 2), LOLs derivados de OLs no modificados (línea 3) y LOLs derivados de los OLs modificados por OlsD (línea 4). Ácidos grasos (AGs).

8.2.2. OlsD también modifica in vitro a los 2-OH-OL

Al realizar el ensayo enzimático con OlsD usando los [^{14}C]2-OH-OLs como sustrato se observó que OlsD también es capaz de convertir 33 % del sustrato al producto NL2 (fig. 12). Lo que sugiere que esta enzima tiene la flexibilidad para reconocer como sustratos a OLs hidroxilados en el C2 de su acilo esterificado. El uso de 2-OH-OLs como sustrato también se observó in vivo [González-Silva et al., 2011].

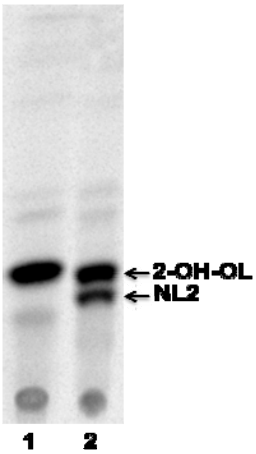


Figura 12. Modificación in vitro de [^{14}C]2-OH-OL por OlsD. 2-OH-OL incubado con: extractos crudos libres de células de *E. coli* BL21 (DE3) x pLysS x pET16b (vector vacío) (línea 1), extractos crudos libres de células de *E. coli* BL21 (DE3) x pLysS x pNG40 (línea 2). 2-OH-OL y NL2 se indican.

8.2.3. La mutante en *bcam1214* derivada de *B. cenocepacia* J2315 aún forma los derivados 2-hidroxilados de los OLs y PE

El análisis de hibridación tipo Southern de varias colonias candidatas a mutantes en el gen *bcam1214* mostró que *bcam1214* se había remplazado en su mayor parte por el gen *cat* (fig. 13), como se esperaba que ocurriera. Lo anterior quedó demostrado debido a que la sonda hibridó en las candidatas a mutantes con un fragmento de 2130 pb, como se esperaba. Aunque también hibridó en las candidatas a mutantes con un fragmento de alrededor de 6.5 kb, que no se esperaba. En la capa silvestre la sonda hibridó solamente con un fragmento de 1654 pb, como se esperaba. Lo anterior sugiere que la construcción que se uso para mutar a *bcam1214* provocó rearreglos en los genomas de las células en las cuales se introdujo. Probablemente por eso la sonda hibridó en las mutantes con un fragmento de alrededor de 6.5 kb, que no esperaba que hibridara. Una mutante se seleccionó (se nombró NG4) para analizar su perfil de lípidos de membrana. El perfil de lípidos de membrana de la cepa NG4 (datos no mostrados) fue igual al de la cepa silvestre y aún forma los derivados 2-OH de los OLs y de PE. Lo anterior sugiere que *bcam1214* no está involucrado en modificar a los OLs ni a PE en *B. cenocepacia* J2315 como lo indicaban los datos de su expresión heteróloga. Posiblemente BCAM1214 esté involucrado en hidroxilar al lípido A de *B. cenocepacia* J2315 en ciertas condiciones de crecimiento o a alguna proteína de esa bacteria como lo hacen proteínas similares a LpxO como las aspartil/asparaginil β-hidroxilasas de mamíferos.

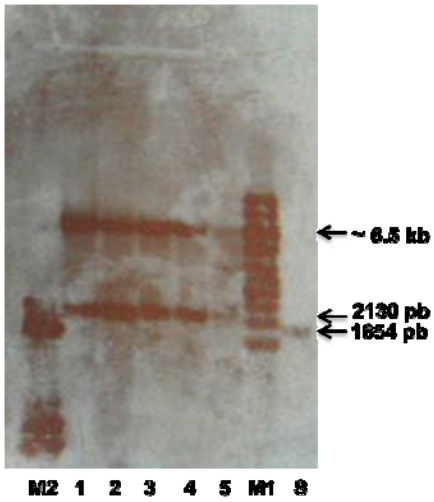


Figura 13. Perfil de hibridación tipo Southern de candidatas a mutantes en el gen *bcam1214*. Marcador molecular de 1 kilobase (kb) (Invitrogen) (línea M2), DNA genómico digerido con *Nco*I y *Pst*I de: candidata a mutante en *bcam1214* NG3 (línea 1), candidata a mutante en *bcam1214* NG4 (línea 2), candidata a mutante en *bcam1214* NG5 (línea 3), candidata a mutante en *bcam1214* NG6 (línea 4), candidata a mutante en *bcam1214* NG7 (línea 5), marcador molecular de 1 kb (Fermentas) (línea M1), cepa silvestre *B. cenocepacia* J2315 (línea S).

8.2.4. La expresión de los genes *bcal0511* y *bcam2775* de *B. cenocepacia* J2315 que codifican para productos homólogos a PHYH sugiere que estos genes no están involucrados en modificar a los OLs ni a PE

La enzima PHYH de humano cataliza la 2-hidroxilación del ácido fitánico proveniente del consumo de ciertos alimentos [Jansen et al., 1997; Jansen et al., 2000]. La secuencia de PHYH (338 aminoácidos) se obtuvo del banco de genes (geneBank), la cual está depositada con el número de acceso CAG46852. Posteriormente realizamos una búsqueda en el genoma de *B. cenocepacia* J2315 (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/b_cenocepacia) utilizando la secuencia de PHYH. En el genoma de *B. cenocepacia* J2315 encontramos dos ORFs homólogos a PHYH: BCAL0511 (257 aminoácidos) con 26 % de identidad, 42 % de similitud y un valor E de 1.7×10^{-15} con respecto PHYH de humano y BCAM2775 (291 aminoácidos) con 28 % de identidad, 43 % de similitud con un traslape de 145 aminoácidos con PHYH de humano y un valor E de 2.5×10^{-05}.

El gen *bcal0511* se localiza en el cromosoma 1 y *bcam2775* en el cromosoma 2. Los productos de ambos genes están anotados como dioxigenasas putativas. Para conocer si uno de estos productos tenía la capacidad de modificar a PE, los plásmidos pNG26 (contiene a *bcam2775*) y pNG18 (contiene a *bcal0511*) se introdujeron a la cepa de *E. coli* BL21 (DE3) x pLysS, se indujo su expresión con IPTG y se verificó en geles desnaturalizantes de poliacrilamida al 10 % (fig. 14). En el extracto total de proteínas de la cepa BL21 (DE3) x pLysS x pNG26 se observó una proteína con una abundancia relativa mayor a todas las demás proteínas de aproximadamente 29 kDa, que probablemente se trata de BCAL0511. Las predicciones indican que BCAL0511 tiene un tamaño de 28.7 kDa. También, en el extracto total de proteínas de la cepa BL21 (DE3) pLysS pNG18 se observó una proteína con una abundancia relativa mayor a todas las demás proteínas de aproximadamente 32 kDa, que probablemente se trata BCAM2775. Las predicciones indican que BCAM2775 tiene un tamaño de 32.6 kDa. El resultado anterior confirmó la expresión de ambas proteínas. Sin embargo, los perfiles lipídicos de los cultivos paralelos marcados con acetato de sodio [1-^{14}C] derivados de las cepas que contenían los plásmidos pNG26 y pNG18

fueron similares al perfil de la cepa que contenía el plásmido vacío (pET9a), en la que solamente se observó la presencia de PE, PG y CL (datos no mostrados). El resultado anterior sugiere que ninguna de las dos proteínas BCAL0511 ni BCAM2775 están involucradas en hidroxilar a PE de *E. coli*. Por eso se espera que BCAL0511 o BCAM2775 tampoco estén involucradas en la 2-hidroxilación de PE de *B. cenocepacia* J2315.

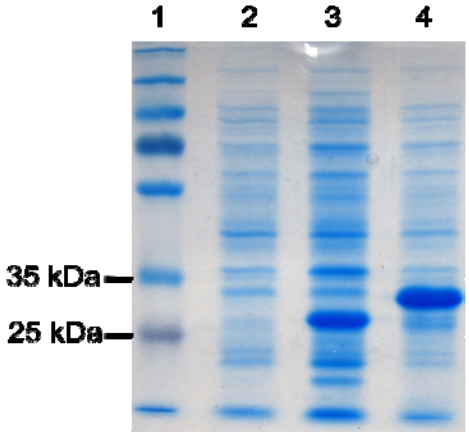


Fig. 14. Análisis de extractos crudos libres de células por SDS-PAGE. Marcador de tamaño molecular de proteínas (LabAid™, PageRuler™ Plus Prestained Protein Ladder, #SM1811) (línea 1). Extractos de *E. coli* BL21 (DE3) x pLysS x pET16b el vector de expresión vacío (línea 2), de *E. coli* BL21 (DE3) x pLysS x pNG26 expresa a BCAL0511 (línea 3), de *E. coli* BL21 (DE3) x pLysS x pNG18 expresa a BCAM2775 (línea 4). Las bandas más prominentes de los carriles 3 y 4 probablemente corresponden a las proteínas BCAL0511 y BCAM2775, respectivamente.

El plásmido pNG27 (contiene a *bcal0511*) se movilizó a las cepas de *S. meliloti* CS111 pNG25 (la cual, no forma PE ni sus derivados metilados y sobreproduce OL) y a *B. cenocepacia* J2315. La cepa CS111 x pNG25 x pNG27 mostró un perfil de lípidos de membrana similar a la cepa CS111 x pNG25. De igual manera la cepa *B. cenocepacia* J2315 x pNG27 (contiene a *bcam2775*) tuvo un perfil cualitativo y cuantitativo similar a la cepa *B. cenocepacia* J2315 pJG16 (datos no mostrados). El plásmido pNG20 (contiene *bcam2775*) se movilizó a la cepa *S. meliloti* 1021 x pILAS03 (sobreproduce OL) y a *B. cenocepacia* J2315. La cepa *S. meliloti* 1021 x pILAS03 x pNG20 mostró un perfil de lípidos de membrana similar a la cepa *S. meliloti* 1021 x pILAS03 x pJG16, de igual manera la cepa *B. cenocepacia* J2315 pNG20 tuvó un perfil cualitativo y cuantitativo similar a la cepa *B. cenocepacia* J2315 pJG16. Estos resultados sugieren que ninguno de los dos productos BCAL0511 ni BCAM2775 están involucrados en hidroxilar a los OLs ni a ningún otro lípido de membrana de las cepas derivadas de *S. meliloti* 1021 o de *B. cenocepacia* J2315. Si BCAL0511 o BCAM2775 estuvieran involucrados en modificar algún lípido se esperaba que

al introducir a pNG27 o a pNG20 en las cepas derivadas de *S. meliloti* 1021 se formara un nuevo lípido y al introducirlos en *B. cenocepacia* J2315 se esperaba que se incrementara considerablemente la producción de algún lípido de los preexistentes. Lo anterior se esperaba debido a que tenemos el antecedente de que al introducir un gen bajo la regulación del promotor T7 en *S. meliloti* 1021 o en *B. cenocepacia* J2315 es suficiente para que el gen se exprese constitutivamente.

8.2.5. Los genes *olsBs* de rizobiales no complementan a la mutante NG1 derivada de *B. cenocepacia* J2315

Los genes *olsB* de *S. meliloti* 1021 y de *R. tropici* CIAT899 no complementaron a la mutante NG1 (datos no mostrados). Lo anterior se demostró fehacientemente porque el pJG21 obtenido de la cepa NG1 si complementó a la mutante AAK1 (fig. 15). No sabemos porque los *olsB* rizobiales no complementaron a la mutante NG1. Nuestra hipótesis a comprobar fue que el gen $olsB_{Sm}$ no interacciona correctamente con la $AcpP_{Bc}$ involucrada en el primer paso de la síntesis de los OLs, por lo tanto, expresando a $olsB_{Sm}$ y $acpP_{Sm}$ posiblemente se sintetizarían los OLs en la mutante NG1. Para comprobar la hipótesis anterior los plásmidos pIML21 y pNG33 se movilizaron a las mutantes AAK1 y NG1, y se analizó su perfil de lípidos de membrana, la cepa AAK1 x pIML21 mostró un perfil de lípidos similar a la cepa AAK1, como se esperaba; y la cepa AAK1 x pNG33 formó OL también como se esperaba (datos no mostrados). Sin embargo, la cepa NG1 x pIML21 y NG1 x pNG33 no formaron OLs (datos no mostrados).

Los resultados anteriores sugieren que el gen *olsB* de *S. meliloti* 1021 no complementa a la mutante NG1 aún cuando se coexpresa con el $acpP_{Sm}$. Posiblemente $AcpP_{Sm}$ no se convirtió a la forma holo en la cepa NG1, para lo cual se requiere la actividad de $AcpS_{Sm}$ (enzima que transfiere el residuo 4-fosfopanteteína de coenzima A a la $AcpP_{Sm}$, para convertirla a su forma activa). Sin el grupo prostético la $AcpP_{Sm}$ no portará grupos acilos para ser transferidos por $OlsB_{Sm}$ a la ornitina y debido a eso posiblemente no se formó el lisolípido de ornitina y por consecuencia tampoco OL. En contraste, $olsB_{Bc}$ sí complementó a la cepa mutante AAK1 (fig. 15). Un fenómeno reportado en *S. meliloti* 1021 y en *B. cenocepacia* J2315 es que la introducción de sus respectivos *olsB* bajo la

regulación del promotor T7, promueve la sobreproducción de los OLs. Este fenómeno no se observó cuando se introdujeron los *olsB* rizobiales en la cepa *B. cenocepacia* J2315, pero sí cuando se introdujo el $olsB_{Bc}$ en la cepa AAK1. Lo que reafirma y es consistente con nuestros resultados de que los *olsB* rizobiales no son funcionales en *B. cenocepacia* J2315.

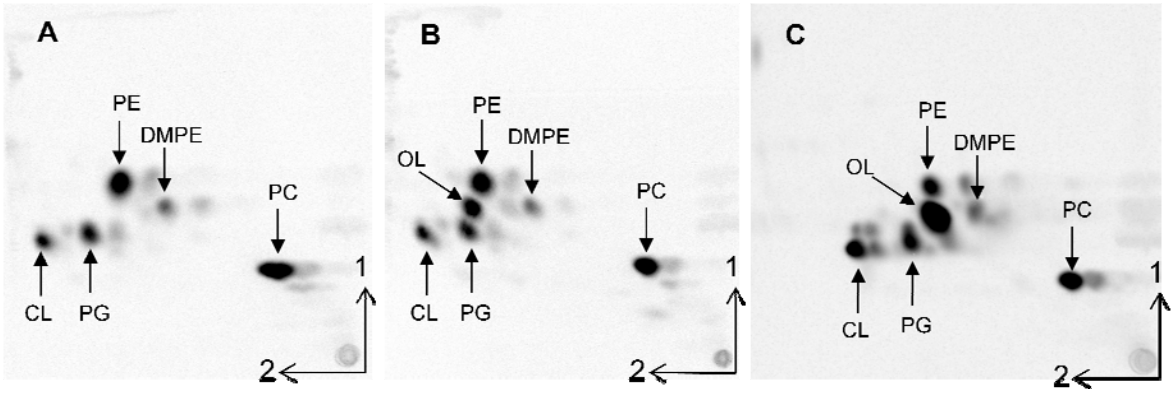


Figura 15. Separación de lípidos totales de membrana por 2D-TLC marcados con acetato de sodio [1-^{14}C] de cepas derivadas de la complementación con $olsB_{Sm}$ y $olsB_{Bc}$ de la mutante en *olsB* de *S. meliloti* 1021 (AAK1). (A) AAK1 x pJG16 el plásmido está vacío, (B) AAK1 x pJG21 el plásmido contiene al gen $olsB_{Sm}$, el plásmido pJG21 se extrajo de la cepa NG1 x pNG21 y (C) AAK1 x pNG24 el plásmido contiene al gen $olsB_{Bc}$. Los lípidos cardiolipina (CL), fosfatidilglicerol (PG), lípido de ornitina (OL), fosfatidiletanolamina (PE), dimetilfosfatidiletanolamina (DMPE) y fosfatidilcolina (PC) se indican.

8.2.6. Sensibilidad a antibióticos de cepas derivadas de *B. cenocepacia* J2315

B. cenocepacia J2315 es un patógeno oportunista resistente a muchos antibióticos y a altas concentraciones de ellos. Para saber sí los OLs están involucrados directa o indirectamente en el mecanismo de resistencia a antibióticos se probó la sensibilidad de las cepas J2315, AQ3, AQ3 pJG16, AQ3 pSphx4, NG1, NG1 pNG28 y NG1 pNG24 a 18 antibióticos con diferentes blancos de acción (tabla 1, pag. 48), entre ellos polimixina B y bacitracina que actúan sobre la membrana. La polimixina B es un péptido catiónico antimicrobiano que se une a los grupos cargados negativamente de las membranas de las bacterias, los agregados de péptidos en la membrana externa rompen la integridad de la membrana, lo que provoca la muerte celular [Lewenza et al., 2011]. La bacitracina es una

mezcla de polipéptidos cíclicos que interfiere con la desfosforilación del isoprenilo C_{55}-pirofosfato, una molécula que transporta los elementos estructurales del peptidoglicano en la membrana celular bacteriana, inhibiendo la formación de la pared celular de las bacterias [Stone y Strominger, 1971]. En el ensayo de sensibilidad a antibióticos no se observaron halos de inhibición, excepto en el ácido nalidíxico que inhibió el crecimiento uniformemente en todas las cepas probadas. Los halos de inhibición que se observaron fueron de pocos milímetros.

8.2.7. La coexpresión en la cepa CS111 de la versión corta de *olsC* y *olsB* de *B. cenocepacia* J2315 formaron un nuevo lípido

Para determinar cuál de las dos versiones (corta o larga) de *olsC* es funcional o si ambas lo son se clonaron las dos versiones independientemente en el plásmido pNG23 que contiene al gen *olsB* de *B. cenocepacia* J2315. Los plásmidos resultantes pNG29 y pNG30 se ligaron al pRK404 resultando los plásmidos pNG31 y pNG32, respectivamente, los cuales se movilizaron a la cepa CS111, así como el pNG24. La cepa CS111 no forma PE, MMPE ni DMPE. El gen *olsB* promoverá la sobreproducción de los OLs no modificados. Por lo tanto, si ambas versiones fueran funcionales se esperaba que las cepas CS111 x pNG31 y CS111 x pNG32 formaran adicionalmente OLs como los OLs P1. El perfil de lípidos de membrana de la cepa CS111 x pNG24 consistió de CL, PG, PC y una alta cantidad de OLs (fig. 16A), como se esperaba. El perfil de lípidos de la cepa CS111 x pNG32 (datos no mostrados) fue similar al perfil de la cepa CS111 pNG24, lo anterior sugiere que la versión larga de *olsC* no es funcional. En contraste, la cepa CS111 x pNG31 si formó un lípido adicional que tiene una movilidad en 2D-TLC similar a los OLs P1 de *R. tropici* CIAT899 (fig. 16B). Lo anterior sugiere que la versión corta de *olsC* sí es funcional. Desconocemos porque no fue funcional la versión larga de OlsC en la cepa CS111 x pNG32 pero posiblemente se debe a que esta versión se pliega mal y por lo tanto es degradada.

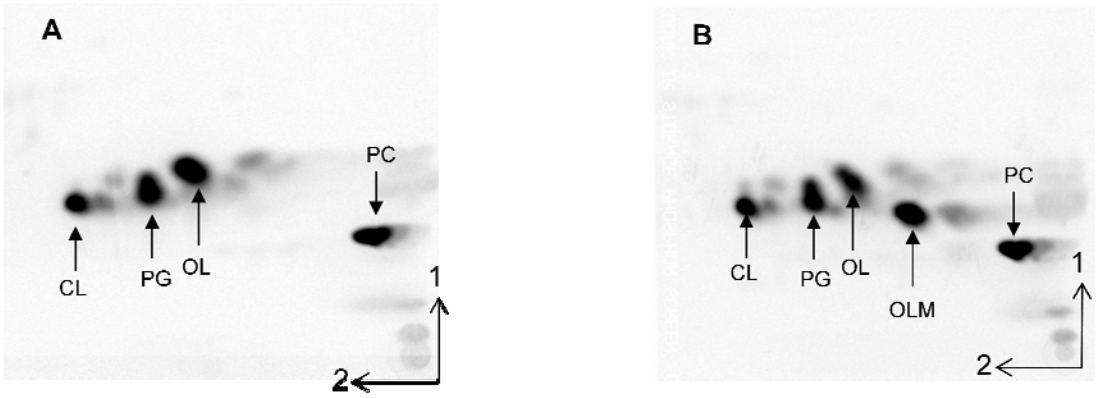


Figura 16. Separación de lípidos totales de membrana por 2D-TLC marcados con acetato de sodio [1-^{14}C] de cepas derivadas de la cepa CS111. (A) CS111 x pNG24 el plásmido contiene el gen *olsB*$_{Bc}$ y (B) CS111 x pNG31 el plásmido contiene el gen *olsB*$_{Bc}$ y la versión corta del gen *olsC* de *R. tropici* CIAT899. Los lípidos cardiolipina (CL), fosfatidilglicerol (PG), lípido de ornitina (OL), lípido de ornitina modificado dependiente de *olsCc* (OLM) y fosfatidilcolina (PC) se indican.

8.2.8. OlsC con cola de histidinas es funcional y aparentemente es una proteína soluble

Los genes que se clonan en el vector pET16b como *Nde*I/*Bam*HI producen proteínas con una cola de 10 histidinas adicionales en el extremo N-terminal. El gen *olsC* se clonó en el pET16b produciendo el plásmido pNG42, el cual subsecuentemente se ligó al pRK404, produciendo el plásmido pNG43. El pNG43 se introdujo en la mutante en *olsC* (899-*olsCΔ1*) derivada de *R. tropici* CIAT899 y después se analizó su perfil de lípidos de membrana. OlsC es la proteína reponsable de hidroxilar los dos OLs menos polares (S1 y S2) en *R. tropici* CIAT899 para convertirlos en otros dos más polares (P1 y P2), por lo tanto, una mutante en dicho gen no forma los OLs P1 ni P2 [Rojas-Jimenez et al., 2005; Vences-Guzmán et al., 2011]. Nosotros observamos que la mutante en *olsC* (899-*olsCΔ1*) con el plásmido pNG43 restauró la producción de los OLs P1 y P2. Eso sugiere que OlsC con la cola de histidinas es funcional (fig. 17).

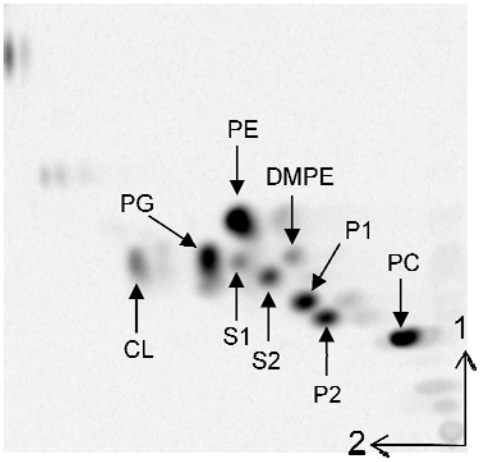


Figura 17. Separación de lípidos totales de membrana por 2D-TLC marcados con acetato de sodio [1-^{14}C] de la cepa 899-*olsCΔ1* x pNG43, el plásmido contiene el gen *olsC*. Los lípidos cardiolipina (CL), fosfatidilglicerol (PG), lípido de ornitina estándar (S1), lípido de ornitina hidroxilado en la ornitina (S2), lípido de ornitina hidroxilado en el acilo esterificado (P1), lípido de ornitina hidroxilado en la ornitina y en el acilo esterificado (P2), fosfatidiletanolamina (PE), dimetilfosfatidiletanolamina (DMPE) y fosfatidilcolina (PC) se indican.

Por otro lado, los extractos crudos líbres de células de las cepas BL21 (DE3) x pLysS x pET16b y BL21 (DE3) x pLysS x pNG42, así como las fracciones soluble e insoluble (membranas) provenientes de los extractos libres de células de la última cepa se utilizaron para hacer el ensayo enzimático. En el ensayo se observó un porcentaje de conversión mayor (57 %) cuando se usó la fracción de proteína soluble (fig. 18A). El porcentaje de conversión cuando se utilizaron extractos crudos y la fracción de membranas es de 38 % y 23 %, respectivamente. El resultado anterior sugiere que la proteína olsC es una proteína soluble que no está asociada a las membranas de *R. tropici* CIAT899, como también lo predice un análisis con el algoritmo TMHMM [Krogh et al., 2001] (fig. 18B).

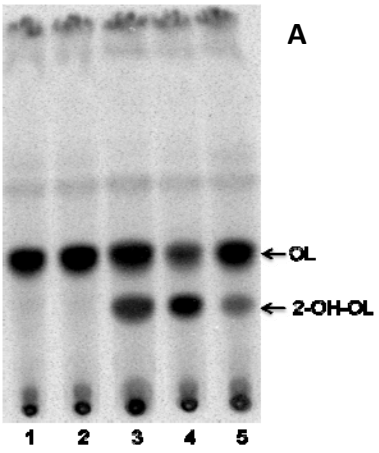
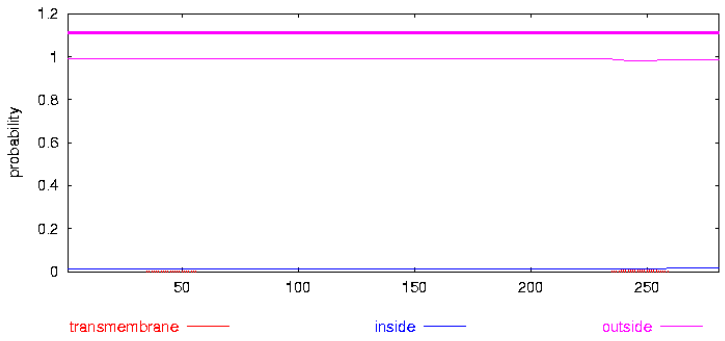


Figura 18. OlsC es una proteína soluble que introduce un grupo hidroxilo en el C2 del acilo esterificado de los OLs. A. OL incubado con extractos libre de células de: *E. coli* BL21 (DE3) x pLysS x pET16b (vector vacío) (línea 1), BL21 (DE3) x pLysS x pNG42 (expresa a OlsC) (línea 3), la fracción de proteína soluble de *E. coli* BL21 (DE3) x pLysS x pNG42 (línea 4), la fracción de membrana de *E. coli* BL21 (DE3) x pLysS x pNG42 (línea 5) y solamente bufer (línea 2), se incubaron con [^{14}C]OL a una concentración final de 169 µM por 2 h. Los lípidos de ornitina no modificados (OLs) y los OL 2-hidroxilados (2-OH-OLs) se indican. **B.** Predicción de hélices transmembranales de OlsC con el algoritmo TMHMM [Krogh et al., 2001].



8.2.9. Los homólogos a SMc02490 se encuentran fusionados con homólogos a OlsB en muchas bacterias

Recordemos que *olsB* y *smc02490* en algunas bacterias coexisten o se encuentran fusionados [Geiger et al., 2010]. En un análisis reciente la fusión de homólogos a OlsB con homólogos de SMc02490 se detectó en 50 especies de bacterias distribuidas en los géneros *Klebsiella* (3), *Dickeya* (3), *Pectobacterium* (3), *Cronobacter* (2), *Erwinia* (1), *Citrobacter* (1), *Serratia* (1), *Enterobacter* (1), *Cellvibrio* (1), *Shewanella* (13), *Alteromonas* (1), *Pseudoalteromonas* (1), *Teredinibacter* (1), *Idiomarina* (1), *Psychromonas* (1), *Stenotrophomonas* (2), *Thiomicrospira* (1), *Kangiella* (1), *Marinomonas* (1), *Aeromonas* (2), *Tolumonas* (1), *Desulfovibrio* (4), *Arcobacter* (1), *Sulfurospirillum* (1), *Magnetococcus* (1) y *Akkermansia* (1).

SMc02490 es similar a miembros de la superfamilia *N*-aciltransferasa (NAT). Los miembros esta superfamilia de enzimas catalizan la transferencia en su mayoría de un grupo acilo a un sustrato y están implicados en una variedad de funciones, que van desde la resistencia bacteriana a los antibióticos a los ritmos circadianos en los mamíferos. Los miembros incluyen *N*-acetiltransferasas relacionadas con GCN5 (GNAT), tales como las enzimas aminoglucósidos *N*-acetiltransferasas, histona *N*-acetiltransferasas, y la serotonina *N*-acetiltransferasa, que catalizan la transferencia de un grupo acetilo a un sustrato. Otros miembros de la superfamilia incluyen la arginina/ornitina *N*-succiniltransferasa, miristoil-CoA: proteína *N*-miristoiltransferasa, y la sintasa de acil homoserina lactona que tienen un mecanismo catalítico similar pero difieren en los tipos de grupos acilo transferidos.

Leucil/fenilalanil-tRNA-proteína transferasa y las peptidiltransferasas no ribosomales FemXAB que catalizan reacciones similares a peptidiltransferasas también están incluidas.

Se ha reportado que en medio mínimo limitado en fosfatos se incrementa la transcripción del *smc02490* como también lo hacen otros genes involucrados en la biosíntesis de lípidos de membrana sin fósforo en cepas de *S. meliloti* [Krol y Becker, 2004]. También en medio mínimo limitado en fosfatos se han detectado cambios en los ácidos grasos provenientes de los lípidos de membrana de *S. meliloti*. La composición porcentual relativa del ácido lactobacilico (ácido *cis*-11,12-metilene octadecanoico) este ácido contiene un grupo ciclopropano y aumentó 2 veces en la condición de crecimiento limitada en fosfatos, el aumento produjo una disminución modesta en todos los otros ácidos grasos, especialmente el *cis*-vaccénico, este es precursor del ácido graso con ciclopropano [Basconcillo y McCarry, 2008]. Aunque no se estudió de que lípidos de membrana provenía el ácido lactobacilico, éste ha sido detectado como acilo secundario de los OLs, los cuales se incrementan en *S. meliloti* cuando se crece en limitación de fosfatos [Geiger et al., 1999]. Los datos anteriores sugieren que OlsB y SMc02490 sí podrían estar involucrados en la biosíntesis de los OLs en condiciones de crecimiento limitadas en fosfatos.

8.2.10. Las mutantes en el gen *smc02490* de *S. meliloti* 1021

Mediante el análisis de hibridación tipo Southern (fig. 19) se demostró que 5 candidatas a mutantes en el gen *smc02490*, tenían remplazado el gen *smc02490* por el gen *cat* que confiere resistencia a cloranfenicol. Debido a que tenían el perfil de hibridación esperado descrito en procedimientos experimentales de los resultados adicionales. Una mutante, NG8, se seleccionó para estudios posteriores. El plásmido pNG39 se transfirió a la cepa NG8, así como a la cepa silvestre para posteriores estudios que se realizaran en medio mínimo limitado en fosfatos, debido a que se ha reportado que en esa condición se incrementa la transcripción del gen *smc02490*. Aunque para verificar si NG8 todavia produce OL también podríamos introducirle el plásmido pJG21 (expresa a $olsB_{Sm}$) y crecerla en un medio de cultivo con concentraciones de fosfato no limitantes.

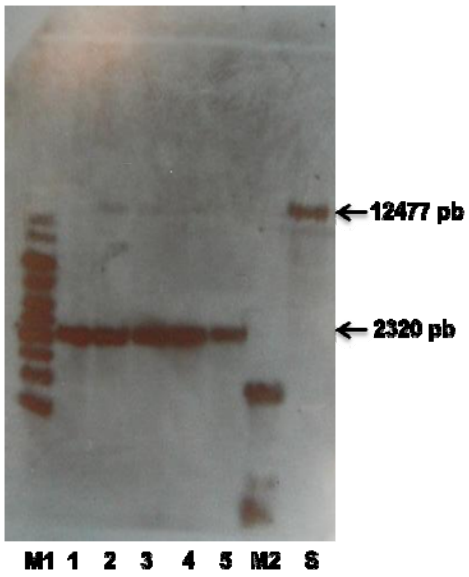


Figura 19. Perfil de hibridación tipo Southern de candidatas a mutantes en el gen *smc02490*. Marcador molecular de 1 kb (Fermentas) (línea M1), DNA genómico digerido con *Eco*RI de: candidata a mutante en *smc02490* NG8 (línea 1), candidata a mutante en *smc02490* NG9 (línea 2), candidata a mutante en *smc02490* NG10 (línea 3), candidata a mutante en *smc02490* NG11 (línea 4), candidata a mutante en *smc02490* NG12 (línea 5), marcador molecular de 1 kb (Invitrogen) (línea M2), cepa silvestre *S. meliloti* 1021 (línea S).

9. Discusión

Los lípidos de ornitina estándar consisten de un residuo del aminoácido ornitina y de dos ácidos grasos. Un ácido graso está amidificado a la ornitina y el otro está esterificado al C3 del acilo amidificado. En algunas bacterias, los OLs estándar (sin modificaciones con grupos polares) se incrementan cuando se crecen en condiciones limitadas en nutrimentos como el fosfato [Geiger et al., 1999], cationes divalentes [Wee y Wilkinson, 1988] o cuando se interrumpe la ruta biosintética de otros lípidos de membrana sin fósforo como el sulfonolípido [Pitta et al., 1989] y el diacilgliceril *N,N,N*-trimetilhomoserina [López-Lara et al., 2005].

Sin embargo, muchas bacterias, entre ellas nuestro modelo de estudio, el patógeno oportunista *B. cenocepacia* J2315, sintetizan OLs aún cuando son crecidas en medios de cultivo con abundante fósforo y cationes divalentes [Palacios-Chaves et al., 2011; Vences-Guzman et al., 2011; González-Silva et al., 2011]. Esto sugiere que la regulación de los genes involucrados en la biosíntesis de los OLs es diferente entre los distintos grupos de bacterias que los poseen. La biosíntesis de los OLs estándar se elucidó en *S. meliloti* 1021 y las enzimas OlsA, OlsB y la acil-ACP están involucradas [Weissenmayer et al., 2002; Gao et al., 2004]. Adicionalmente, algunos grupos de bacterias producen enzimas que tienen la capacidad de hidroxilar los OLs estándar en distintas partes de la molécula produciendo nuevas clases más polares de OLs. La biosíntesis de las clases hidroxiladas conocidas se dilucidó en *R. tropici* CIAT899 e incluye a las enzimas OlsC [Rojas-Jiménez et al., 2005] y OlsE [Vences-Guzmán et al., 2011] además de OlsA, OlsB y la acil-ACP.

Se ha observado que en *R. tropici* CIAT899 los lípidos de ornitina estándar y hidroxilados incrementan la tolerancia de la bacteria a estreses como pH ácido y altas temperaturas, además de estar involucrados en la eficiencia simbiótica [Vences-Guzmán et al., 2011]. En un estudio anterior también se reportó que *Burkholderia cepacia* NCTC 10661 forma 2-OH-PE además de los 2-OH-OLs y que ambas clases de lípidos 2-hidroxilados se incrementaban cuando esta bacteria se crecía en altas temperaturas [Taylor et al., 1998]. Un fenómeno similar ocurre en *B. cenocepacia* J2315 (datos no mostrados).

Al inicio de este trabajo nos planteamos identificar el gen que codifica para la enzima que hidroxila a los OLs estándar de *B. cenocepacia* J2315 para convertirlos en los 2-OH-OLs, los cuales son análogos a la clase de OLs P1 de *R. tropici* CIAT899. Nosotros buscamos e identificamos en el genoma de *B. cenocepacia* J2315 dos genes (*bcam1214* y *olsD*) que codificaban para proteínas similares a LpxO de *S. typhimurium,* se clonaron en el vector de expresión pET9a y subsecuentemente en el pRK404. La transcripción de los genes quedó regulada por el promotor T7. Después los plásmidos que contenían los genes se introdujeron en *S. meliloti* 1021 y en *B. cenocepacia* J2315. Los genes regulados por el promotor T7 al introducirlos en *S. meliloti* 1021 y en *B. cenocepacia* J2315 se transcriben eficientemente. Al introducir el plásmido que contenía al gen *olsD* en *S. meliloti* 1021 se formó un nuevo lípido (NL) positivo a la tinción con ninhidrina. Sin embargo, la mutante en *olsD* (AQ3) derivada de *B. cenocepacia* J2315 aún formó las dos clases de lípidos de ornitina (OLs y 2-OH-OLs) y no mostró ninguna alteración en el perfil de lípidos de membrana respecto a la cepa parental cuando se crecieron en condiciones normales (LB a 30 ^{o}C en agitación). Eso sugería que OlsD estaba involucrada en biosintetizar otra nueva clase de lípidos, y que no se expresa en las condiciones normales de crecimiento.

Además, al introducir el plásmido que contenía al gen *olsD* en *B. cenocepacia* J2315 se formaron dos nuevos lípidos adicionales (NL1 y NL2) positivos a la tinción con ninhidrina. Los nuevos lípidos se movieron diferentemente en 2D-TLC a todos los lípidos de membrana previamente caracterizados de esa bacteria. Eso era consistente con la presunción de que los lípidos dependientes de *olsD* eran diferentes a los lípidos de membrana conocidos de *B. cenocepacia* J2315.

Por análisis de espectrometría de masas se mostró que los lípidos NL1 y NL2 que se formaron en *B. cenocepacia* J2315 y NL en *S. meliloti* 1021 dependientes de OlsD eran versiones de OLs hidroxilados en un carbono del acilo amidificado de OLs preexistentes en ambos organismos. Lo anterior, también se confirmó al analizar la movilidad en TLC de los lisolípidos (LOLs) derivados de los OLs estándar y de los OLs modificados por OlsD. Los lisolípidos derivados de los OLs modificados por OlsD se movieron menos en la TLC respecto a los LOLs derivados de los OLs estándar. Esto sugería que los LOL derivados de

los OLs modificados por OlsD eran más polares, probablemente porque OlsD introduce el grupo hidroxilo en el acilo amidificado o en el residuo de ornitina, los cuales, forman el LOL.

La existencia en la naturaleza de OLs hidroxilados en un carbono de su acilo amidificado no había sido reportada previamente. Probablemente debido a que el perfil de lípidos de membrana de la cepa silvestre no había sido analizado en la condición que se expresa el gen *olsD*. Nosotros observamos pequeñas cantidades de los nuevos lípidos dependientes OlsD (NL1 y NL2) cuando la cepa silvestre *B. cenocepacia* J2315 se creció en LB a pH 4 a 30 ^{o}C en agitación, los cuales, no fueron observados en la mutante AQ3 crecida en las mismas condiciones. Una baja producción (menor a 1.5 %) de cada uno los OLs NL1 y NL2 también se observó cuando se introdujo el plásmido que contenía el gen *olsD* en la cepa mutante en *OlsD* (AQ3) [González-Silva et al., 2011]. El dato anterior es sorprendente debido a que existe el antecedente de que los genes regulados por el promotor T7 se transcriben eficientemente en *B. cenocepacia* J2315 y la cantidad de sustratos (OLs y 2-OH-OLs) in vivo parece no ser limitante. Nosotros pensamos que existe la posibilidad de que una fracción considerable de los OLs NL1 y NL2, producidos por la célula tal vez están unidos a una molécula hidrofílica y por esa razón el método de extracción usado puede ser ineficiente y que a eso se debería que observamos pequeñas cantidades de estos lípidos.

Recientemente se ha reportado que los OLs S1 (estándar) y P1 (2-OH-OL, hidroxilado en el C2 del acilo esterificado) se incrementan considerablemente en *R. tropici* CIAT899 cuando se crece en pHs ácidos de 4.5 y 4.0 [Vences-Guzmán et al., 2011]. Esto sugiere que los genes *olsD* de *B. cenocepacia* J2315 y *olsC* de *R. tropici* CIAT899 que están involucrados en síntesis de OLs hidroxilados en el acilo amidificado y en el esterificado, respectivamente, se expresan en condiciones ácidas de crecimiento [González-Silva et al., 2011; Vences-Guzmán et al., 2011]. Estos resultados son consistentes con la presunción de que en ciertas condiciones de crecimiento en pH ácido, altas temperaturas y bajas concentraciones de cationes divalentes, los OLs estándar o modificados podrían remplazar al LPS, porque en esas condiciones la estabilidad del LPS en la membrana no estaría garantizada, debido a la repulsión electrostática entre las moléculas adyacentes de LPSs

con cargas negativas [Nikaido, 2003]. En esas condiciones los OLs hidroxilados en el acilo amidificado y en el esterificado podrían estar formando enlaces de hidrógeno entre moléculas adyacentes. Por lo tanto, los OLs hidroxilados podrían incrementar la impermeabilidad de las membranas a ciertos iones o moléculas.

Al invadir macrófagos de humanos y sobrevivir en su interior *B. cenocepacia* J2315 se podría enfrentar a condiciones adversas que presumiblemente existen en los fagolisosomas de los macrófagos como pH ácido y limitación de nutrimentos entre ellos el Mg^{+} [Guo et al., 1997] además de temperaturas mayores a 37 ^{o}C. Condiciones que favorecen la síntesis de los OLs estándar o sus formas modificadas en diferentes organismos. En la cepa de *Burkholderia cepacia* NCTC 10661 solamente las clases de lípidos de ornitina (OLs y 2-OH-OLs) se detectaron cuando esta cepa se creció en condiciones limitadas en fósforo [Taylor et al., 1998]. Lo que sugiere que en esas condiciones los OLs y sus formas modificadas de *B. cenocepacia* J2315 podrían jugar un papel protagónico e indispensable para la sobrevivencia de esta bacteria en esas condiciones.

Los OLs de muchas bacterias tienen la propiedad de activar el sistema inmune de mamíferos porque son reconocidos como marcadores moleculares asociados a patógenos [Kawai y Yano, 1983; Kawai et al., 1988; Kawai et al., 1988 b; Kawai et al., 1999]. Contradictoriamente, Palacios-Chaves et al. (2011) reportaron recientemente que los OLs de *B. abortus* carecen de un marcador de patrón molecular asociado a patógeno y que no otorgan ventaja a *B. abortus* para multiplicarse intracelularmente [Palacios-Chaves et al., 2011]. Sin embargo, los OLs de *B. cenocepacia* J2315 no son idénticos a los de *B. abortus* y no se ha estudiado si los OLs *B. cenocepacia* J2315 están involucrados de alguna manera en inducir o en resistir la respuesta inmune de mamíferos. Por lo tanto, se necesita evidencia experimental para determinar si los OLs o sus formas modificadas están involucrados en la patogenicidad de esta bacteria.

Además de la diversidad de especies moleculares de OLs generada por las hidroxilaciones, los OLs también son heterogéneos en sus ácidos grasos. Un resultado consistente de este trabajo con otros estudios previos es que el ácido graso amidificado de

los OLs de *B. cenocepacia* J2315 es de C16:0, como también lo es en otros miembros del género *Burkholderia* [Phung et al., 1995; Taylor et al., 1998]. Los ácidos grasos esterificados de los OLs de *B. cenocepacia* J2315 son muy variables e incluyen tamaños de entre C16 a C19 y algunos de ellos tienen insaturaciones que también han sido reportados en otros miembros del género *Burkholderia* [Phung et al., 1995; Taylor et al., 1998]. Basado en nuestros resultados la enzima OlsB de *B. cenocepacia* J2315 pareciera ser una *N*-aciltransferasa específica para transferir acilos de C16 y la OlsB de *S. meliloti* 1021 de C18.

Para obtener más OL modificado por OlsD para usarlo para análisis estructural y determinar la posición de la hidroxilación, así como para comprobar que OlsD depende de los mismos componentes (cofactores) para su actividad que LpxO, nosotros desarrollamos un ensayo asumiendo que OlsD era una dioxigenasa dependiente de $Fe^{2+}/O_2/\alpha$-cetoglutarato. [^{14}C]OL se usó como sustrato aceptor en presencia de los cofactores apropiados y diferentes extractos libres de células provenientes de cepas de *E. coli* y los productos se analizaron por TLC. Al incubar [^{14}C]OL con el bufer y extractos crudos libres de células de *E. coli* que contenían a pNG40 que expresaba OlsD, se observó que se formó el compuesto dependiente de OlsD (*N*-acyl-OH-OL) y que se movió menos en TLC que el sustrato. El compuesto *N*-acyl-OH-OL no se formó cuando [^{14}C]OL y el bufer se incubó con extractos crudos libres de células de *E. coli* que contenían el plásmido vacío. Al separar por ultracentrifugación la fracción soluble de proteínas y la insoluble (membranas) de los extractos crudos libres de células y evaluar su actividad se mostró que mucha de la proteína OlsD está asociada con la fracción de membrana y una menor cantidad con la fracción soluble de proteínas. Análisis de la secuencia de OlsD con el algoritmo TMHMM [Krogh et al., 2001] que predice hélices transmembranales muestra que esta proteína carece de hélices α (fig. 20) que atraviesan la membrana y probablemente a eso se debe que parte de la proteína se disocie de la fracción de membrana. Aunque para OlsD si se predice una pequeña región hidrofóbica en el extremo C-terminal que no atraviesa la membrana (fig. 20), la cual probablemente determina la asociación mayoritaria de OlsD a la membrana.

En el ensayo de LpxO se adicionaron fosfolípidos (0.5 mg/ml) de *E. coli* además de los contenidos en las membranas [Gibbons et al., 2008]. Nosotros para OlsD solamente adicionamos los fosfolípidos (alrededor de 10 µg) que contenían los extractos crudos libres de células provenientes de *E. coli*. La actividad de OlsD sobre los OLs parece ocurrir después de las actividad de OlsA y también de la actividad como OlsC en *B. cenocepacia* J2315 ya que OlsD fue capaz de modificar in vitro y en vivo a los OLs [González-Silva et al., 2011] y a los 2-OH-OLs (fig. 12). Además, OlsD no es específica para hidroxilar OLs de *B. cenocepacia* J2315 en el que acilo amidificado es de C16, sino que también muestra flexibilidad para usar como sustrato in vivo a los OLs de *S. meliloti* 1021 en los cuales el acilo amidificado es de C18 [González-Silva et al., 2011].

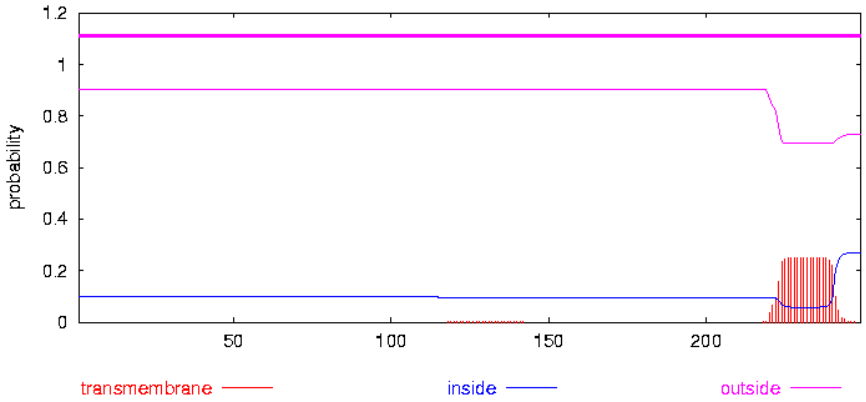


Figura. 20. Predicción de hélices transmembranales de OlsD con el algoritmo TMHMM [Krogh et al., 2001].

Se observó que la actividad de OlsD es dependiente de α-cetoglutarato, la omisión de Fe^{2+} adicional no afecta la actividad de OlsD, y la dependencia de este ion metálico solo se observó cuando se adicionó el quelante de fierro (2,2'-bipyridyl). La omisión de ascorbato disminuyó considerablemente la actividad de OlsD. Gibbons et al. (2008) reportaron que la inclusión de catalasa en el ensayo in vitro para LpxO eliminaba parcialmente el requerimiento de ascorbato con lo que demostraron que ascorbato no es necesario para la actividad de LpxO. Lo anterior es consistente con la actividad in vivo de LpxO en *E. coli* la cual no forma ascorbato [Gibbons et al., 2008]. En ausencia de ditiotreitol (DTT) la actividad OlsD se incrementó más del 50 %. Esto sugiere que el ambiente químico

generado por la presencia de ese compuesto afecta negativamente la actividad de OlsD, lo que posiblemente se debe a que el DTT tiene la capacidad de reducir parte del O_2 disuelto en el bufer a H_2O [Usha y Ramasarma, 1982], y probablemente a que el O_2 se requiere en la reacción de hidroxilación. Consecuentemente el DTT podría provocar que el O_2 disuelto sea un componente limitante para la velocidad de la reacción, posiblemente haciéndola más lenta. Aunque nosotros no evaluamos si OlsD requiere de O_2 para realizar su actividad, debido a la similitud que comparte con LpxO y a que al menos demostramos que dos componentes de la reacción (Fe^{2+}/α-cetoglutarato), al igual que para LpxO, también son necesarios en el proceso catalítico de OlsD, creemos que OlsD también es dependiente de Fe^{2+}/O_2/α-cetoglutarato al igual que LpxO.

Comparando el porcentaje de conversión de sustrato a producto se observó que la enzima OlsC es más eficiente para hidroxilar el C2 del acilo esterificado de los OLs que la enzima OlsD que hidroxila un carbono del acilo amidificado también de los OLs. Cabe recordar que los ensayos enzimáticos de OlsC se realizaron con 0.4 mg/ml de proteína total y los ensayos de OlsD con 1 mg/ml. Cuando nosotros usamos 1 mg/ml para los ensayos de OlsC obtuvimos un porcentaje de conversión mayor a 80 % (datos no mostrados). Otra diferencia de los ensayos presentados es que con OlsD se usó 0.1 % de Triton X-100 y con OlsC 0.2 %.

Recientemente se han identificado algunos de los genes que codifican para las enzimas involucradas en la biosíntesis de los OLs. Sin embargo, no se ha reportado ningún gen involucrado en la biodegradación de esos lípidos. Este tema espera ser estudiado.

10. Conclusiones

En esta tesis doctoral se identificó el gen *olsD* y se caracterizó su producto. Los resultados presentados nos permiten concluir:

1. El gen *olsD* codifica para una enzima que está involucrada en hidroxilar los lípidos de ornitina de *B. cenocepacia* J2315.
2. La enzima OlsD hidroxila el acilo amidificado de los lípidos de ornitina (OLs y 2-OH-OLs) de *B. cenocepacia* J2315 para formar otros dos lípidos adicionales más polares (NL1 y NL2). La presencia de los genes *olsB*, *olsA*, un gen que codifica para una enzima que hace una actividad como OlsC y *olsD* en *B. cenocepacia* J2315 la capacitan para formar cuatro clases diferentes de lípidos ornitina.
3. La hidroxilación por la enzima OlsD ocurre cuando *B. cenocepacia* J2315 se crece en condiciones ácidas y OlsD requiere de Fe^{2+}/α-cetoglutarato para realizar su actividad.
4. Para que se produzca cualquier clase de lípidos de ornitina en *B. cenocepacia* J2315 se requiere que el gen *olsB* esté integro.
5. La hipótesis que nos planteamos es falsa, debido a que ninguno de los dos genes (*bcam1214* o *olsD*) de *B. cenocepacia* J2315 homólogos a LpxO de S. *typhimurium* están involucrados en hidroxilar a los OLs estándar para convertirlos en los 2-OH-OLs.

11. Perspectivas

Los resultados y conclusiones generados en esta tesis doctoral, dan lugar a estudiar los siguientes aspectos:

- Determinar el carbono del acilo amidificado de los OLs que es hidroxilado por OlsD.
- Comprobar si OlsD requiere de O_2 para realizar su actividad.
- Determinar si los lípidos de ornitina en *B. cenocepacia* J2315 están involucrados en la patogenicidad de esta bacteria.
- Estudiar la función que desempeñan los lípidos de ornitina en la membrana de *B. cenocepacia* J2315.

12. Referencias bibliográficas

Asselineau, J. (1991) Bacterial lipids containing amino acids or peptides linked by amide bonds. *Chem. Org. Naturst. 56*, 1-85.

Aygun-Sunar, S., Bilaloglu, R., Goldfine, H., and Daldal, F. (2007) *Rhodobacter capsulatus* OlsA is a bifunctional enyzme active in both ornithine lipid and phosphatidic acid biosynthesis. *J. Bacteriol. 189,* 8564-8574.

Aygun-Sunar, S., Mandaci, S., Koch, H.-G., Murria, I. V. J., Goldfine, H., and Daldal, F. (2006) Ornithine lipid is required for optimal steady-state amounts of *c*-type cytochromes in *Rhodobacter capsulatus*. *Mol. Microbiol. 61*, 418-435.

Basconcillo, L. S., and McCarry, B. E. (2008) Comparison of three GC/MS methodologies for the analysis of fatty acids in *Sinorhizobium meliloti*: Development of a micro-scale, one-vial method. *J. Chromatogr. B. 871,* 22-31.

Basconcillo, L. S., Zaheer, R., Finan, T. M., and McCarry, B. E. (2009) A shotgun lipidomics study of a putative lysophosphatidic acid acyl transferase (PlsC) in *Sinorhizobium meliloti*. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877,* 2873-2882.

Batrakov, S. G., Nikitin, D. I., Mosezhnyi, A. E., and Ruzhitsky, A. O. (1999) A glycine-containing phosphorus-free lipoaminoacid from the gram-negative marine bacterium *Cyclobacterium marinus* WH. *Chem. Phys. Lipids 99,* 139-143.

Batrakov, S. G., Nikitin, D. I., Sheichenko, VI, and Ruzhitsky, A. O. (1998) A novel sulfonic-acid analogue of ceramide is the major extractable lipid of the gram-negative marine bacterium *Cyclobacterium marinus* WH. *Biochim. Biophys. Acta 1391,* 79-91.

Beringer, J. E. (1974) R factor transfer in Rhizobium leguminosarum. *J. Gen. Microbiol. 84,* 188-198.

Bligh, E. G., and Dyer, J. W. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol. 37*, 911-917.

Brady, S. F., and Clardy, J. (2005) *N*-acyl derivatives of arginine and tryptophan isolated from environmental DNA expressed in *Escherichia coli*. *Org. Lett. 7,* 3613-3616.

Brady, S. F., Chao, C. J., and Clardy, J. (2002) New natural product families from an environmental DNA (eDNA) gene cluster. *J. Am. Chem. Soc. 124,* 9968-9969.

Clardy, J., and Brady, S. F. (2007) Cyclic AMP directly activates NasP, an *N*-acyl amino acid antibiotic biosynthetic enzyme cloned from an uncultured β-proteobacterium. *J. Bacteriol. 189,* 6487-6489.

Cullinane, M., Baysse, C., Morrissey, J. P., and O'Gara, F. (2005) Identification of two lysophosphatidic acid acyl transferase genes with overlapping function in *Pseudomonas fluorescens*. *Microbiology 151,* 3071-3080.

Dees, C., and Shively, J. M. (1982) Localization and quantitation of the ornithine lipid of *Thiobacillus thiooxidans. J. Bacteriol. 149*, 798-799.

Donovan, C., and Bramkamp, M. (2009) Characterization and subcellular localization of a bacterial flotillin homologue. *Microbiology 155*, 1786-1799.

Dorrer E., and Teuber, M. (1977) Induction of polymyxin resistance in *Pseudomonas fluorescens* by phosphate limitation. *Arch. Microbiol. 114,* 87-89.

Dowhan, W., Bogdanov, M., and Mileykovskaya, E. (2008) Functional roles of lipids in membranes. In: Vance, D. E., and Vance, J. E., editors. Biochemistry of Lipids, Lipoproteins and Membranes (5th Edition). Amsterdam, The Netherlands: Elsevier. p. 1-37.

Facey, S. J., and Kuhn, A. (2010) Biogenesis of bacterial inner-membrane proteins. *Cell Mol. Life Sci. 67,* 2343-2362.

Galbraith, L., Jonsson, M. H., Rudhe, L. C., and Wilkinson, S. G. (1999) Lipids and fatty acids of *Burkholderia* and *Ralstonia* species. *FEMS Microbiol. Lett. 173*, 359-364.

Gao, J.-L., Weissenmayer, B., Taylor, A. M., Thomas-Oates, J., López-Lara, I. M., and Geiger, O. (2004) Identification of a gene required for the formation of lyso-ornithine lipid, an intermediate in the biosynthesis of ornithine-containing lipids. *Mol. Microbiol. 53*, 1757-1770.

Geiger, O., González-Silva N., López-Lara, I. M., and Sohlenkamp, C. (2010) Amino acid-containing membrane lipids in bacteria. *Prog. Lipid Res. 49,* 46-60.

Geiger, O., Röhrs, V., Weissenmayer, B., Finan, T. M., and Thomas-Oates, J. E. (1999) The regulator gene *phoB* mediates phosphate stress-controlled synthesis of the membrane lipid diacylglyceryl-*N,N,N*-trimethylhomoserine in *Rhizobium (Sinorhizobium) meliloti*. *Mol. Microbiol. 32*, 63-73.

Gibbons, H. S., Kalb, S. R., Cotter, R. J., and Raetz, C. R. H. (2005) Role of Mg^{2+} and pH in the modification of *Salmonella* lipid A after endocytosis by macrophage tumour cells. *Mol. Microbiol. 55*, 425-440.

Gibbons, H. S., Lin, S., Cotter, R. J., and Raetz C. R. H. (2000) Oxygen requirement for the biosynthesis of the *S*-2-hydroxymyristate moiety in *Salmonella typhimurium* lipid A. Function of LpxO, a new Fe2+/α-ketoglutarate-dependent dioxygenase homologue. *J. Biol. Chem. 275*, 32940-32949.

Gibbons, H. S., Reynolds, C. M., Guan, Z., and Raetz, C. R. H. (2008) An inner membrane dioxygenase that generates the 2-hydroxymyristate moiety of *Salmonella* lipid A. *Biochemistry 47*, 2814-2825.

Gomi, K., Kawasaki, K., Kawai, Y., Shiozaki, M., and Nishijima, M. (2002) Toll-like receptor 4-MD-2 complex mediates the signal transduction induced by flavolipin, an amino acid-containing lipid unique to *Flavobacterium meningosepticum*. *J. Immunol. 168,* 2939-2943.

González-Silva, N., López-Lara, I. M., Reyes-Lamothe, R., Taylor, A. M., Sumpton, D., Thomas-Oates, J., and Geiger, O. (2011) The dioxygenase-encoding *olsD* gene from *Burkholderia cenocepacia* causes the hydroxylation of the amide-linked fatty acyl moiety of ornithine-containing membrane lipids. *Biochemistry 50,* 6396-6408.

Gough, J., Karplus, K., Hughey, R., and Chothia, C. (2001) Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure*. J. Mol. Biol. 313,* 903-919.

Gould, T. A., Schweizer, H. P., and Churchill, M. E. A. (2004) Structure of the *Pseudomonas aeruginosa* acylhomoserine lactone synthase LasI. *Mol. Microbiol. 53,* 1135-1146.

Guo, L., Lim, K. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M., and Miller, S. I. (1997) Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. *Science 276,* 250-253.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol. 166,* 557-580.

Heath, R. J., and Rock, C. O. (1998) A conserved histidine is essential for glycerolipid acyltransferase catalysis. *J. Bacteriol. 180,* 1425-1430.

Hoischen, C., Gura, K., Luge, C., and Gumpert, J. (1997) Lipid and fatty acid composition of cytoplasmic membranes from *Streptomyces hygroscopicus* and its stable protoplast-type L form. *J. Bacteriol. 179,* 3430-3436.

Jansen, G. A., Hogenhout, E. M., Ferdinandusse, S., Waterham, H. R., Ofman, R., Jakobs, C., Skjeldal, O. H., and Wanders, R. J. (2000) Human phytanoyl-CoA hydroxylase: resolution of the gene structure and the molecular basis of Refsum's disease. *Hum. Mol. Genet. 9,* 1195-1200.

Jansen, G. A., Ofman, R., Ferdinandusse, S., Ijlst, L., Muijsers, A. O., Skjeldal, O. H., Stokke, O., Jakobs, C., Besley, G. T., Wraith, J. E. and Wanders, R. J. (1997) Refsum disease is caused by mutations in the phytanoyl-CoA hydroxylase gene. *Nat. Genet. 17,* 190-193.

Kadner, R. J. (1996) Cytoplasmic membrane. In: *Escherichia coli* and *Salmonella*. Cellular and Molecular Biology, 2nd ed. In: Neidhardt, F. C., editor. ASM press; Washington, DC. p. 58-87.

Kato, H., and Goto, N. (1997) Adjuvanticity of an ornithine-containing lipid of *Flavobacterium meningosepticum* as a candidate vaccine adjuvant. *Microbiol. Immunol. 41,* 101-106.

Kawahara, K., Seydel, U., Matsuura, M., Danbara, H., Rietschel, E. T., and Zähringer, U. (1991) Chemical structure of glycosphingolipids isolated from *Sphingomonas paucimobilis*. *FEBS Lett. 292,* 107-110.

Kawai, Y, and Akagawa, K. (1989) Macrophage activation by an ornithine-containing lipid or a serine-containing lipid. *Infect. Immun. 57,* 2086-2091.

Kawai, Y. and Yano, I. (1983) Ornithine-containing lipid of *Bordetella pertussis*, a new type of hemagglutinin. *Eur. J. Biochem. 136,* 531-538.

Kawai, Y., Kaneda, K., Morisawa, Y., and Akagawa, K. (1992) Protection of mice from lethal endotoxemia by use of an ornithine-containing lipid or a serine-containing lipid. Infect Immun. 1991 59, 2560-6. Erratum in: *Infect. Immun. 60,* 320.

Kawai, Y., Nakagawa, Y., Matuyama, T., Akagawa, K., Itagawa, K., Fukase, K., Kusumoto, S., Nishijima, M., and Yano, I. (1999) A typical bacterial ornithine-containing lipid *N*α-(D)-[3-(hexadecanoyloxy)hexadecanoyl]-ornithine is a strong stimulant for macrophages and a useful adjuvant. *FEMS Immunol. Med. Microbiol. 23,* 67-73.

Kawai, Y., Watanabe, M., Matsuura, M., Nishijima, M., and Kawahara, K. (2002) The partially degraded lipopolysaccharide of *Burkholderia cepacia* and ornithine-containing lipids derived from some Gram-negative bacteria are useful complex lipid adjuvants. *FEMS Immunol. Med. Microbiol. 34,* 173-179.

Kawai, Y., Yano, I., and Kaneda, K. (1988) Various kinds of lipoamino acids including a novel serine-containing lipid in an opportunistic pathogen *Flavobacterium*. Their structures and biological activities on erythrocytes. *Eur. J. Biochem. 171*, 73-80.

Kawai, Y., Yano, I., Kaneda, K. and Yabuuchi, E. (1988b) Ornithinecontaining lipids of some *Pseudomonas* species. *Eur. J. Biochem. 175,* 633-641

Kawasaki, K., Gomi, K., Kawai, Y., Shiozaki, M., and Nishijima, M. (2003) Molecular basis for lipopolysaccharide mimetic action of Taxol™ and flavolipin. *J. Endotoxin Res. 9*, 301-307.

Kawazoe, R., Monde, K., Reichardt, W., and Okuyama, H. (1992) Lipoamino acids and sulfonolipids in *Cytophaga johnsonae* Stanier strain C21 and six related species of *Cytophaga*. *Arch. Microbiol. 158,* 171-175.

Kawazoe, R., Okuyama, H., Reichardt, W., and Sasaki, S. (1991) Phospholipids and a novel glycine-containing lipoamino acid in *Cytophaga johnsonae* Stanier strain C21. *J. Bacteriol. 173,* 5470-5475.

Keck, M., Gisch, N., Moll, H., Vorhölter, F. J., Gerth, K., Kahmann, U., Lissel, M., Lindner, B., Niehaus, K., and Holst, O. (2011) Unusual outer membrane lipid composition of the gram-negative, lipopolysaccharide-lacking myxobacterium *Sorangium cellulosum* So ce56. *J. Biol. Chem. 286,* 12850-12859.

Knoche, H. W., and Shively, J. M. (1972) The structure of an ornithine-containing lipid from *Thiobacillus thiooxidans*. *J. Biol. Chem. 247,* 170-178.

Kovach, M. E., Elzer, P., Hill, D., Robertson, T., Farris, M., Roop, M., and Peterson, K. M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene 166,* 175-176.

Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M., II, and Peterson, K. M. (1994) pBBR1MCS: A broad-host-range cloning vector. *BioTechniques 16,* 800–802.

Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. L. (2001) Predicting trans-membrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol. 305*, 567-580.

Krol, E., and Becker, A. (2004) Global transcriptional analysis of the phosphate starvation response in *Sinorhizobium meliloti* strains 1021 and 2011. *Mol. Genet. Genomics 272,* 1-17.

Lewenza, S., Falsafi, R., Bains, M., Rohs, P., Stupak, J., Sprott, G. D., and Hancock. R. E. (2011) The *olsA* gene mediates the synthesis of an ornithine lipid in *Pseudomonas aeruginosa* during growth under phosphate-limiting conditions, but is not involved in antimicrobial peptide susceptibility. *FEMS Microbiol. Lett. 320,* 95-102.

López, D., and Kolter, R. (2011) Functional microdomains in bacterial membranes. *Genes & Development 24*, 1893-1902.

López-Lara, I. M., Gao, J.-L., Soto, M. J., Solares-Pérez, A., Weissenmayer, B., Sohlenkamp, C., Verroios, G. P., Thomas-Oates, J., Geiger, O. (2005) Phosphorus-free membrane lipids of *Sinorhizobium meliloti* are not required for the symbiosis with alfalfa but contribute to increased cell yields under phosphorus-limiting conditions of growth. *Mol. Plant-Microbe Interact. 18*, 973-982.

López-Lara, I. M., Sohlenkamp, C., and Geiger, O. (2003) Membrane lipids in plant-associated bacteria: their biosyntheses and possible functions. *Mol. Plant-Microbe Interact. 16,* 567-579.

Madigan M. T., and Martinko J. M. (2006) Brock biology of microorganisms. Pearson Prentice Hall, 11th ed. U. S. A.

Mahenthiralingam, E., Urban, T. A., and Goldberg, J. B. (2005) The multifarious, multireplicon *Burkholderia cepacia* complex. *Nat. Rev. Microbiol. 3*, 144-156.

Maneerat, S., Bamba, T., Harada, K., Kobayashi, A., Yamada, H., and Kawai, F. (2006) A novel crude oil emulsifier excreted in the culture supernatant of a marine bacterium, *Myroides* sp. strain SM1. *Appl. Microbiol. Biotechnol. 70,* 254-259.

Martínez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P., and Pardo, M. A. (1991) *Rhizobium tropici,* a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int. J. Syst. Bacteriol. 41,* 417-4126.

Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E., and Ausubel, F. M. (1982) Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *J. Bacteriol. 149,* 114-122.

Minnikin, D. E., and Abdolrahimzadeh, H. (1974) The replacement of phosphatidylethanolamine and acidic phospholipids by an ornithine-amide lipid and a minor phosphorus-free lipid in *Pseudomonas fluorescens* NCMB 129. *FEBS Lett. 43,* 257-260.

Nelson, D. L. and Cox, M. M. (2010) Lehninger principles of Biochemistry. Freeman, 4th ed. U. S. A.

Nikaido, H. (1996) Outer membrane. In: *Escherichia coli* and *Salmonella.* Cellular and Molecular Biology, 2nd ed. In: Neidhardt, F. C., editor. ASM press; Washington, DC. p. 29-47.

Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev. 67*, 593-656.

Nikitin, D. I., Oranskaya, M. S., Pitryuk, I. A., Chernykh, N. A., and Lysenko, A. M. (1994) A new ring-forming bacterium *Arcocella aquatica* gen. et sp. nov. *Microbiology (Moscow) 63,* 87-90 (Engl. Transl.)

Okemoto, K., Hanada, K., Nishijima, M., and Kawasaki, K. (2008) The preparation of a lipidic endotoxin affects its biological activities. *Biol. Pharm. Bull. 31,* 1952-1954.

Okuyama, H., and Monde, K. (1996) Identification of an ornithine-containing lipid from *Cytophaga johnsonae* Stanier strain C21 by ^{1}H-NMR. *Chem. Phys. Lipids 83,* 169-173.

Palacios-Chaves, L., Conde-Álvarez, R., Gil-Ramírez, Y., Zúñiga-Ripa, A., Barquero-Calvo, E., Chacón-Díaz, C., Chaves-Olarte, E., Arce-Gorvel, V., Gorvel, J. P., Moreno, E., de Miguel, M. J., Grilló, M. J., Moriyón, I., and Iriarte M. (2011) *Brucella abortus* ornithine lipids are dispensable outer membrane components devoid of a marked pathogen-associated molecular pattern. *PLoS ONE 6,* e16030.

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.

Pitta, T. P., Leadbetter, E. R., and Godchaux, III, W. (1989) Increase of ornithine amino lipid content in a sulfonolipid-deficient mutant of *Cytophaga johnsonae*. *J. Bacteriol. 171,* 952-957.

Raetz, C. R. H., and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu. Rev. Biochem. 71,* 635-700.

Raetz, C. R. H., Reynolds C. M., Trent M. S., and Bishop, R. E. (2007) Lipid modification systems in Gram-negative bacteria. *Annu. Rev. Biochem. 76,* 295-329.

Raj, H. D., and Maloy, S. R. (1990) Proposal of *Cyclobacterium marinus* gen. nov., comb. nov. for a marine bacterium previously assigned to the genus *Flectobacillus*. *Int. J. Syst. Bacteriol. 40,* 337-347.

Rock, C. O. (2008) Fatty acid and phospholipid metabolism in prokaryotes. In: Vance, D. E., and Vance, J. E., editors. Biochemistry of Lipids, Lipoproteins and Membranes (5th Edition). Amsterdam, The Netherlands: Elsevier. p. 59-96.

Rojas-Jiménez, K., Sohlenkamp, C., Geiger, O., Martínez-Romero, E., Werner, D., and Vinuesa, P. (2005) A CIC chloride channel homolog and ornithine-containing membrane lipids of *Rhizobium tropici* CIAT899 are involved in symbiotic efficiency and acid tolerance. *Mol. Plant-Microbe Interact. 18*, 1175-1185.

Rumley, M. K., Therisod, H., Weissborn, A. C., and Kennedy, E. G. (1992) Mechanism of regulation of biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. *J. Biol. Chem. 267,* 11806-11810.

Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandoval-Calderón, M., Geiger, O., Guan, Z., Barona-Gómez, F., and Sohlenkamp, C. (2009) A eukaryote-like cardiolipin synthase is present in *Streptomyces coelicolor* and in most Actinobacteria. *J. Biol. Chem. 284,* 17383-17390.

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.

Schüler, D. (2004) Molecular analysis of a subcellular compartment: the magnetosome membrane in *Magnetospirillum gryphiswaldense*. *Arch. Microbiol. 181,* 1-7.

Shih, G. C., Kahler, C. M., Swartley, J. S., Rahman, M. M., Coleman, J., Carlson, R. W., and Stephens, D. S. (1999) Multiple lysophosphatidic acid acyltransferases in *Neisseria meningitidis*. *Mol. Microbiol. 32,* 942-952.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med. 189,* 1777-1782.

Shiozaki, M., Deguchi, N., Ishikawa, T., Haruyama, H., Kawai, Y., and Nishijima, M. (1998a) Revised structure of flavolipin and synthesis of its isomers. *Tetrahedron Lett. 39,* 4497-4500.

Shiozaki, M., Deguchi, N., Mochizuki, T., Wakabayashi, T., Ishikawa, T., Haruyama, H., Kawai, Y., and Nishijima, M. (1998b) Revised structure and synthesis of flavolipin. *Tetrahedron 54,* 11861-11876.

Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in Gram-negative bacteria. *Nat. Biotechnol. 1,* 784–791.

Sohlenkamp, C., de Rudder, K. E. E., and Geiger, O. (2004) Phosphatidylethanolamine is not essential for growth of *Sinorhizobium meliloti* in complex culture media. *J. Bacteriol. 186,* 1667-1677.

Stone, K. J., and Strominger, J. L. (1971) Mechanism of action of bacitracin: complexation with metal ion and c_{55}-isoprenyl pyrophosphate. *Proc. Nat. Acad. Sci. 68,* 3223-3227.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol. 185*, 60-89.

Tahara, Y., Kameda, M., Yamada, Y., and Kondo, K. **(**1976b) An ornithine-containing lipid isolated from *Gluconobacter cerinus*. *Biochim. Biophys. Acta 450*, 225-230.

Tahara, Y., Shinmoto, K., Yamada, Y., and Kondo, K. (1978) Enzymatic synthesis of tauro-ornithine lipid in *Gluconobacter cerinus*. *Agr. Biol. Chem. 42,* 205-206.

Tahara, Y., Yamada, Y., and Kondo, K. (1976a) A new lysine-containing lipid isolated from *Agrobacterium tumefaciens*. *Agr. Biol. Chem. 40,* 1449-1450.

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.

Usha, D., S., and Ramasarma T. (1987) Hemin-mediated oxidation of dithiothreitol reduces oxygen to H20. *Mol. Cell. Biochem. 77,* 111-120.

Van Wagoner, R. M., and Clardy, J. (2006) FeeM, an *N*-acyl amino acid synthase from an uncultured soil microbe: Structure, mechanism, and acyl carrier protein binding. *Structure 14,* 1425-1435.

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 *Rhizobium tropici* CIAT899. *Mol. Microbiol. 79,* 1496-1514.

Vollmer, W., and Bertsche, U. (2008) Murein (peptidoglycan) structure, architecture and biosynthesis in *Escherichia coli*. *Biochim. Biophys. Acta 1778*, 1714-1734.

Watson, W. T., Minogue, T. D., Val, D. L., von Bodman S. B., and Churchill, M. E. A. (2002) Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. *Mol. Cell. 9,* 685-694.

Wee, S., and Wilkinson, B. J. (1988) Increased outer membrane ornithine-containing lipid and lysozyme penetrability of *Paracoccus denitrificans* grown in a complex medium deficient in divalent cations. *J. bacterial. 170,* 3283-3286.

Weissenmayer, B., Gao, J.-L., López-Lara I. M., and Geiger O. (2002) Identification of a gene required for the biosynthesis of ornithine-derived lipids. *Mol. Microbiol. 45*, 721-733.

West, J., Tompkins, C. K., Balantac, N., Nudelman, E., Meengs, B., White, T., Bursten, S., Coleman, J., Kumar, A., Singer, J. W., and Leung, D. W. (1997) Cloning and expression of two human lysophosphatidic acid acyltransferase cDNAs that enhance cytokine-induced signaling responses in cells. *DNA Cell. Biol. 16,* 691-701.

Zhang, H. M., Li, Z., Tsudome, M., Ito, S., Takami, H., and Horikoshi, K. (2005) An alkali-inducible flotillin-like protein from *Bacillus halodurans* C-125. *Protein J. 24*, 125-131.

Zhang, X., Ferguson-Miller, S. M., and Reid, G. E. (2009) Characterization of ornithine and glutamine lipids extracted from cell membranes of *Rhodobacter sphaeroides*. *J. Am. Soc. Mass. Spectrom. 20*, 198-212.