UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
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ESTUDIOS SOBRE LAS MODIFICACIONES QUE PUEDEN SUFRIR LOS LÍPIDOS DE ORNITINA EN BACTERIAS DE LA FAMILIA *Rhizobiaceae*

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I. RECONOCIMIENTOS

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III. Abreviaturas

2D: en dos dimensiones
ACP: proteína acarreadora de grupos acilo
AcpS: holo-ACP sintasa
CL: cardiolipina
CoA: coenzima A
DGTS: diacilgliceriltrimetilhomoserina
DMPE: dimetilfosfatidiletanolamina
GL: lípido de glicina
Kdo: ácido 2-ceto-3-desoxi-D-mano-octulosónico
LL: lípido de lisina
LOL: lisolípido de ornitina
LPS: lipopolisacáridos
MDO: oligosacáridos derivados de la membrana
ME: membrana externa
MI: membrana interna
MMPE: monometilfosfatidiletanolamina
OL: lípido de ornitina
P: fósforo
P1: lípido de ornitina hidroxilado en el carbono 2 del ácido graso esteríficado
P2: lípido de ornitina hidroxilado en el aminoácido ornitina y en el carbono 2 del ácido graso esteríficado
PAMPs: patrones moleculares asociados a patógenos
PC: fosfatidilcolina
PCR: la reacción en cadena de la polimerasa
PE: fosfatidiletanolamina
PG: fosfatidilglicerol
PI: fosfatidilinositol
S1: lípido de ornitina
S2: lípido de ornitina hidroxilado en el aminoácido ornitina
SGL: lípido de glicinaserina
TLC: cromatografía en capa fina
Uma: unidades de masa atómica

IV. Índice de figuras

V. Resumen

Todas las células vivas están delimitadas por membranas que funcionan como barreras de permeabilidad selectiva. Estas membranas están formadas por bicapas lipídicas en las que se embeben diversas proteínas. La biosíntesis y función de los lípidos de membrana se han estudiado detalladamente en las bacterias modelo *Escherichia coli* y *Bacillus subtilis*. En ambos casos los lípidos de membrana mayoritarios son los fosfolípidos fosfatidilglicerol (PG), fosfatidiletanolamina (PE) y cardiolipina (CL); de los cuales se conocen bien sus vías de biosíntesis (Heath *et al*., 2002). En las membranas de algunas bacterias se encuentran también fosfatidilcolina (PC) y fosfatidilinositol (PI), (López-Lara *et al*., 2003; Geiger *et al*., 2010). Además de los fosfolípidos se han descrito lípidos de membrana sin fósforo, como el sulfolípido sulfoquinovosildiacilglicerol (SL), diacilgliceriltrimetilhomoserina (DGTS) y diferentes lípidos de ornitina (OL) (López- Lara *et al*., 2003; Geiger *et al*., 2010).

Se han descrito cuatro diferentes tipos de OL, en *Rhizobium tropici* CIAT899, llamados S1, S2, P1 y P2 (Rojas-Jiménez *et al* 2005). Rojas-Jiménez *et al*. (2005) identificaron el gen *olsC* que codifica para la hidroxilasa OlsC, la cual introduce un grupo hidroxilo en los OLs S1 y S2, generando la formación de los OLs P1 y P2, respectivamente. Por otra parte, no se conocía el gen que codifica para la enzima responsable de la modificación de los OLs S1 y P1 a S2 y P2, respectivamente. En el presente trabajo de tesis se estudió el papel que desempeñan los OLs en la tolerancia a diferentes tipos de estrés, en la simbiosis o en la patogenicidad, en las bacterias de *R. tropici* CIAT899 y *Agrobacterium tumefaciens*. Para conocer la función de los OLs se trabajó sobre la identificación del gen involucrado en la biosíntesis de los OLs S2, P2, y en la elucidación de las estructuras de los

lípidos S2 y P1. Una vez que el lípido de ornitina S2 se purificó, el análisis por espectrometría de masas mostró que el S2 posee una modificación en el residuo ornitina del OL, sin embargo, no se logró determinar la posición exacta del grupo hidroxilo. Por otro lado, para el OL P1 se determinó que el grupo hidroxilado está presente en el carbono 2 del ácido graso esterificado.

Para identificar al gen responsable de la hidroxilación de la ornitina (*olsE*), se realizó un tamizaje de expresión funcional. Para lo cual se empleó un banco de cósmidos de *R. tropici* CIAT899, que se movilizó a *S. meliloti* 1021, para posteriormente analizar la presencia del OL modificado en las transconjugantes. Para encontrar el gen *olsE* dentro del cósmido, el inserto alojado en el mismo se subclonó en un plásmido de amplio rango de hospedero. Una vez identificado el gen de interés, se realizó un alineamiento básico local (BLAST), mediante el cual se determinó que la proteína OlsE pertenece a la superfamilia de hidroxilasas de ácidos grasos, que se caracterizan por tener dos iones de hierro en su centro activo. Para determinar la función de los OLs, en *R. tropici* CIAT899, se construyeron mutantes deficientes en *olsE* y mutantes doble deficientes en *olsC/olsE*. La caracterización de las mismas, bajo condiciones de acidez y de temperaturas elevadas, permitió concluir que los lípidos de ornitina están involucrados en la resistencia a temperaturas altas y pHs bajos.

El análisis de la composición lipídica de la membrana del patógeno de plantas *Agrobacterium tumefaciens,* indicó que forma dos diferentes OLs. Para determinar el papel de los OLs en la tolerancia al estrés y en la patogenicidad de *A. tumefaciens*, se construyeron y se caracterizaron mutantes en los genes *olsB* y *olsE*. Estas mutantes carecen por completo de los OLs (Δ*olsB*) o sólo forman el OL no hidroxilado (Δ*olsE*). Ambas mutantes se caracterizaron bajo diferentes condiciones de estrés y también en ensayos de infección con discos de papa. No se observaron

cambios en el crecimiento de las mutantes, bajo las distintas condiciones de estrés abiótico, con respecto a la cepa silvestre. Por otro lado, la ausencia de los OLs agrobacteriales promueve la formación de tumores en las plantas huésped en tiempos más cortos.

VI. Summary

All living cells are surrounded by membranes that act as selective permeability barriers. These membranes are composed of lipid bilayers in which different proteins are embedded. Biosynthesis and function of the membrane lipids have been studied in detail in the model bacteria *Escherichia coli* and *Bacillus subtilis*. In both cases the major membrane lipids are the phospholipids phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and cardiolipin (CL), for which the biosynthetic pathways are well known (Heath *et al*., 2002). In the membranes of some other bacteria also the lipids phosphatidylcholine (PC) and phosphatidylinositol (PI) can be found (López-Lara *et al*. 2003; Geiger *et al*., 2010). In addition to phospholipids non-phosphorus lipids such as sulfoquinovosyldiacilglicerol sulfolipid (SL), diacylglyceriltrimetilhomoserina (DGTS) and different ornithine lipids (OL) can be present in the membrane of some bacteria. The latter are only synthesized in large quantities when the bacteria are grown under phosphorus limiting conditions (López-Lara *et al*. 2003; Geiger *et al*., 2010).

In *Rhizobium tropici* CIAT899 four different types of OL, called S1, S2, P1 and P2 have been identified (Rojas-Jimenez *et al* 2005). Rojas-Jimenez *et al*. (2005) identified the *olsC* gene encoding the OL hydroxylase OlsC which introduces a hydroxyl group into the OLs S1 and S2, leading to the formation of the OLs P1 and P2, respectively. On the other hand, the gene that codes for the enzyme responsible for the modification of the OLs S1 and S2 into P1 and P2, respectively is not known. In the present work we studied whether OLs may play a role in tolerance to various stresses, or in symbiosis or pathogenicity in *R. tropici* CIAT899 and *Agrobacterium tumefaciens*. In order to clarify the role of hydroxylated OLs in these organisms, we identified the unknown gen for the biosynthesis

of OLs S2 and P1 and determined the lipid structures of the OLs P1 and S2. As a first step, the ornithine lipids P1 and S2 were purified and analyzed by mass spectrometry. The results indicated that the modification in S2 is a hydroxylation introduced in the ornithine head group. In the case of P1, OlsC introduces the hydroxyl group in the C2 position of the esterified fatty acid. To find the gene responsible for the hydroxylation within the ornithine head group (OlsE) a functional expression screening was performed. A cosmid bank from *R. tropici* CIAT899 was moved to *S. meliloti* 1021 and the transconjugants were analyzed for the presence of a modified OL. The DNA insert of a cosmid containing *olsE* was subcloned to indenting the *olsE* gene. OlsE belongs to the superfamily of fatty acid hydroxylases which is characterized by having two iron ions in its active center. Mutant deficient in *olsE* and double mutants deficient in *olsE* and *olsC* were constructed. Characterizing the mutants under acidic and high temperature conditions, it was concluded that ornithine lipids contribute to the resistance to high temperature and low pH in *R. tropici* CIAT899.

Analyzing the membrane lipid composition of the plant pathogen *Agrobacterium tumefaciens* we noticed that two distinct OLs are formed. It was studied whether OLs may play a role in stress tolerance and pathogenicity of *A. tumefaciens*. For this study *A. tumefaciens* mutants deficient in *olsB* and *olsE* were constructed and characterized. These mutants are devoid of OLs (Δ*olsB*) or form only the non-hydroxylated form of OL (Δ*olsE*). Both mutants were characterized under conditions of abiotic stress and in infection assays with potato discs. Remarkably, the absence of OLs promotes tumor formation in the host plants in shorter times, while no differences in growth were observed when comparing the mutants with the wild type under abiotic stress conditions.

1. Introducción

1.1. Membranas bacterianas

La membrana celular (también llamada membrana plasmática) separa el contenido del medio exterior y también funciona como una barrera selectiva para la entrada o la salida de moléculas. No obstante, las membranas no son simples límites estáticos, que separan unas regiones de otras, por el contrario son sistemas dinámicos encargados de muchas funciones, como producir ATP, transportar sustancias de modo selectivo al interior o al exterior de la célula, entre otras. Por otro lado, no todas las membranas son idénticas, ni homogéneas, de modo que los ejemplos de las funciones mencionados se observan en diferentes tipos de membrana.

Las bacterias se pueden dividir en dos grupos principales, las Gram positivas y las Gram negativas, ambos grupos se diferencian en la estructura de su envoltura celular (Fig. 1). Una característica de las Gram positivas es la presencia de una pared celular formada por varias capas de peptidoglicano. Además, tienen una membrana interna y el ácido teicoico, el cual es un polímero de glicerol o ribitol unidos mediante enlaces fosfodiéster; su presencia es una característica única de las bacterias Gram positivas (Madigan *et al.*, 2006).

Por otro lado, las bacterias Gram negativas contienen dos tipos de membranas, la membrana interna (MI) y la membrana externa (ME). Entre las dos membranas se encuentra un espacio osmóticamente activo denominado espacio periplasmático compuesto por una pared celular delgada de peptidoglicano, oligosacáridos derivados de la membrana (MDO), también llamados glucanos cíclicos, proteínas involucradas en el transporte de metabólitos, entre otros (Fig.1) (Rock,

2008). La composición de la ME es distinta de la MI. La MI está compuesta por fosfolípidos y proteínas, mientras que la ME además está compuesta de LPS que forma la monocapa externa de esta membrana. En la ME también se encuentran embebidas las porinas, que son unas proteínas específicas involucradas en el trasporte de compuestos de peso molecular elevado.

Otra característica particular de las bacterias Gram negativas es la presencia del lípido-A, la cual puede modificarse en condiciones de estrés o durante la interacción con su hospedero eucariota (Raetz *et al*., 2007) (Fig. 2). En *Salmonella typhimurium* y *Pseudomona aeruginosa* se ha descrito la 2-hidroxilación de un residuo esterificado de ácido miristico del lípido-A (Fig. 2). En el caso de *S. typhimurium* se ha hipótetizado que esta hidroxilación es importante para la patogénesis de esta bacteria (Gibbons *et al*., 2000; Gibbons *et al*., 2008). La 2-hidroxilación la cataliza la dioxigenasa LpxO dependiente de $Fe2+/O2/\alpha$-cetoglutarato para producir el 2-OH-lípido-A-Kdo2 y ocurre después de que el residuo acilo se une a la molécula del lípido A, (Fig. 2) (Gibbons *et al*., 2000; Gibbons *et al*., 2008). La expresión del gen *lpxO* se induce cuando *S. typhimurium* crece en bajas concentraciones de Mg^{2+} (Gibbons *et al*., 2005).

Nikaido y Gibbons *et al*. (2003; 2008), propusieron que las hidroxilaciones sobre el lípido-A podrían incrementar los enlaces de hidrógeno, entre las moléculas adyacentes de los lípidos que las poseen, incrementando la estabilidad e impermeabilidad de la ME a ciertos compuestos en algunas condiciones de crecimiento (Nikaido, 2003; Gibbons *et al.,* 2008).
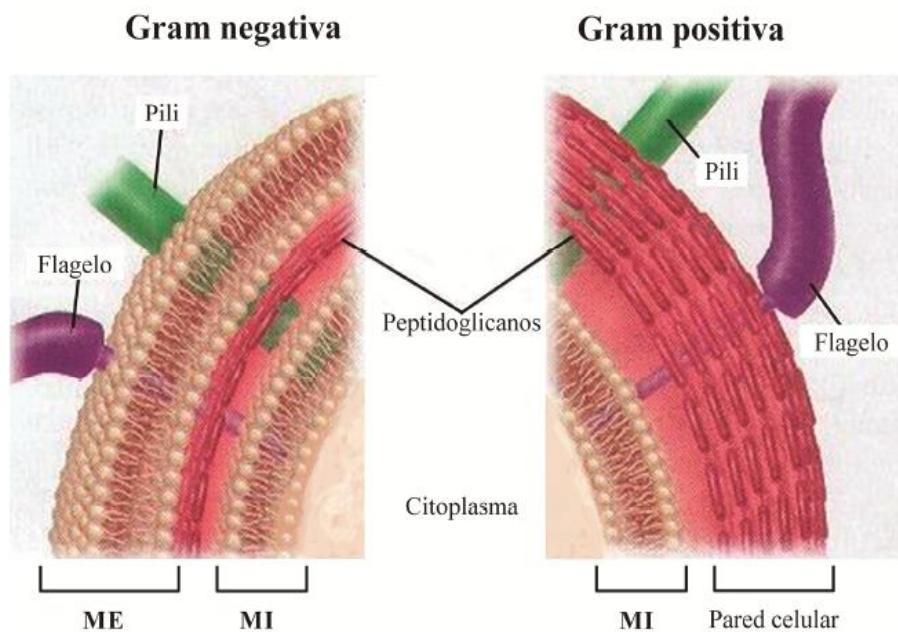
Figura 1. Representación esquemática de la envoltura celular de bacterias Gram negativas y Gram positivas. Las bacterias Gram negativas presentan las siguientes características: una membrana citoplasmática o membrana interna (MI) compuesta principalmente de fosfolípidos, una capa delgada de peptidoglicanos (Más delgada que en las bacterias Gram positivas), y una membrana externa (ME) que contiene lipopolisacáridos (LPS). Entre la MI y la ME existe un espacio denominado espacio periplásmico. No obstante, las bacterias Gram positivas generalmente presentan las siguientes características: la MI, una capa gruesa de peptidoglicanos, y la presencia del ácido teicoico, el cual es una característica única de las bacterias Gram positivas. En el caso de que se presenten, los pili y los flagelos se encuentran embebidos a las dos membranas. Figura tomada y modificada de http://www.biologycorner.com.
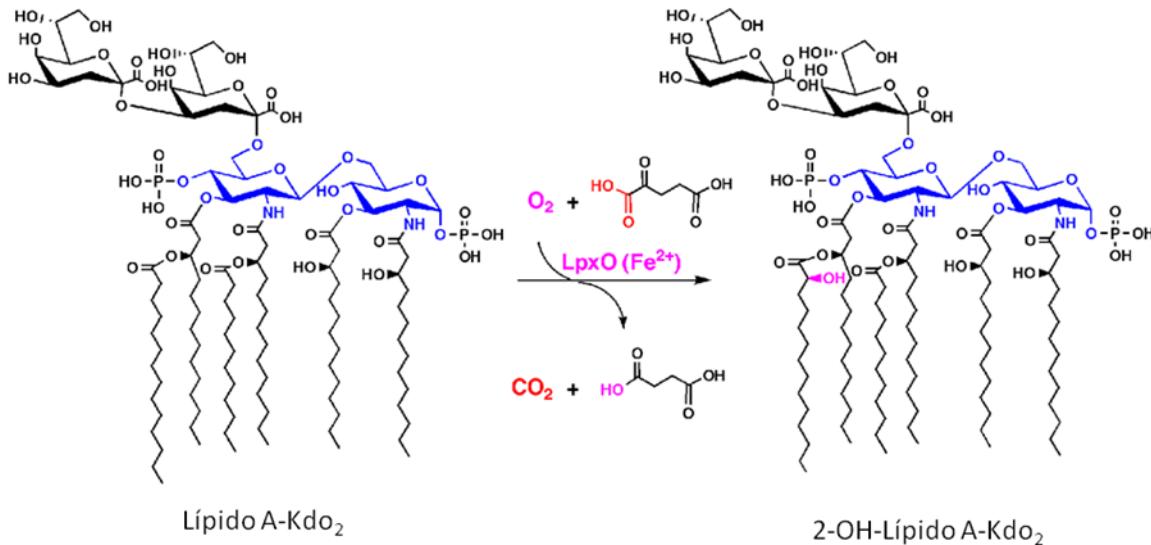
Figura 2. Estructura del lípido- A-Kdo2 de *E. coli* y la reacción catalizada por LpxO en *S. typhimurium* propuesta por Gibbons *et al*. (2008). El LpxO cataliza la 2-hidroxilación del residuo de miristato unido al C3 del acilo que está unido a la posición 3′ del lípido-A-Kdo2 hexaacilado para producir el 2-OH- lípido-A-Kdo2. Modificado de Gibbons *et al*. (2008).

1.2. Lípidos de membrana de bacterias

La función estructural de los lípidos de membrana es esencial para los organismos. Estos lípidos son anfifílicos y consisten de dos cadenas hidrofóbicas largas de acilo o alquilo y un grupo cabeza hidrofílico (Fig. 3) (Dowhan, 2008). En la bacteria modelo *Echerichia coli*, los principales lípidos de membrana son los glicerofosfolípidos: fosfatidiletanolamina (PE), fosfatidilglicerol (PG) y cardiolipina (CL) (Fig. 3) (Rock, 2008). Otros glicerofosfolípidos tales como fosfatidilserina (PS), fosfatidilcolina (PC) y fosfatidilinositol (PI), no se encuentran, ni se acumulan en *E. coli*, aun así, están presentes en grupos específicos de bacterias. Cabe mencionar, que formas deaciladas de fosfolípidos, llamados liso-fosfolípidos, se han encontrado en la membrana celular de algunas bacterias (Hoischen et. al., 1997; Sandoval- Calderón *et al*., 2009).
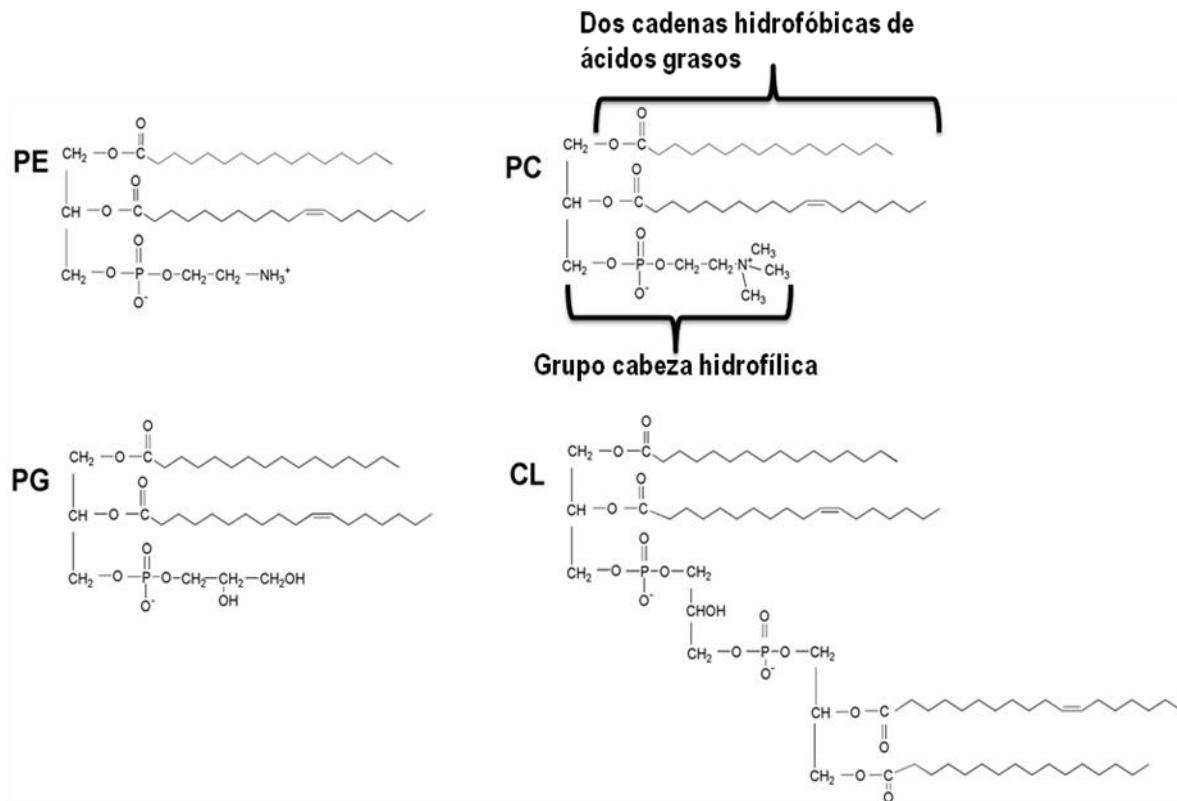
Figura 3. Estructuras de algunos fosfolípidos presentes en bacterias. Fosfatidiletanolamina (PE), fosfatidilglicerol (PG), cardiolipina (CL) y fosfatidilcolina (PC). En el caso de la PC la parte hidrofóbica y la parte hidrofílica del fosfolípido está señalada.

1.3. Biosíntesis de fosfolípidos en bacterias

En *E. coli* se han reportado dos vías para la biosíntesis de glicerol-3-fosfato (G3P). Una de ellas lo produce directamente a partir de glicerol por la actividad de una glicerol cinasa (GlpK), mientras que la otra vía es a través de la reducción de dihidroxiacetona fosfato, catalizada por la G3P deshidrogenasa (GpsA) (Cronan y Rock, 1996).

Se han descrito dos rutas diferentes para el primer paso en la formación de ácido fosfatídico (PA). En *E. coli* se inicia con la acilación de G3P mediante la G3P aciltransferasa (PlsB), reacción por muchos años se pensaba que era la común en las bacterias. Sin embargo, recientemente se ha demostrado que en la gran mayoría de las bacterias la acilación de G3P no la cataliza la PlsB, sino lo hace a través de la nueva vía PlsX/PlsY (Cronan y Rock, 1996, Lu *et al*., 2006) (Fig. 4). PlsX cataliza la conversión de la forma acilada de la proteína acarreadora de grupos acilo (acil-ACP) y fosfato inorgánico a ACP y acil-fosfato. En un segundo paso, PlsY transfiere el grupo acilo de acil-fosfato a G3P formando 1-acil-G3P (ácido lisofosfatídico). La segunda acilación para la biosíntesis del PA catalizada por PlsC ocurre en todas las bacterias estudiadas hasta el momento (Cronan y Rock, 1996) (Fig. 4). Posteriormente, PA es activado a CDP-diacilglicerol, considerado como el intermediario central activado en bacterias, para la formación de los distintos fosfolípidos de la membrana. La conversión de PA a difosfato de citidina-diacilglicerol (CDP-DAG) la cataliza la CDP-DAG sintasa (CdsA) (Fig. 4) (Rock, 2008).

El fosfolípido aniónico PG se sintetiza en dos pasos a partir de CDP-DAG. La fosfatidilglicerofosfato sintasa (PgsA) transfiere *sn*-glicerol- 3-fosfato a CDP-DAG, generando como productos fosfatidilglicerofosfato (PGP) y CMP. Luego PGP se defosforila rápidamente por una PGP

fosfatasa para la formación de PG. Con respecto al anterior, en *E. coli* se han reportado dos familias de fosfatasas que pueden tener esa actividad (PgpA y PgpB) (Rock, 2008). En la síntesis de CL, la cardiolipina sintasa (Cls), del tipo procariota, cataliza la condensación de dos moléculas de PG, liberando glicerol en una reacción de trans-esterificación.

La síntesis del fosfolípido zwiteriónico PE se da en dos pasos a partir de CDP-DAG. Inicialmente, la PS sintasa (PssA) condensa el CDP-DAG con el aminoácido L-serina, para formar fosfatidilserina (PS) y liberar CMP. En el segundo paso ocurre la descarboxilación de PS, catalizado por la PS descarboxilasa (Psd), dando como producto PE y CO_2 (Rock, 2008). Se han reportado dos vías para la síntesis de PC: la ruta de metilación y la ruta de la fosfatidilcolina sintasa. En la primer vía se sintetiza PC a través de tres metilaciones sucesivas de PE, utilizando el donador de grupos metilo *S*-adenosil-L-metionina (SAM) y la enzima fosfatidiletanolamina *N*-metiltransferasa (PmtA), para formar monometil-PE, dimetil-PE y PC (de Rudder *et al*., 2000). En la vía de la fosfatidilcolina sintasa (Pcs), la Pcs cataliza la condensación de colina con CDP-DAG para formar CMP y PC (de Rudder *et al*., 1997; de Rudder *et al*., 1999 y Sohlenkamp *et al*., 2000). La vía de Pcs es una vía exclusiva dentro de las eubacterias (Sohlenkamp *et al*., 2003) (Fig. 4).
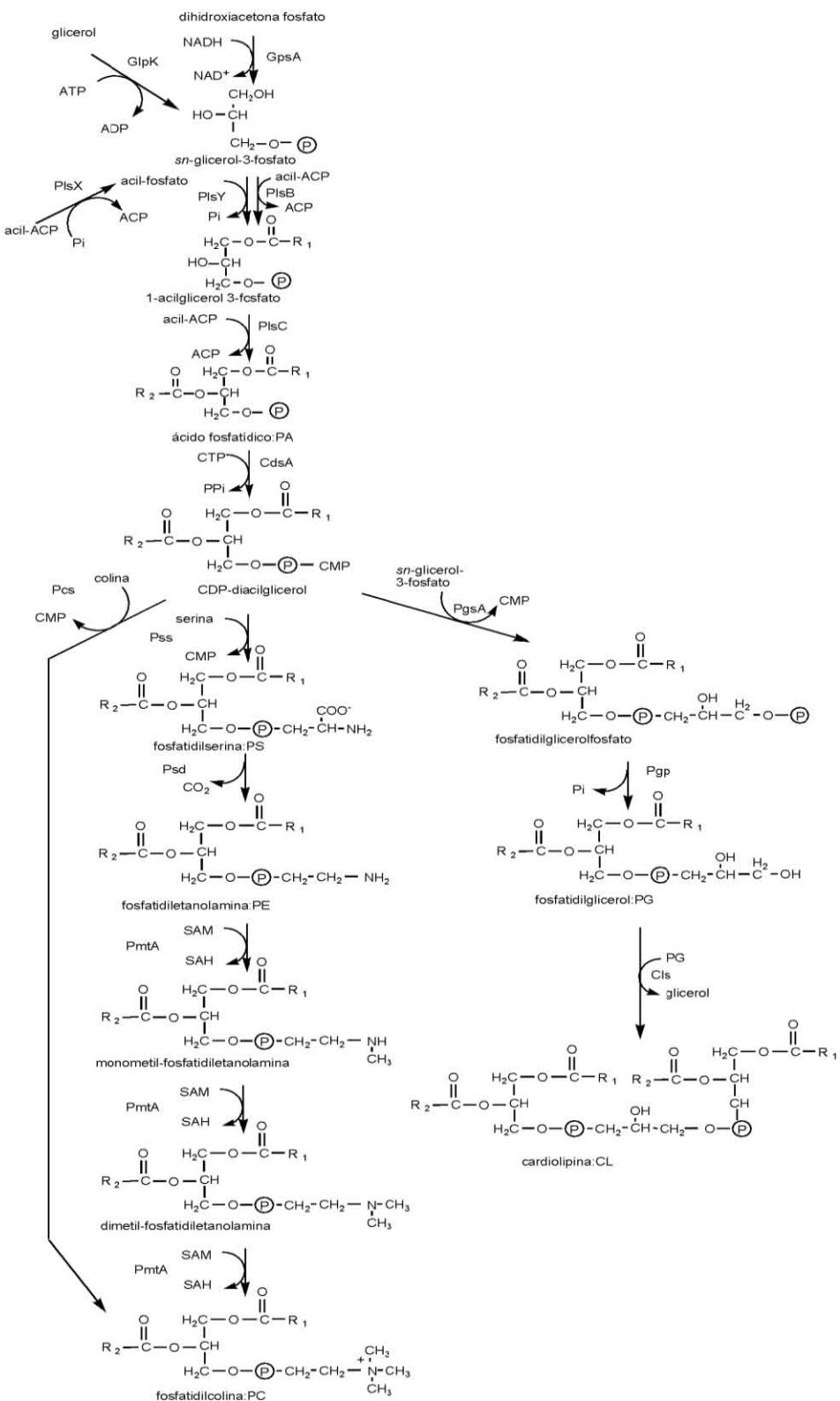
Figura 4. Biosíntesis de glicerofosfolípidos en eubacterias. Los principales fosfolípidos de *E. coli* son PE, PG y CL. *S. meliloti* sintetiza PC además de los fosfolípidos que contiene *E. coli*. Tomada y modificada de López-Lara *et al*., 2003.

Además de los fosfolípidos existen otros lípidos de membrana que carecen de fósforo, ejemplo de ellos son: (I) el sulfolípido sulfoquinovosildiacilglicerol (SL), (II) la diacilgliceriltrimetilhomoserina (DGTS), que es un lípido de betaína y (III) los lípidos de ornitina (OLs) (López- Lara *et al*., 2003; Geiger *et al*., 2010).

1.4 Biosíntesis de lípidos de membrana sin fósforo en bacterias

El fósforo (P) en forma de fosfato inorgánico participa en la transferencia de energía, así como en el metabolismo del nitrógeno y del carbono. Sin embargo, uno de los grandes problemas del fósforo es su baja biodisponibilidad en los suelos, debido a su baja solubilidad y alta adsorción en el suelo (Bieleski, 1973). La respuesta bacteriana bajo limitación de fosfato más estudiada es la del sistema de dos componentes PhoU y PhoB (Ulrich, *et al*., 2005). El regulador de respuesta PhoB reconoce las cajas Pho en los promotores de los genes regulados por la limitación de fosfato, mientras que PhoU regula negativamente las cajas Pho. Uno de los procesos celulares inducidos en estas condiciones es la remodelación en la membrana. Se ha propuesto la degradación de los fosfolípidos de la membrana, de obtención de P, para poder ocuparl fósforo en ellos contenido en otros procesos celulares, como por ejemplo la síntesis de ácidos nucléicos (Ulrich, *et al*., 2005).

Cuando *Sinorhizobium meliloti* 1021 se cultiva en medios con concentraciones elevadas de fosfato inorgánico, sus principales lípidos de membrana son PG, CL, PE y PC. En contraste, cuando se cultiva en medios con concentraciones limitantes de fosfato inorgánico, la mayor parte de sus fosfolípidos son reemplazados por lípidos de membrana sin fosforo como el sulfolípido

sulfoquinovosildiacilglicerol (SL), la diacilgliceriltrimetilhomoserina (DGTS) y los lípidos de ornitina (OLs) (Geiger *et al*., 1999).

Por otra parte, en la bacteria fotosintética *Rhodobacter sphaeroides*, en condiciones de crecimiento limitantes de fosfato inorgánico, las funciones de PG pueden ser reemplazadas por SL, y las de PC por el lípido de betaína DGTS, y las de PE por los OLs. Adicionalmente, forma un glucolípido que no contienen fósforo, que posee en su grupo cabeza glucosa o galactosa (Benning *et al*., 1995).

Por otro lado, también se ha observado que tanto en *Pseudomonas fluorescens* como en *Pseudomonas diminuta*, bajo esa condición de estrés, los fosfolípidos de membrana son parcialmente reemplazados por glucolípidos y por los OLs (Minnikin y Abdolrahimzadeh, 1974 y Minnikin *et al*., 1974).

1.4.1. Biosíntesis de sulfolípidos

El sulfolípido sulfoquinovosil-diacilglicerol (SL) se encuentra ampliamente distribuido en organismos fotosintéticos, desde las bacterias hasta las plantas (Benning *et al*., 1993). Los SL no son esenciales para la fotosíntesis, pero se ha observado que son necesarios para el crecimiento de *R. sphaeroides* y las cianobacterias en condiciones limitantes de fósforo. Se ha observado en *R. sphaeroides* una correlación inversa entre la cantidad de fosfolípidos y los sulfolípidos en condiciones limitantes de fosfato. Esto ha sugerido que el lípido aniónico SL sustituye al lípido aniónico PG en condiciones limitantes de fosfato inorgánico (Benning *et al*., 1993). La presencia de SL también se ha detectado en bacterias rizobias de distintas especies, entre ellos: *S. meliloti*,

Rhizobium leguminosarum y *Sinhizobium fredii* NGR234. Si bien en *S. meliloti* se desconoce la función de los SLs, se ha demostrado que su biosíntesis depende de la condición limitante de fosfato inorgánico (López-Lara *et al*., 2005).

En *R. sphaeroides* se requieren cuatro genes estructurales para la biosíntesis de sulfolípidos (*sqdA*, *sqdB*, *sqdC* y *sqdD*). El gen *sqdB* está altamente conservado en las bacterias y tiene un ortólogo en plantas, SQD1, que codifica para una enzima implicada en la biosíntesis de UDP-sulfoquinovosa desde UDP-glucosa y sulfito como donador de azufre (Sanda *et al*., 2001). Por otro lado, los genomas de algunas archeas (*Thermoplasma* y *Sulfolobus*), también tienen homólogos de SqdB (López-Lara *et al*., 2003), no obstante, aún no se ha demostrado la presencia de SL en archeas. Por otra parte, las mutantes de *S. meliloti*, deficientes de *sqdB,* aún son capaces de formar nódulos fijadores de nitrógeno con su planta hospedera alfalfa (Weissenmayer *et al*., 2000).

El gen *sqdA* codifica para una aciltransferasa cuya función bioquímica no se conoce en detalle (Benning, 2007). Finalmente, se piensa que los productos de *sqdC* y *sqdD* catalizan la transferencia de sulfoquinovosa de UDP-sulfoquinovosa a DAG, sin embargo, sigue sin definirse bien el mecanismo (Benning, 2007).

1.4.2. Biosíntesis de lípidos de betaína

El lípido de betaína, diacilgliceril-*N,N,N*-trimetilhomoserina (DGTS), forma parte de las membranas tanto en los organismos eucariotas inferiores, como en las algas verdes, los musgos y los helechos, así como, en los hongos (Künzler y Eichenberger, 1997 y Furlong *et al*., 1986).

En el grupo de las α-proteobacterias se ha identificado a DGTS como un lípido sin fosfato, que reemplaza a PC en condiciones limitantes de fosfato inorgánico (Benning *et al*., 1995; Geiger *et al*., 1999). Aparentemente, existe una correlación inversa entre el contenido de PC y la de DGTS (López-Lara *et al*., 2005). Estos lípidos son zwiteriónicos, lo que sugiere que pueden ser intercambiables para funciones esenciales en esas bacterias.

Se estima que la presencia de lípidos de betaína tipo DGTS es limitada en bacterias. La primera α-proteobacteria en la que se describió la formación de DGTS fue en *R. sphaeroides* (Benning *et al*., 1995). Se encuentran homólogos de BtaA y BtaB en algunos órdenes de α-proteobacterias como: *Rhodobacterales*, (los géneros *Rhodobacter, Roseobacter, Sagittula y Stappia*)*, Esfingomonadales*, (los géneros *Sphingomonas y Erythrobacter*) y *Rhizobiales*, (los géneros *Rhizobium, Agrobacterium, Sinorhizobium, Ochrobactrum, Mesorhizobium, Beijerinckia, Rhodopseudomonas*) y algunos miembros de *Planctomycetes* como *Planctomyces, Blastopirellula* y *Rhodopirellula* (Geiger *et al*., 2010).

En las α-proteobacterias, la biosíntesis de DGTS involucra dos pasos: (I) La enzima BtaA (una S-adenosilmetionina/diacilglicerol 3-amino-3-carboxipropil transferasa) convierte al DAG en diacilgliceril-homoserina (DGHS), mediante la formación del enlace éter, donde SAM funciona como un donador del grupo homoserina. (II) La S-adenosilmetionina/diacilglicerol-homoserina-N-metiltransferasa (BtaB) metila tres veces a DGHS para formar la DGTS (Klug y Benning, 2001).

Con respecto a la función de DGTS, López-Lara *et al*., 2005, propusieron que estos lípidos favorecen el crecimiento de ciertas bacterias simbiontes, como *S. meliloti*, en condiciones limitantes

de fósforo. Por otro lado, en patógenos oportunistas la presencia de DGTS depende del entorno donde se encuentre, ya que en *Brucella* spp, que es un patógeno intracelular obligado, tanto DGTS como los genes de la biosíntesis del mismo no están presentes debido a la abundancia de fosfato en el medio. En contraste, se ha reportado la presencia de DGTS en el patógeno oportunista de vida libre *Ochrobacter anthropi*, que habita en suelos donde generalmente las condiciones de fosfato son limitantes (Geiger *et al.*, 2010).

1.4.3. Lípidos de ornitina

1.4.3.1. Distribución y estructura de los lípidos de ornitina

Los lípidos de ornitina (OLs) se encuentran ampliamente distribuidos entre las bacterias Gram negativas. Por otro lado, se ha reportado su presencia en algunas bacterias Gram positivas, como *Streptomyces*, y también en *Mycobacterium*, pero están ausentes en arqueas y eucariontes.

La estructura de los OLs presente en diferentes bacterias es la α-*N*- (aciloxi-acil)-ornitina (López-Lara *et al.*, 2003, Knoche y Shively, 1972; Geiger *et al.*, 1999). Existen otros lípidos análogos a los OLs (Fig. 5), que en lugar de contener un residuo de ornitina presentan otro aminoácido como lisina, glicina o glutamato, y se denominan como: lípidos de lisina (LLs), lípidos de glicina (GLs), y lípidos de glutamina (Geiger *et al.*, 2010).

La estructura de los OLs, que se ha elucidado por resonancia magnética nuclear, es consistente con la descrita 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). 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).

Actualmente se conoce poco sobre las funciones que desempeñan los OLs, en las bacterias que los poseen. Las mutantes de *S. meliloti* 1021 y de *P. aeruginosa,* deficientes en la biosíntesis de OLs, no muestran ninguna alteración sobresaliente en su fenotipo macroscópico, cuando se

compara con la cepa silvestre (Weissenmayer *et al*., 2002; López- Lara *et al*., 2005; Lewenza *et al*., 2011).

1.4.3.2. Biosíntesis de los lípidos de ornitina

La biosíntesis de los OLs describió por primera vez en *S. meliloti*. En el primer paso, participa la *N*-aciltransferasa OlsB, que cataliza la transferencia de un grupo acilo 3- hidroxilado, desde una proteína acarreadora de acilos (ACP), al grupo α-amino de la ornitina, formando el lisolípido de ornitina (LOL). En el segundo paso, la *O*-aciltransferasa OlsA cataliza la transferencia de otro grupo acilo, desde una ACP, al grupo 3-hidroxilo del LOL, formando el OL (Gao *et al*., 2004; Weissenmayer *et al*., 2002) (Fig. 5).

1.4.3.2.1 Aciltransferasas OlsB y OlsA

La transcripción del gen *olsB* en *S. meliloti* 1021, que está bajo el control del regulador transcripcional PhoB, se induce cuando se cultiva en condiciones limitantes en fosfato (Krol y Becker, 2004). La expresión constitutiva de *olsB* en *S. meliloti* 1021, incrementa la formación de OLs de manera independientemente a la concentración de fosfato presente en el medio (Gao *et al*., 2004). Sin embargo, muchas otras bacterias como *Brucella abortus*, *Burkholderia cepacia, Rhizobium tropici* CIAT899 y *Thiobacillus thiooxidans* sintetizan OLs, en cantidades relativamente altas, aún cuando se cultivan en medios ricos en fosfato (Palacios-Chaves *et al*., 2011, Taylor *et al*., 1998, Rojas-Jiménez *et al*., 2005, Dees y Shively 1982). Mutantes deficientes en el gen *olsB* derivadas de *S. meliloti* 1021, *Rhodobacter capsulatus* y *Brucella abortus* son incapaces de formar

OLs. (Gao *et al*., 2004; Aygun-Sunar *et al*., 2006; Palacios-Chaves *et al*., 2011). Por otro lado, la expresión independiente de OlsB en una cepa de *E. coli,* que carece de los genes para la biosíntesis de los OLs, induce la formación de pequeñas cantidades de LOL (Gao *et al*., 2004).

Los resultados anteriores permitieron proponer que OlsB es una *N*-aciltransferasa. Esta hipótesis se corroboró mediante ensayos enzimáticos, donde se demostró que OlsB condensa una molécula de 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 (Fig. 5). Posteriormente, se realizó un análisis bioinformático, que indicó que la enzima OlsB está conformada por 296 aminoácidos, carece de hélices transmembranales y es soluble en agua. La caracterización funcional de OlsB permitió asignar una función específica para un grupo de proteínas ortólogas (COG3176), previamente asignadas como hipotéticas o como hemolisinas putativas (Gao *et al*., 2004).

Por otro lado, con respecto a OlsA se cuenta con muy poca información. Weissenmayer *et al*., 2002 realizaron un análisis bioinformático que mostró que OlsA de *S. meliloti* 1021 es una proteína de 292 aminoácidos, con una probable hélice transmembranal cercana al extremo *N*-terminal. Existen varios trabajos referentes a la construcción de mutantes deficientes en el gen *olsA,* de *S. meliloti* 1021, *R. capsulatus*, *Brucella abortus* y *Pseudomonas aeruginosa*. En todos los casos se observó que las bacterias mutantes fueron incapaces de formar OLs. (Aygun-Sunar *et al*., 2006; Palacios-Chaves *et al*., 2011; Lewenza *et al*., 2011).
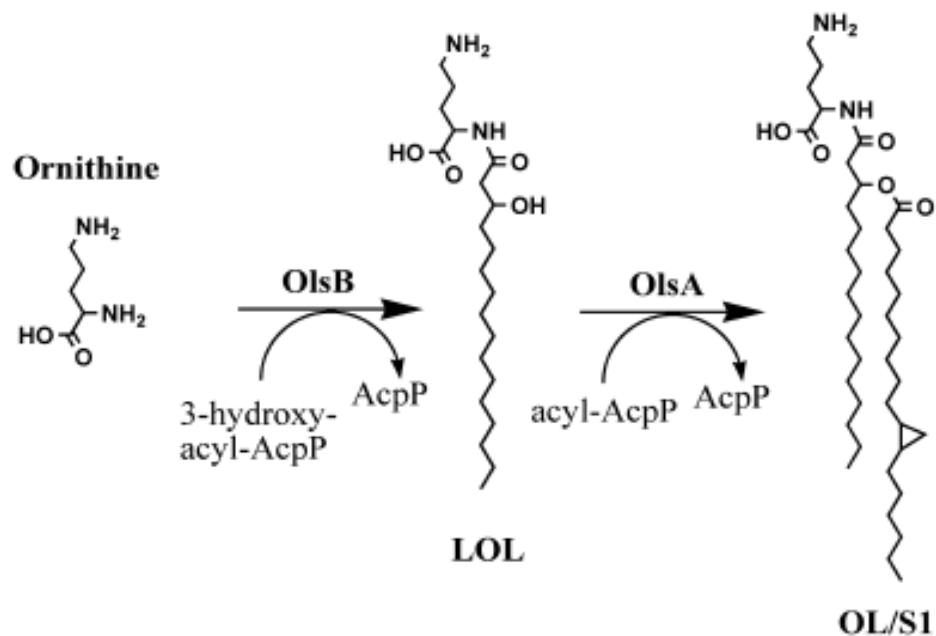
Figura 5. Biosíntesis y estructura del lípido de ornitina. Tomada de Vences-Guzmán *et al*. (2012).

2. Antecedentes

Rhizobium tropici CIAT899 es una bacteria tolerante a pH bajo y a temperaturas elevadas, es un simbionte de plantas leguminosas de gran importancia alimenticia como el frijol (Martínez-Romero *et al*., 1991). Esta cepa forma cuatro clases moleculares de OLs que se han nombrando S1, S2, P1 y P2 (Fig. 6B) (Rojas-Jiménez *et al*., 2005). Mutantes deficientes en el gen *olsC* (899-*olsC*Δ), no forman los OLs P1 ni P2 (Fig. 6C). El producto codificado por el gen *olsC* es una hidroxilasa, homóloga putativa a LpxO, que puede convertir los OLs (S1 y S2) menos polares, a las formas más polares, (P1 y P2) (Rojas-Jiménez *et al*., 2005). Mediante un análisis bioinformático se observó, que la proteína OlsC de *R. tropici* CIAT899 contiene 281 aminoácidos y es soluble en agua (Rojas-Jiménez *et al*., 2005).

Previamente, Dees y Shively (1982) reportaron que cuando la bacteria *Thiobacillus thiooxidans*, crece en ambientes ácidos, forma lípidos de ornitina, los cuales se encuentran localizados mayoritariamente en la ME, y que estos mismos podrían estar involucrados en la resistencia a la acidez. Rojas-Jiménez *et al*. (2005) reportaron que la cepa mutante 899-*olsC*Δ, en condiciones de acidez (pH 4.5), creció de forma similar a la cepa silvestre, ya que ambas cepas alcanzaron una D.O.$_{600\ nm}$ de 1.6. Sin embargo, la complementación de dicha mutante, a pH 4.5, con un fragmento que contenía a *olsC* en un vector de alto número de copias, afectó significativamente el crecimiento de dicha cepa, ya que el máximo crecimiento observado nunca fue mayor a una D.O.$_{600\ nm}$ de 0.4 (Rojas-Jiménez *et al*., 2005). Estos antecedentes indicaban que existe una correlación entre la presencia de OLs y la respuesta a este tipo de estrés.

Por otro lado, también se observó que la cepa mutante 899-*olsCΔ1* formó el doble de nódulos en plantas de frijol (peso/número de nódulos), tras 21 días de inoculación, sin embargo, estaban poco desarrollados y carecían de lenticelas, además de que fijaban menos nitrógeno que la cepa silvestre. (Rojas-Jiménez *et al*., 2005). Cabe mencionar que no se conoce el gen ni la enzima responsable de la conversión de los OLs S1 y P1 a S2 y P2 (Fig. 6A).

Con respecto a la función de los OLs en la patogénesis existe la posibilidad de que estén involucrados en la resistencia al estrés impuesto por el sistema inmune del hospedero. Los OLs al igual que el lípido-A, están en la membrana externa, por lo que podrían tener una función análoga (Dees y Shively, 1982). Se ha reportado que el sistema inmune de las plantas reconoce la estructura del lipooligosacárido del lípido-A de las bacterias patógenas de las plantas, como *Xanthomonas campestris* (Silipo *et al* 2005). Probablemente, el lípido-A y el núcleo de la estructura de dicha molécula desencadenan respuestas celulares defensivas en las plantas en contra de *X. campestris*.
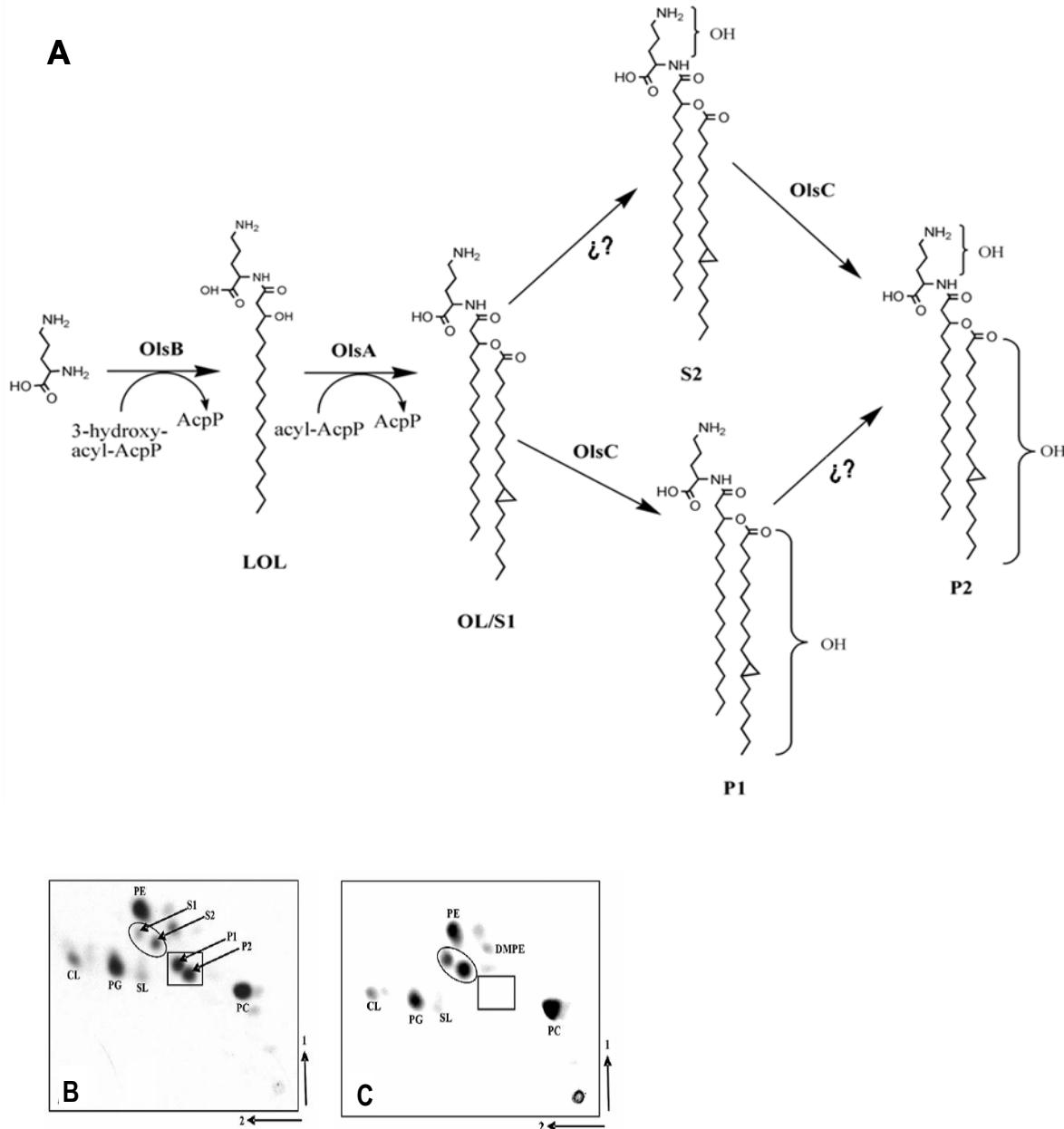
Figura. 6. **A:** Modelo propuesto para la biosíntesis de los OLs en *R. tropici* CIAT899, obsérvese que la enzima responsable de la conversión de S1 a S2 y de P1 a P2 se desconoce. **B y C:** Cromatografía en capa fina (TLC) de dos dimensiones **B:** mostrando la separación de lípidos de la cepa *R. tropici* CIAT899 marcados con [¹⁴C] acetato. **C:** mostrando la separación de lípidos de la cepa mutante 899-*olsC*-1 marcados con [¹⁴C] acetato (Rojas-Jiménez *et al.* 2005). PG: fosfatidilglicerol, CL: cardiolipina, PC: fosfatidilcolina, PE: fosfatidiletanolamina, MMPE: monometil PE, DMPE: dimetil PE, S1: lípido de ornitina no modificado y S2, P1 y P2: lípidos de ornitina modificados.

Antecedentes directos de nuestro laboratorio mostraron que la cepa de *Agrobacterium tumefaciens* C58, cuyo genoma está secuenciado, forma un OL con el mismo Rf que el OL S2 de *R. tropici* CIAT899, sugiriendo que posiblemente se trata del mismo tipo de modificación estructural del OL en ambos casos. Posteriormente, se hicieron búsquedas tipo BLAST en el genoma de *A. tumefaciens* C58 usando como sonda las secuencias de OlsC de *R. tropici* CIAT899, pero no se lograron identificar homólogos cercanos.

Por lo anteriormente mencionado se propone, que los OLs desempeñan un papel en la respuesta a condiciones de estrés, durante el establecimiento de la simbiosis y de la patogenicidad. La falta de conocimiento sobre al biosíntesis de los OLs ha sido uno de los principales obstáculos para caracterizar la función de los mismos.

3. Relevancia e impacto

Los OLs pueden sufrir una gran variedad de modificaciones estructurales en organismos como *Rhizobium tropici* CIAT899, *Agrobacterium tumefaciens*, *Burkholderia cepacia* o *Brucella melitensis.* Sin embargo, la función y la elucidación estructural exacta de las distintas variedades de OLs, así como las enzimas responsables de algunas de estas modificaciones, continúa siendo un problema a resolver. La descripción de la biosíntesis de las formas modificadas de OLs, y de la función de estos lípidos en las bacterias, contribuirá a entender mejor las interacciones de éstas con sus hospederos eucariotas.

4. Hipótesis

Con base en las observaciones de la composición lipídica de una cepa simbiótica, *Rhizobium tropici* CIAT899, y de otra patógena, *Agrobacterium tumefaciens* C58, se propone que existe un gen que codifica para la(s) enzima(s) responsable(s) en la modificación del lípido de ornitina de S1 a S2.

5. Objetivos

De *R. tropici* CIAT899 y *A. tumefaciens* C58:

1. Purificar las variantes estructurales de sus OLs y elucidar su estructura química.

2. Identificar el gen o los genes que codifican para la(s) enzima(s) que realizan la conversión del S1 a S2.

3. Identificar la función de las diferentes formas de los OLs bajo distintas condiciones de estrés abiótico en vida libre, y en asociación con su hospedero.

6. Resultados

Los resultados obtenidos durante el desarrollo del presente trabajo de tesis se dividieron en tres secciones:

> La primer sección corresponde al artículo "Hydroxylated ornithine lipids increase stress tolerance in *Rhizobium tropici* CIAT899". Publicado en Molecular Microbiology. 2011; 79:1496- 1514. Vences-Guzmán, M. A., Guan, Z., Ormeño-Orrillo, E., González-Silva, N., López-Lara, I. M., Martínez-Romero, E., Geiger, O., and Sohlenkamp, C.

La segunda sección es acerca de un artículo de revisión, donde se incluyeron los resultados de las posibles funciones de las hidroxilasas de OLs, "Ornithine lipids and their structural modifications: from A to E and beyond". Publicado en FEMS Microbiology Letters 2012; 355 (1): 1-10. Vences-Guzman, M. A., Geiger, O., and Sohlenkamp, CLa tercera sección trata sobre el artículo "Agrobacteria lacking ornithine lipids induce more rapid tumour formation Environmental Microbiology". 2013; 15(3):895-906. Vences-Guzmán, M. A., Guan, Z., Bermudez-Barrientos, J. R., Geiger O., and Sohlenkamp, C.

6.1. Artículo 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 *Rhizobium tropici* CIAT899.** Molecular Microbiology. 79:1496-1514.

Los OLs están constituidos por un residuo de ornitina y dos ácidos grasos, y están ampliamente distribuidos en las bacterias Gram negativas. El primer ácido graso presenta una hidroxilación en el carbono 3 y está unido al grupo α-amino de la ornitina por un enlace amido, el otro ácido graso está esterificado al grupo hidroxilo libre del ácido graso amidificado. Adicionalmente, en algunas bacterias los OLs pueden presentar algún tipo de modificaciones como por ejemplo hidroxilaciones en el ácido graso esterificado. Se ha reportado que esta modificación está involucrada en la tolerancia al estrés.

Rhizobium tropici CIAT899 es una bacteria resistente a diferentes tipos de estrés, como pH bajo y altas temperaturas, y forma nódulos en las raíces de plantas de frijol, en los cuales fija nitrógeno. Si bien se sabía que *R. tropici* CIAT899 forma cuatro clases de OLs, se desconocía tanto la función de los mismos como sus respectivas rutas de biosíntesis.

En esta investigación se logró describir que la biosíntesis de los OLs, en *R. tropici* CIAT899, se incrementa bajo condiciones de pH ácido (4.0) y temperatura elevada (42°C) y que la membrana externa está enriqueca de éstos.

Adicionalmente, mediante un escrutinio de expresión funcional, se identificó a la hidroxilasa OlsE de OLs, la cual en combinación con la hidroxilasa OlsC llevan a cabo la biosíntesis de los OLs modificados en *R. tropici* CIAT899. A diferencia de otras hidroxilaciones descritas, la hidroxilación que cataliza OlsE ocurre en el residuo de ornitina. También se describió que la enzima OlsC introduce un grupo hidroxilo en el carbono 2 del ácido graso esterificado.

Con el objetivo de determinar la función de los OLs, tanto de los modificados o como los que no, se construyeron y caracterizaron mutantes deficientes en OlsE, OlsC y dobles mutantes OlsC/OlsE. Se observó que las mutantes, derivadas de *R. tropici* CIAT899, deficientes en la hidroxilación de OLs, codificado por OlsC, son más susceptibles al estrés ácido y a la temperatura elevada.

En cuanto a la simbiosis en plantas de frijol, las tres mutantes que carecían de las hidroxilasas de OLs presentaron alteraciones, en relación al peso/número de nódulos, con respecto a la cepa silvestre. Por un lado, la mutante en OlsC formó el doble de nódulos, pero fijó solamente el 50% de nitrógeno. Mientras que las mutantes en OlsE y las dobles mutantes OlsC/OlsE formaron el mismo número de nódulos, pero también fijaron menos nitrógeno (33 y 25% respectivamente) (ver figura suplementaría S2 del artículo).

En conclusión, el gen *olsE* codifica para una enzima que está involucrada en la hidroxilación de los OLs S1 y P1 de *R. tropici* CIAT 899. OlsC junto con OlsE son responsables de formar los OLs modificados S2 y P2. Los resultados obtenidos permitieron proponer que estos lípidos contribuyen en la resistencia de diferentes tipos de estrés abiótico (pH ácido, temperaturas altas), además se encontró que tienen un papel en la interacción planta-bacteria

6.2. Artículo 2

En esta revisión se tocaron varios puntos relevantes acerca de los OLs, así como las enzimas que participan en la biosíntesis de estos lípidos, las enzimas que participan en su modificación; también se discute sobre su distribución en bacterias y su probable función.

El primer reporte sobre los dos genes estructurales *olsA* y *olsB*, involucrados en la biosíntesis de OLs no modificados, fue en *S. meliloti*. No se ha descrito hasta ahora la presencia de OLs en ningún eucariota, ni tampoco se han encontrado genes homólogos a *olsA* u *olsB* en las secuencias de genomas de eucariotas. Alrededor de 25% de las especies bacterianas secuenciadas tienen homólogos de *olsB*. Sin embargo, están ausentes en algunas especies en las que se ha reportado la presencia de OLs, como en *Serratia* sp, *Sorangium cellulosum* y *Flavobacterium* sp. Considerando estos antecedentes, se propuso que existe una nueva ruta metabólica de síntesis de OLs, independiente de OlsB, en *Serratia* sp, *Sorangium cellulosum* y *Flavobacterium* sp. y probablemente en algunas bacterias más.

Por otro lado, *Burkholderia cenocepacia* J2315 es una bacteria Gram negativa conocida por ser patógeno oportunista de humanos. Una característica distintiva del género *Burkholderia* es su repertorio de lípidos polares de membrana, entre los que se encuentran: la fosfatidiletanolamina (PE), los OLs y sus respectivos derivados hidroxilados en el carbono 2 del ácido graso esterificado

(2-OH-PE y 2-OH-OL, respectivamente). Se ha propuesto que el OL 2-hidroxilado, podría ser factor de virulencia. Para *Salmonella typhimurium* existe el antecedente de que los residuos miristato esterificados del lípido A, pueden presentar la modificación 2-OH, la cual es realizada por la enzima LpxO. Sin embargo, la hidroxilasa responsable de incorporar la sustitución 2-OH en los OLs de *B. cenocepacia* J2315 no se conocía. En el año 2011, González-Silva *et al.* describieron las enzimas OlsD y LpxO1 en *B. cenocepacia*, las cuales son homólogos del LpxO de *Salmonella typhimurium*. OlsD introduce la hidroxilación en el ácido graso amidificado del OL, mientras que la función de LpxO1 aún no está clara, pero sabe que no hidroxila OLs (González-Silva *et al.* 2011).

Al analizar las secuencias de los genomas del género *Brucella* se encontró que las *Brucellas* patógenas, como *B. abortus*, *B. suis*, *B. ovis* y *B. melitensis,* las cuales son agentes intracelulares estrictos, contienen un gen que codifica para un OlsC disfuncional. Mientras que *Brucellas* atípicas, aisladas del suelo, o su pariente cercano *Ochrobactrum*, presentan un gen *olsC* que sí codifica para un OlsC funcional. Estas últimas tienen en común que pueden crecer bien en vida libre además de ser patógenos oportunistas.

La membrana de las bacterias del género *Brucella* tiene un papel muy importante en la virulencia. Su estructura posee ciertas características que interfieren con el reconocimiento eficiente, durante la respuesta inmune innata del hospedero. Durante la etapa temprana de la infección, *Brucella* pasa por el compartimento lisosomal, el cual tiene un pH ácido, por lo que se podría inferir que la 2-hidroxilación introducida en el OL por OlsC, le confiere resistencia a la acidez como en el caso de *R. tropici*. Sin embargo, como ya se mencionó, OlsC es disfuncional en las cepas patógenas de *Brucella*, por lo que la resistencia se debe a otro componente de la bacteria. Es probable que la

presencia de la 2-hidroxilación en el OL de las *Brucellas* atípicas, confiera más inmunogenicidad y por lo tanto, ya no puedan evadir la eliminación por la respuesta inmune del hospedero.

6.3. Artículo 3

Vences-Guzmán, M. A., Guan, Z., Bermudez-Barrientos, J. R., Geiger O., and Sohlenkamp, C. 2013. **Agrobacteria lacking ornithine lipids induce more rapid tumour formation.** Environmental Microbiology 15(3): 895-906.

Los OLs están conformados por un residuo de ornitina y dos ácidos grasos: el primero está unido al grupo α-amino de la ornitina, mediante un enlace amido, mientras que el segundo se encuentra esterificado al C3 del ácido graso amidificado. Se han descrito distintas ubicaciones de las hidroxilaciones en los OLs: en el ácido graso esterificado, en el ácido graso amidificado, y en la ornitina. Estas modificaciones se han relacionado con una mayor tolerancia al estrés y a la competencia simbiótica en diferentes organismos como *R. tropici* CIAT899 o *B. cenocepacia*. El análisis de la composición lipídica de la membrana del patógeno de plantas *Agrobacterium tumefaciens*, mostró que forma dos OLs diferentes. Con el objetivo de determinar la función de los OLs en la tolerancia al estrés y en la patogenicidad en *A. tumefaciens*, se construyeron y se caracterizaron mutantes en los genes *olsB* y *olsE*. Estas mutantes carecen por completo de los OLs (Δ*olsB*) o sólo forman el OL sin modificar (Δ*olsE*). Por otro lado, no se observaron diferencias en el crecimiento de las mutantes con respecto a la cepa silvestre, bajo distintas condiciones de estrés abiótico. Adicionalmente, la carencia de los OLs agrobacteriales promueve, en menor tiempo, la formación de tumores en la planta huésped en ensayos de transformación, empleando discos de papa.

7. Discusión General

A continuación se presenta una discusión general de los datos obtenidos en esta tesis doctoral. Sin embargo, cada publicación tiene la discusión de los datos presentados en cada una de ellas.

Los lípidos de membrana bacterianos se clasifican en dos grandes grupos, los que poseen el grupo fosfato y los que no lo tienen. Dentro de estos últimos se encuentran los OLs, cuya distribución es mayoritaria en el grupo de las bacterias Gram negativas.

Los OLs están conformado por un grupo acilo que está unido al grupo α-amino de la ornitina mediante un enlace amido, y por un segundo grupo acilo ligado por enlace éster al C3 del acilo amidificado.

La biosíntesis de los OLs requiere de las enzimas OlsA, OlsB y la acil-ACP en S. *meliloti*. En el presente trabajo se comprobó que homólogos de dichas enzimas también están involucradas en la biosíntesis de los OLs en *Rhizobium tropici* CIAT899 y en *Agrobacterium tumefaciens*. Pero también se encontró que en algunas bacterias, los OLs se incrementan bajo condiciones limitantes de nutrientes 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 (Pitta *et al*., 1989 y López-Lara *et al*., 2005). Alrededor del 25% de las especies bacterianas secuenciadas tienen homólogos de *olsB*. Sin embargo, estos homólogos de OlsB están ausentes en algunas especies que forman OLs, como las cepas de *Serratia* sp, *Sorangium cellulosum*, y *Flavobacterium* sp. (Vences-Guzmán *et al*., 2012).

Estos datos indican que existe una segunda ruta metabólica de síntesis de lípidos de ornitina independiente de OlsB en *Serratia* sp, *Sorangium cellulosum* y *Flavobacterium* sp.

Con respecto a la regulación en otras bacterias, entre ellas nuestros modelos de estudio *R. tropici* CIAT899 y *A. tumefaciens*, los sintetizan aún en condiciones de fósforo abundante y cationes divalentes (Vences-Guzmán *et al*., 2011, 2012, 2013); Esto sugiere que la regulación de los genes involucrados en la biosíntesis de los OLs es diferente entre los distintos grupos de bacterias como *R. tropici*, *A. tumefaciens, B. cenocepacia* y *Brucella abortus*; sin embargo, aún se desconoce cuál es la regulación transcripcional de la biosíntesis de los OLs, sin embargo observamos un aumento en la formación de los OLs en limitación de fósforo en *R. tropici* y *A. tumefaciens.*

Recientemente se han reportado algunos grupos de bacterias como *R. tropici* CIAT899, *B. cenocepacia* J2315, *Serratia* sp, *Sorangium cellulosum*, *Flavobacterium* sp., *Thiobacillus, Gluconobacter, Streptomyces, Ralstonia* y *A. tumefaciens* que producen enzimas con la capacidad de hidroxilar los OLs en distintas partes de la molécula produciendo nuevas clases más polares de OLs (Rojas-Jiménez *et al*., 2005, Vences-Guzmán *et al*., 2011, 2012, 2013 y González-Silva *et al*., 2011). En la biosíntesis de las clases hidroxiladas conocidas en *R. tropici* CIAT899, *B. cenocepacia* J2315 y *A. tumefaciens* participan las enzimas OlsC en *R. tropici* CIAT899 (Rojas-Jiménez *et al*., 2005), OlsE en *R. tropici* CIAT899, en *A. tumefaciens* (Vences-Guzmán *et al*., 2011, 2013) y OlsD en *B. cenocepacia* J2315 (González-Silva *et al*., 2011) (Fig. 7)*,* además de OlsA, OlsB y la acil-ACP en las bacterias ya mencionadas. Tomando en cuenta conjuntamente los resultados anteriores y de este trabajo proponemos un modelo para la biosíntesis de los OLs hidroxilados en *R. tropici* CIAT899 que consta de las cuatro enzimas OlsA, OlsB, OlsC y OlsE (Vences-Guzmán *et al*., 2011, 2012) (Fig. 7). Las enzimas OlsA y OlsB son las responsables de la formación del OL no modificado (S1). La

enzima OlsE realiza una hidroxilación en el aminoácido ornitina del S1 formando así el S2, la enzima OlsC realiza una hidroxilación en el carbono 2 de la cadena esterificada del S1 formando así el P1. Por último, se piensa que OlsC hidroxila el S2 y que OlsE probablemente también hidroxila a P1, en ambos casos causando la formación de P2 (Fig. 7) (Vences-Guzmán *et al*., 2011).
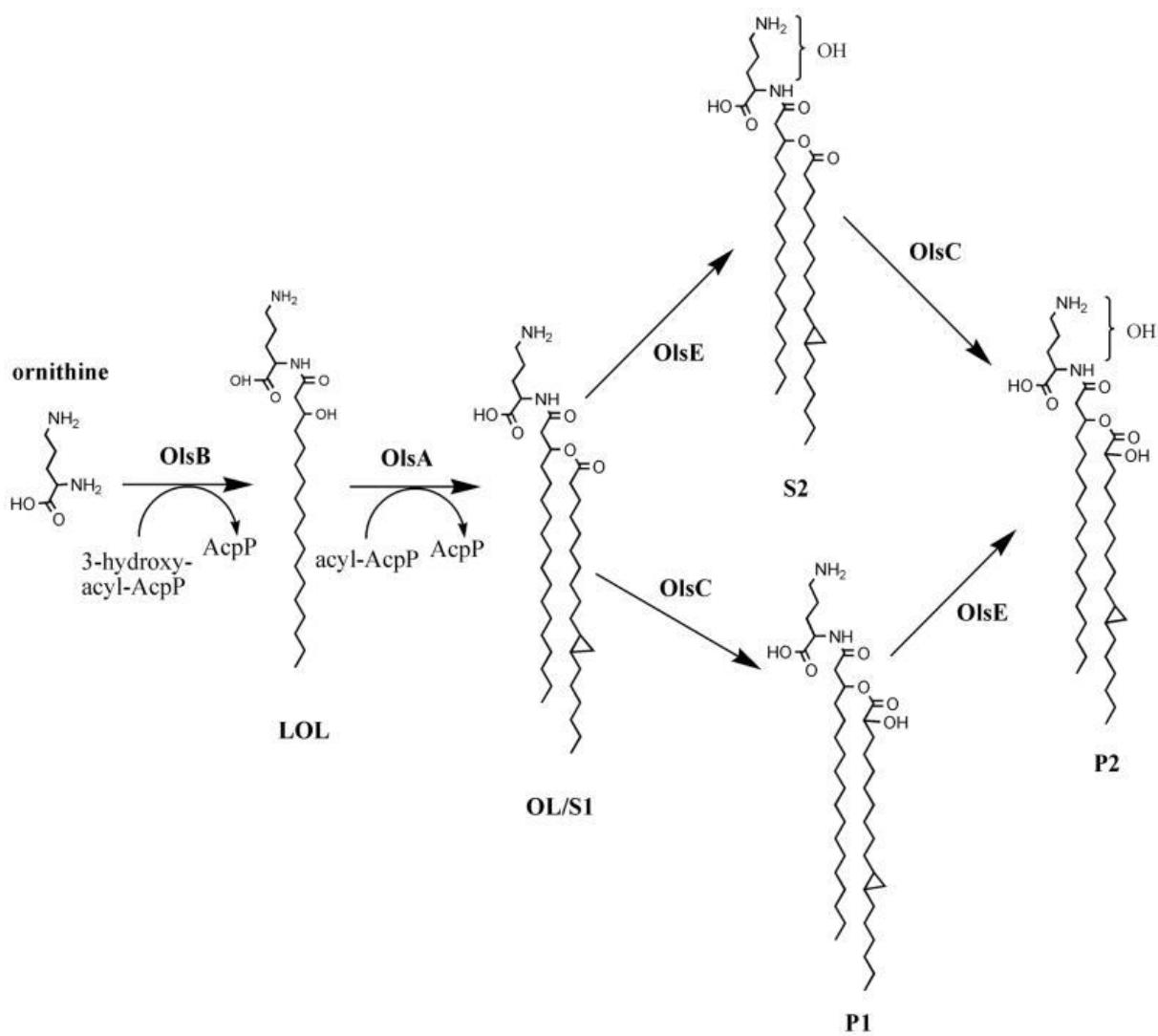
Figura. 7. Biosíntesis de los lípidos de ornitina en *Rhizobium tropici* CIAT899. Los genes que codifican para las enzimas OlsB y OlsA se identificaron por primera vez en *S. meliloti* 1021 (Weissenmayer *et al*., 2002; Gao *et al*., 2004), mientras que el gen que codifica para la hidroxilasa OlsC de OLs se describió por primera vez en *R. tropici* CIAT899 (Rojas-Jiménez *et al*., 2005). En este trabajo se determinó que OlsC hidroxila al C2 del ácido graso secundario y también se identificó el gen que codifica para la hidroxilasa OlsE, la cual introduce un grupo hidroxilo en el residuo de ornitina de los OLs. Liso-lípido de ornitina (LOL), lípido de ornitina (OL). Tomada de Vences-Guzmán *et al*., (2011).

Las hidroxilasas de los OLs descritas hasta ahora, pertenecen a dos familias diferentes. OlsE pertenece a la familia de las desaturasas por tener un patrón de varias histidinas conservadas. Dentro de esta familia se encuentra la superfamilia de las hidroxilasas de ácidos grasos. Las hidroxilasas de OLs OlsC de *R. tropici* CIAT899 y OlsD de *B. cenocepacia* J2315 pertenecen a la familia de βhidroxilasas, dependientes de α-cetoglutarato. González-Silva *et al*. (2011) describieron la enzimas OlsD de *B. cenocepacia*, que es responsable para una hidroxilación en el ácido graso amidificado del OL (Fig.8). Sin embargo, la hidroxilasa de *B. cenocepacia* J2315 responsable la síntesis del OL 2-OH, probablemente pertenezca a una tercera familia.

Es posible que la presencia de grupos 2-OH adicionales en los OLs podrían incrementar el número de los enlaces de hidrógeno, entre las moléculas adyacentes de lípido A y de OLs, aumentando así la estabilidad de la membrana externa y consecuentemente disminuyendo la permeabilidad de la membrana a ciertos compuestos.

Recientemente, en el filo de los planctomicetos, bacterias acuáticas encontradas en agua dulce, salobre y marina, se reportaron tres nuevos lípidos de ornitina metilados (mono-, di-, y tri metilados) en la posición de ϵ-nitrógeno del grupo cabeza de la ornitina (Fig. 8). No se conocen las funciones de estos lípidos, ni se conocen las enzimas involucradas en su síntesis. Se sugiere que las tres clases de lípidos de ornitina metilados, se pueden formar de manera análoga a la metilación de PE para formar PC mediante la enzima fosfatidiletanolamina *N*-metiltransferasa (PmtA) (Moore *et al*., 2013).
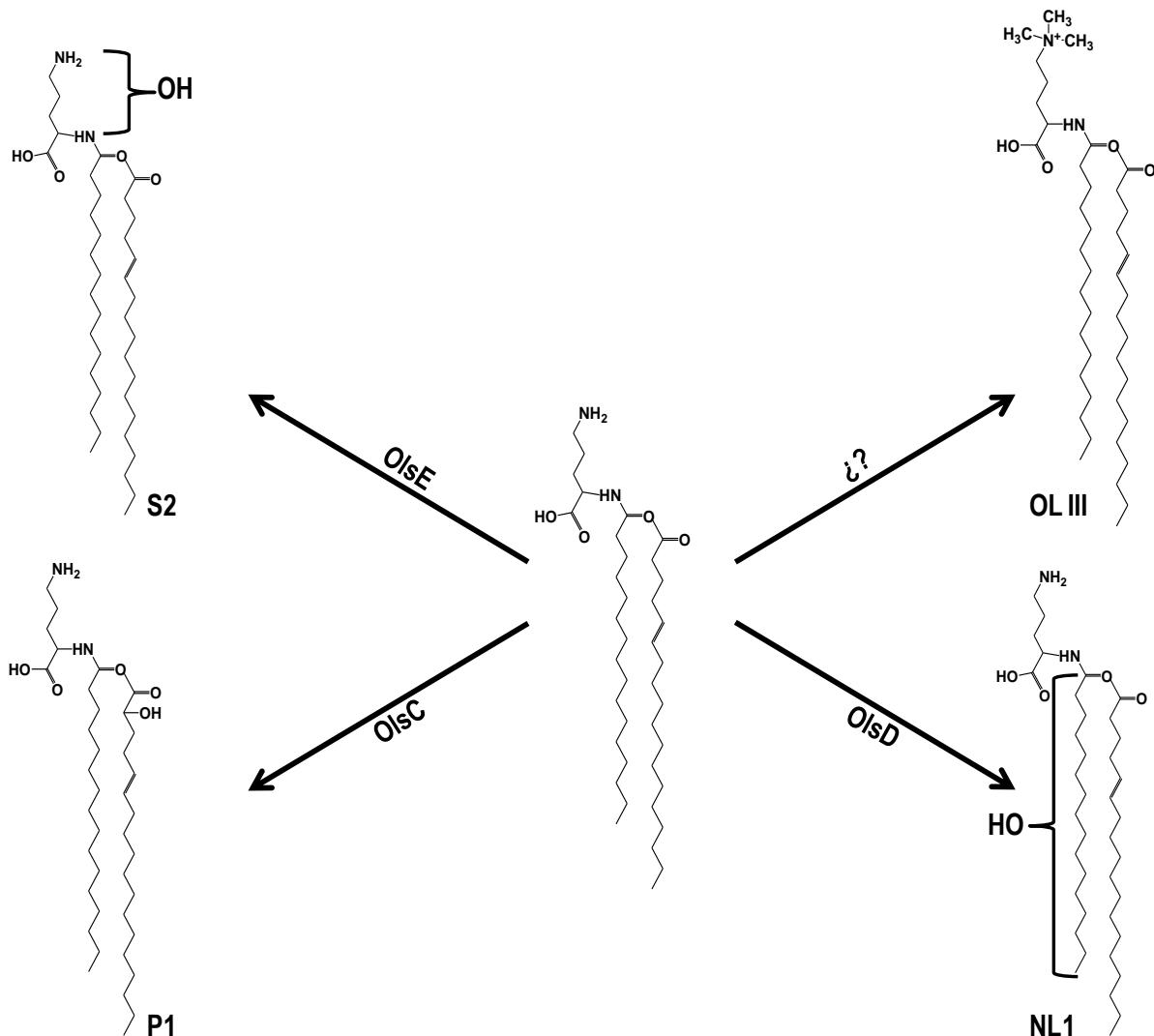
Figura. 8. Modificaciones que pueden sufrir los lípidos de ornitina. Hay tres diferentes hidroxilaciónes de los lípidos de ornitina (OL) que se han descubierto por ahora. El OL S1 no modificado puede ser hidroxilado por la enzima OlsC conduciendo a la formación del lípido de ornitina P1; también el lípido S1 puede ser hidroxilado por la enzima OlsD, así formando el lípido de ornitina NL1; El lípido S1 puede ser hidroxilado por la enzima OlsE conduciendo a la formación del S2. Estos lípidos hidroxilados pueden ser sujetos a una segunda hidroxilación: por ejemplo el lípido de ornitina P2 es un lípido hidroxilado dos veces por las enzimas OlsC y OlsE (esta estructura no se muestra en la figura). Los paréntesis indican que la posición exacta del grupo hidroxilo introducido no se conoce aun. El OL III es un OL metilado en el grupo amino primario libre de la ornitina. Por el momento no se sabe cuál es la enzima o enzimas que causan estas metilaciones.

Los OLs tienen una función importante para las bacterias tanto en vida libre como en asociación simbiótica o patogenica. Sin embargo, los reportes referentes a la misma aún son insipientes.

Hasta el momento, hay pocos avances acerca del papel de los grupos hidroxilo en los OLs. Por ejemplo, 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). Otro ejemplo es la bacteria *Thiobacillus thiooxidans*, que crece en ambientes muy ácidos y que debido a la localización de los OLs en la ME se especuló que esos lípidos podrían estar involucrados en la resistencia a acidez (Dees y Shively, 1982). Con respecto a la localización de los OLs en la ME, lo mismo observamos para *R. tropici* CIAT899. Rojas-Jiménez *et al*. (2005) reportaron que la cepa 899-*olsCΔ* 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). Rojas-Jiménez *et al*. (2005) reportaron que al cultivar en medio TY, y marcar los lípidos radiactivamente, la cepa 899-*olsCΔ* complementada, formó mayoritariamente los OLs P1 y P2, y una ausencia casi total de S1 y S2.

Con respecto a la simbiosis, la cepa 899-*olsCΔ* derivada de *R. tropici* CIAT899 formó nódulos poco desarrollados en las plantas de frijol 21 días después de la inoculación con la bacteria. Estos nódulos carecían de lenticelas y además fijaron solamente la mitad de nitrógeno que la cepa silvestre (Rojas-Jiménez *et al*., 2005).

Por otro lado, los hallazgos de este trabajo de tesis, indican que mutantes en *olsC* que no hidroxilan los OLs son más susceptibles a estrés térmico y al ambiente ácido, mientras que las otras dos mutantes, en *olsE* y *olsC-olsE*, 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). 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 o los OLs modificados podrían remplazar al LPS, porque en esas condiciones la estabilidad del mismo en la membrana no sería la adecuada, debido a la repulsión electrostática entre las moléculas adyacentes del LPS con cargas negativas (Nikaido, 2003). Bajo esas condiciones, los OLs hidroxilados en el acilo amidificado y esterificado podrían estar formando enlaces de hidrógeno entre moléculas adyacentes; por lo tanto, podrían incrementar la impermeabilidad de las membranas a ciertos iones o moléculas (Nikaido, 2003).

Referente a la patogénesis y mediante ensayos de transformación en discos de papa, observamos que la falta de los OLs agrobacteriales promueven la formación temprana de tumores en las plantas huésped Vences-Guzmán *et al*., 2013. Con base en estos resultados y en lo reportado por Tischer *et al.* (2012), proponemos que los lípidos de ornitina de *Mycobacteruim tuberculosis*, que se encuentran en la membrana externa interaccionando con el lípido A, le confieren a esta bacteria la capacidad de ser más inmunogénica, y por lo tanto que no pueda evadir el sistema inmune de su hospedero, de tal manera que puede ser eliminada de manera más eficiente.

Las bacterias patógenas del género *Brucella* son patógenos intracelulares. En el hombre causan una enfermedad febril, septicémica e infecciones focalizadas. Durante el proceso infeccioso, los patrones moleculares de superficie del patógeno activan el sistema inmune del hospedero y normalmente las bacterias son fagocitadas por los macrófagos y canaizadas al compartimento lisosomal para digerirlas. Palacios-Chaves et al. en el 2011, describieron la presencia de OLs en *Brucella* y reportaron que el genoma de *Brucella abortus* contiene el gen *olsC,* el cual contiene una mutación que cambia el marco de lectura del gen, lo que causa que la proteína codificada no sea funcional. Durante un análisis exhaustivo de las secuencias de los genomas bacterianos secuenciados, buscando genes que codifican para enzimas involucradas en la biosíntesis y modificación de OLs, observamos que también existen algunas bacterias del género *Brucella* que tiene un OlsC funcional. Este hallazgo es interesante, ya que en Brucellas patógenas intracelulares estrictas como *B. abortus*, *B. suis*, *B. ovis* o *B. melitensis*, el *olsC* presente es disfuncional. Mientras que en Brucellas aisladas del suelo, las cuales pueden ser patógenos oportunistas, o en su pariente cercano *Ochrobactrum*, el gen *olsC* codifica para un OlsC funcional (Vences-Guzmán *et al*., 2012).

Durante la respuesta inmune innata del hospedero, *Brucella* es fagocitada por los macrófagos y alojada en el compartimento lisosomal, el cual tiene un pH ácido. Con base en lo observado para *R. tropici* CIAT899, la 2-hidroxilacion introducida en el OL por OlsC la protege contra el estrés por acidez y a la temperatura elevada. El *olsC* de *Brucella* podría tener un papel similar; sin embargo, recordemos que su *olsC* no es funcional, por lo que la mutación en este gen podría tener una función durante la virulencia.

La evidencia experimental generada en el presente trabajo de tesis contribuye en varios niveles al conocimiento de los OLs. Desde su biosíntesis y distribución, hasta la función de los mismos en bacterias de vida libre y en la que interactúan con un hospedero. Adicionalmente, se propone la existencia de una nueva ruta de biosíntesis de OLs independiente de OlsB. También se plantea que la presencia de la hidroxilasa OlsC en el género *Brucella* pudiera tener alguna función bajo ciertas condiciones de estrés. Por otro lado, se ha reportado a la metilación como un nuevo tipo de modificación sobre los OLs; sin embargo, se desconoce tanto como la biosíntesis de las enzimas responsables como la función de las mismas. Si bien el campo de los OLs no es totalmente incipiente, aun faltan muchos puntos que elucidar. Por ejemplo, es necesario determinar la presencia de OLs modificados en organismos en donde aún no se han descrito y la caracterización estructural de los mismos. También sería importante determinar la función de los OLs, tanto en bacterias de vida libre como en asociación con su hospedero, bien sea simbiosis o patogénesis, así como extrapolar estos conocimientos al ámbito agrícola y médico.

8. Conclusiones

En esta tesis se emplearon las bacterias *R. tropici* CIAT899 y *A. tumefaciens* C58*, como modelos de estudio. En *R. tropici* CIAT899 se logró la identificación del gen *olsE* como una OL hidroxilasa y se caracterizó las mutantes deficientes en las hidroxilasas: OlsE, OlsC y OlsE/OlsC, bajo diferentes condiciones de estrés abiótico. Para el caso de *A. tumefaciens* C58 se caracterizaron las mutantes deficientes en la síntesis de OLs.

1. El gen *olsE* codifica para la enzima OlsE, la cual incorpora una grupo OH en los OLs S1 y P1 de *R. tropici* CIAT899 y S1 de *A. tumefaciens* C58.

2. La enzima OlsE hidroxila el aminoácido ornitina de los lípidos de ornitina (S1 y P1) de *R. tropici* CIAT899 para formar otros dos lípidos adicionales más polares (S2 y P2).

3. Para que se produzca cualquier clase de lípidos de ornitina en *R. tropici* CIAT899 y *A. tumefaciens* C58 se requiere que el gen *olsB*.

4. Los lípidos de ornitina confieren resistencia a *R. tropici* CIAT899 bajo condiciones de estrés abiótico como pHs ácidos y temperaturas elevadas.

5. Los lípidos de ornitina se encuentran mayoritariamente en la membrana externa de *R. tropici* CIAT899.

6. Los lípidos de ornitina de *R. tropici* CIAT899 tienen un papel importante en la interacción planta-bacteria.

7. La ausencia de los lípidos de ornitina agrobacteriales promueven la formación de tumores en la planta hospedera más rápidamente.

8. Este trabajo permite confirmar la hipótesis de que hay un gen en *Rhizobium tropici* CIAT899 que codifica para una enzima responsable de la modificación del lípido de ornitina S1 a S2 llamado *olsE*. También se demuestra que si existe un gen ortólogo en *A. tumefaciens* C58.

9. Perspectivas

Los resultados y conclusiones generados en esta tesis doctoral, dan sustento para proponer las siguientes perspectivas:

- Determinar la posición exacta de la hidroxilación del OL S2, incorporada por la enzima OlsE de *Rhizobium tropici* CIAT899.

- Implementar ensayos enzimáticos para caracterizar cinéticamente a la enzima OlsE de *Rhizobium tropici* CIAT899.

- Determinar las características biofísicas de los OLs hidroxilados de *Rhizobium tropici* CIAT899, mediante ensayos con liposomas.

- Determinar que otros factores además de OlsC le confieren resistencia a la acidez a *Rhizobium tropici* CIAT899.

- Determinar cual es el papel de la enzima OlsC en *Brucella*, durante la patogénesis.

- Caracterizar a nivel bioquímico y funcional la nueva ruta de biosíntesis de OLs en *Serratia* sp, *Sorangium cellulosum*, y *Flavobacterium* sp.

- Identificar el gen que codifica para la enzima responsable de la 2-hidroxilación del ácido graso esterificado del OL en *Burkholderia cenocepacia*.

- Identificar las enzimas involucradas en la biosíntesis de OLs metilados.

9.0 Cepas y plásmidos, y sus características relevantes

Vences-Guzmán, *et al* 2011 Mol. Microbiol. 79, 1496-1514.	Hydroxylated ornithine lipids increase stress tolerance in *Rhizobium tropici* CIAT899
Rhizobium tropici CIAT899	Cepa silvestre Nal[R]
MAV04	Δ*olsE*, derivada de *R. tropici* CIAT899
MAV05	Δ*olsE*, Δ*olsC*, derivada de *R. tropici* CIAT899
899-*olsCΔ1*	Δ*olsC*, derivada de *R. tropici* CIAT899
pNG23	Gen *olsB* de *Burkholderia cenocepacia* clonado como fragmento NdeI/HindIII en pET17b
pNG25	Gen *olsB* de *B. cenocepacia* cortado con BglII/HindIII, proviene del plásmido pNG23 clonado como fragmento BamHI/HindIII al plásmido pBBR1-MCS3
pCCS98	Gen *olsC* de *R. tropici* CIAT899 clonado como fragmento NdeI/BamHI en pET9a
pCos94	Cósmido derivado del pVK102 que contiene el gen *olsE*
pEMAV01	Lado flanqueante 1-kb río arriba del gen *olsE* de *R. tropici* CIAT899 clonado como fragmento SmaI/BamHI en el plásmido pUC18
pEMAV02	Lado flanqueante 1-kb río abajo del gen *olsE* de *R. tropici* CIAT899 clonado como fragmento BamHI/HindIII en el plásmido pUC18
pEMAV03	Lados flanqueantes 1-kb río arriba y abajo del gen *olsE* de *R. tropici* CIAT899 clonado como fragmento SmaI/HindIII en el plásmido pUC18
pPMAV04	Vector suicida para la deleción del gen *olsE* de *R. tropici* CIAT899
pURMAV03	Fragmento de 3.5 kb derivado del pCos94 clonado como fragmento PstI/PstI en pUC18
pERMAV04	Fragmento de 3.5 kb derivado del pCos94 clonado como fragmento PstI/PstI en pRK404
pERMAV05	Plásmido pET17b clonado como fragmento HindIII en pRK404
pERMAV06	Plásmido pET9a clonado como fragmento BamHI en pRK404
pEMAV07	ORF1 clonado como fragmento NdeI/BamHI en pET9a
pEMAV08	ORF2 clonado como fragmento NdeI/BamHI en pET9a
pEMAV09	Gen *olsE* clonado como fragmento NdeI/BamHI en pET9a
pERMAV11	pEMAV07 clonado como fragmento BamHI en pRK404
pERMAV12	pEMAV08 clonado como fragmento BamHI en pRK404
pERMAV13	pEMAV09 clonado como fragmento BamHI en pRK404
pERMAV15	pCCS98 clonado como fragmento BamHI en pRK404
pEMAV16	Gen *olsC* clonado como fragmento BamHI/BglII en el plásmido pEMAV09 cortado como fragmento BamHI
pERMAV17	pEMAV16 clonado como fragmento BamHI en pRK404

Miguel Ángel Vences-Guzmán, *et al* 2013. Environmental Microbiology 15(3), 895-906.	**Agrobacteria lacking ornithine lipids induce more rapid tumour formation**
Agrobacterium tumefaciens **A208**	Cepa silvestre Rim^R
MAV07	Δ*olsE*, derivada de *A. tumefaciens* A208
MAV08	Δ*olsB,* derivada de *A. tumefaciens* A208
pUMAV18	Lado flanqueante 1-kb río arriba del gen *olsE* de *A. tumefaciens* A208 clonado como fragmento EcoRI/BamHI en el plásmido pUC18
pUMAV19	Lado flanqueante 1-kb río abajo del gen *olsE* de *A. tumefaciens* A208 clonado como fragmento BamHI/XbaI en el plásmido pUC18
pUMAV20	Lados flanqueantes 1-kb río arriba y abajo del gen *olsE* de *A. tumefaciens* A208, clonado como fragmento EcoRI/XbaI en el plásmido pUC18
pPMAV21	Vector suicida para la deleción del gen *olsE* de *A. tumefaciens* A208
pUMAV23	Lado flanqueante 1-kb río arriba del gen *olsB* de *A. tumefaciens* A208 clonado como fragmento SmaI/BamHI en el plásmido pUC18
pUMAV24	lado flanqueante 1-kb río abajo del gen *olsB* de *A. tumefaciens* A208 clonado como fragmento BamHI/HindIII en el plásmido pUC18
pUMAV25	lados flanqueantes 1-kb río arriba y abajo del gen *olsE* de *A. tumefaciens* A208 clonado como fragmento SmaI/HindIII en el plásmido pUC18
pPMAV26	Vector suicida para la deleción del gen *olsB* de *A. tumefaciens* A208
pEMAV27	ORF *Atu0318* de *A. tumefaciens* A208 clonado como fragmento NdeI/HindIII en pET17b
pERMAV28	pEMAV27 clonado como fragmento HindIII en pRK404
pEMAV29	ORF *Atu0344* de *A. tumefaciens* A208 clonado como fragmento NdeI/BamHI en pET9a
pERMAV30	pEMAV29 clonado como fragmento BamHI en pRK404

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

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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.

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).
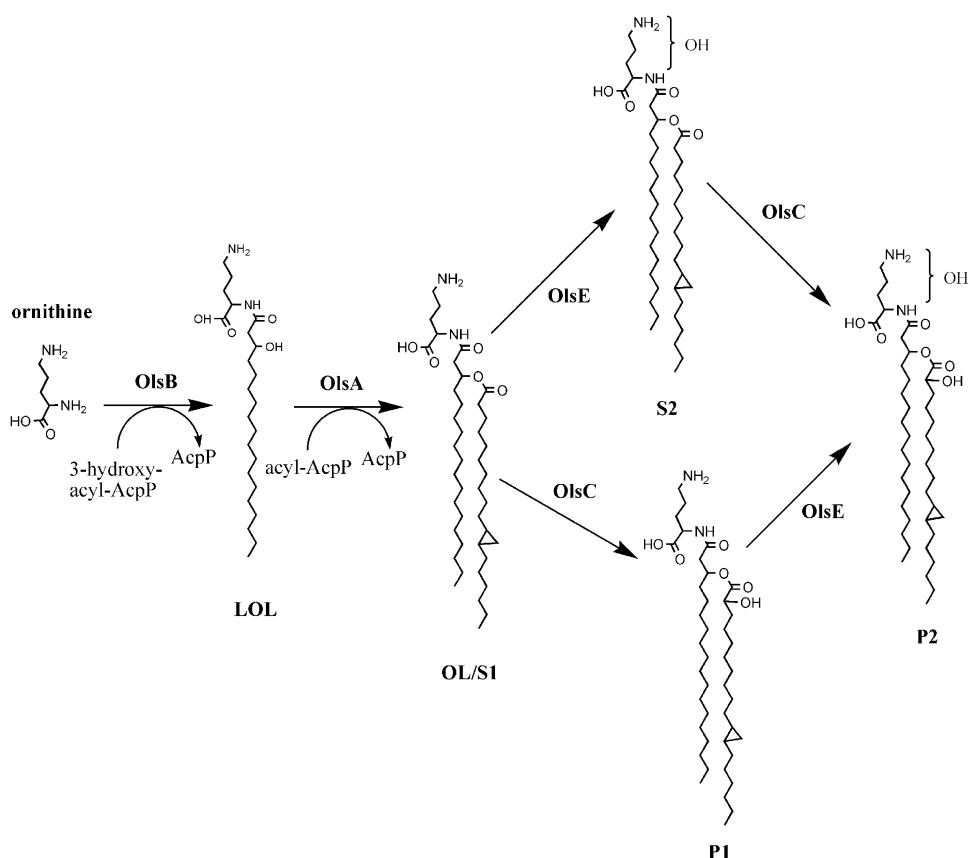
Fig. 1. Biosynthesis of ornithine lipids in *Rhizobium tropici* CIAT899. The genes coding for OlsB and OlsA have been first identified in *Sinorhizobium meliloti*, whereas the gene encoding the OL hydroxylase OlsC has been described first in *R. tropici*. Here we describe that the hydroxylation introduced 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 associ-

ated 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
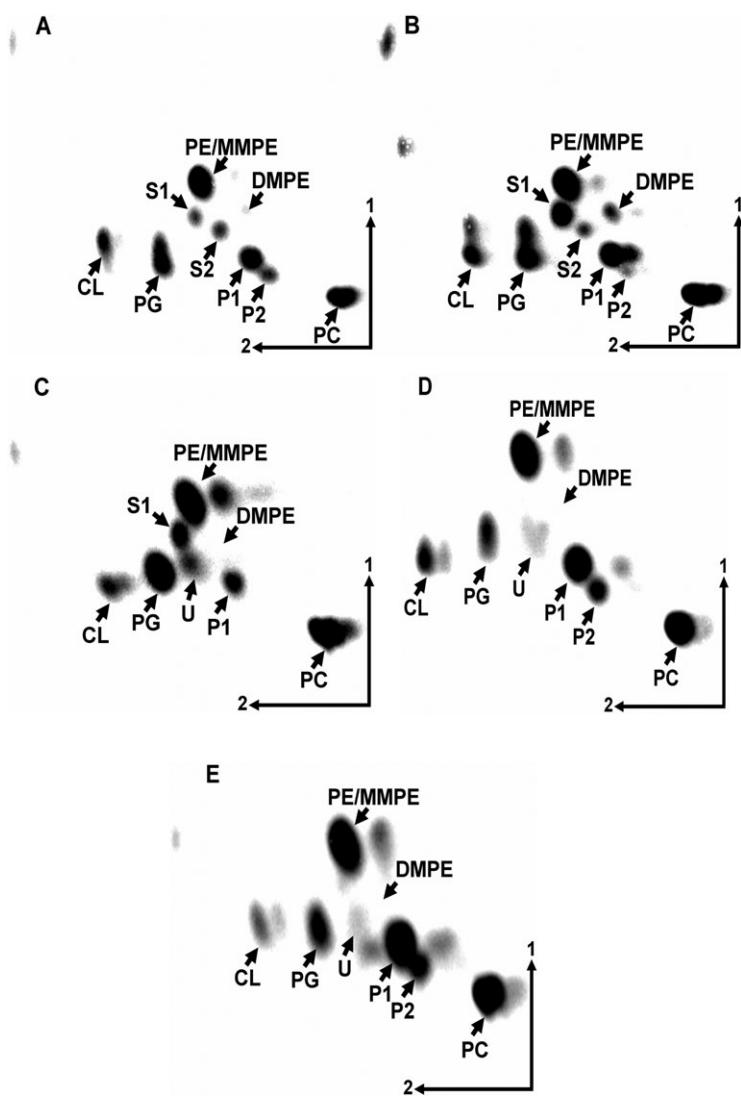
Fig. 2. Separation of [^{14}C]acetate-labelled lipids from *Rhizobium tropici* CIAT899 grown in complex TY medium at 30°C (A), at 37°C (B), at 42°C (C), at 30°C at pH 4.5 (D) or at 30°C at pH 4.0 (E) by two-dimensional thin-layer chromatography. The phospholipids phosphatidylethanolamine (PE), phosphatidylcholine (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.

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)

Lipid	30°C				37°C				42°C			
	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)

Lipid	pH 7.0				pH 4.5				pH 4.0			
	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.

A

B

C

D
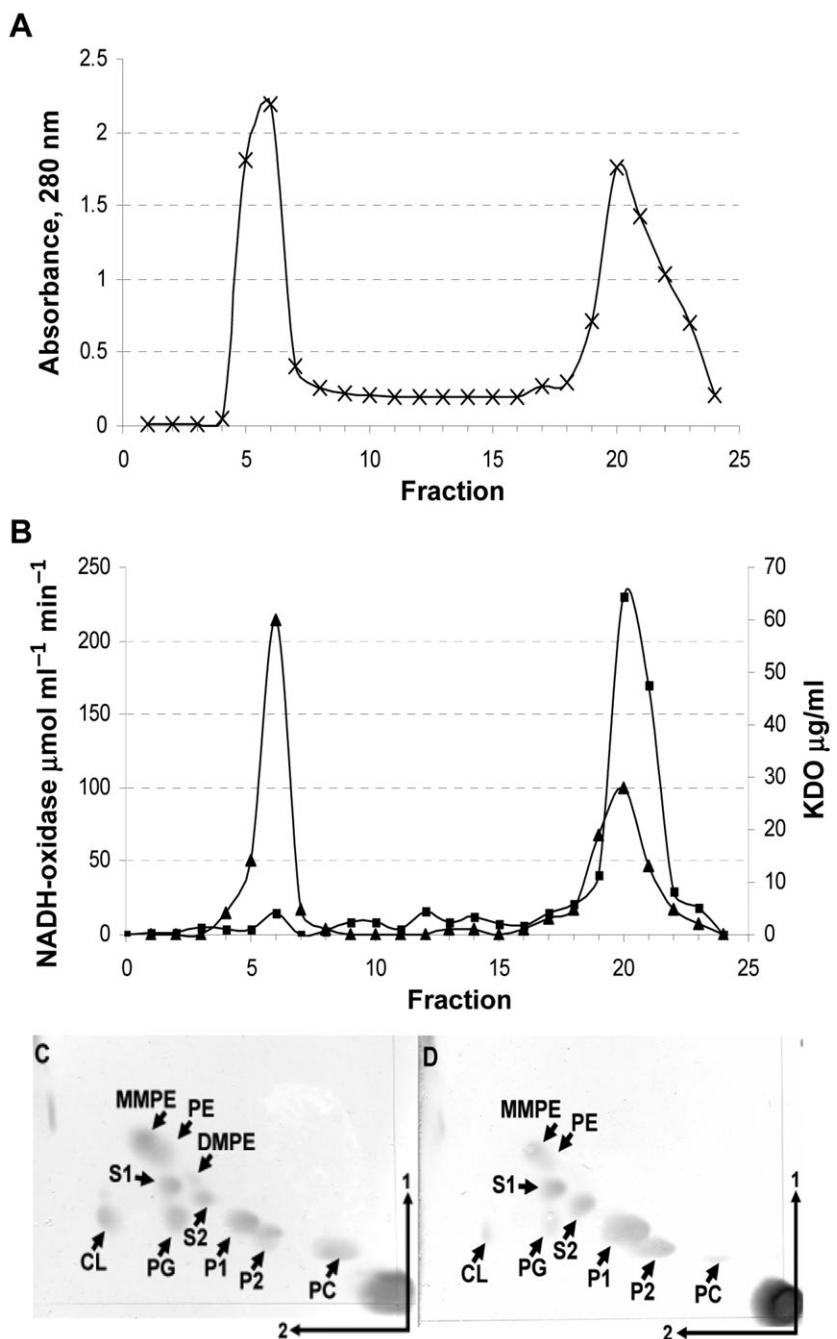
Fig. 3. Localization of OLs in membranes of wild-type *Rhizobium tropici* CIAT899. A and B. Results of a sucrose density gradient centrifugation of cell membranes of *R. tropici* CIAT899. (A) A280 readings of the gradient fractions. (B) 2-Keto-3-oxyoctonate content (closed triangles) and NADH oxidase (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)

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 [14C]acetate showed that ORF3 codes for the putative hydroxylase OlsE which is responsible for the formation of S2 (Fig. 4).
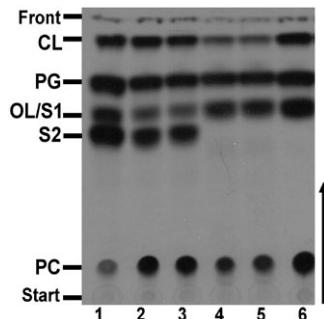
Fig. 4. Expression cloning of *olsE* from *R. tropici*. Lipids of *Sinorhizobium meliloti* CS111.pNG25 containing different plasmids or cosmids were radiolabelled with [14C]acetate and separated by one-dimensional TLC. The following strains were analysed: CS111.pNG25.pCos94 (cosmid, lane 1), CS111.pNG25.pERMAV04 (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 (ΔolsCΔolsE) 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 (ΔolsE) and MAV05 (ΔolsCΔolsE) were also complemented. When *olsE* was present *in trans* in MAV04 again formation of S2 and P2 was detected and
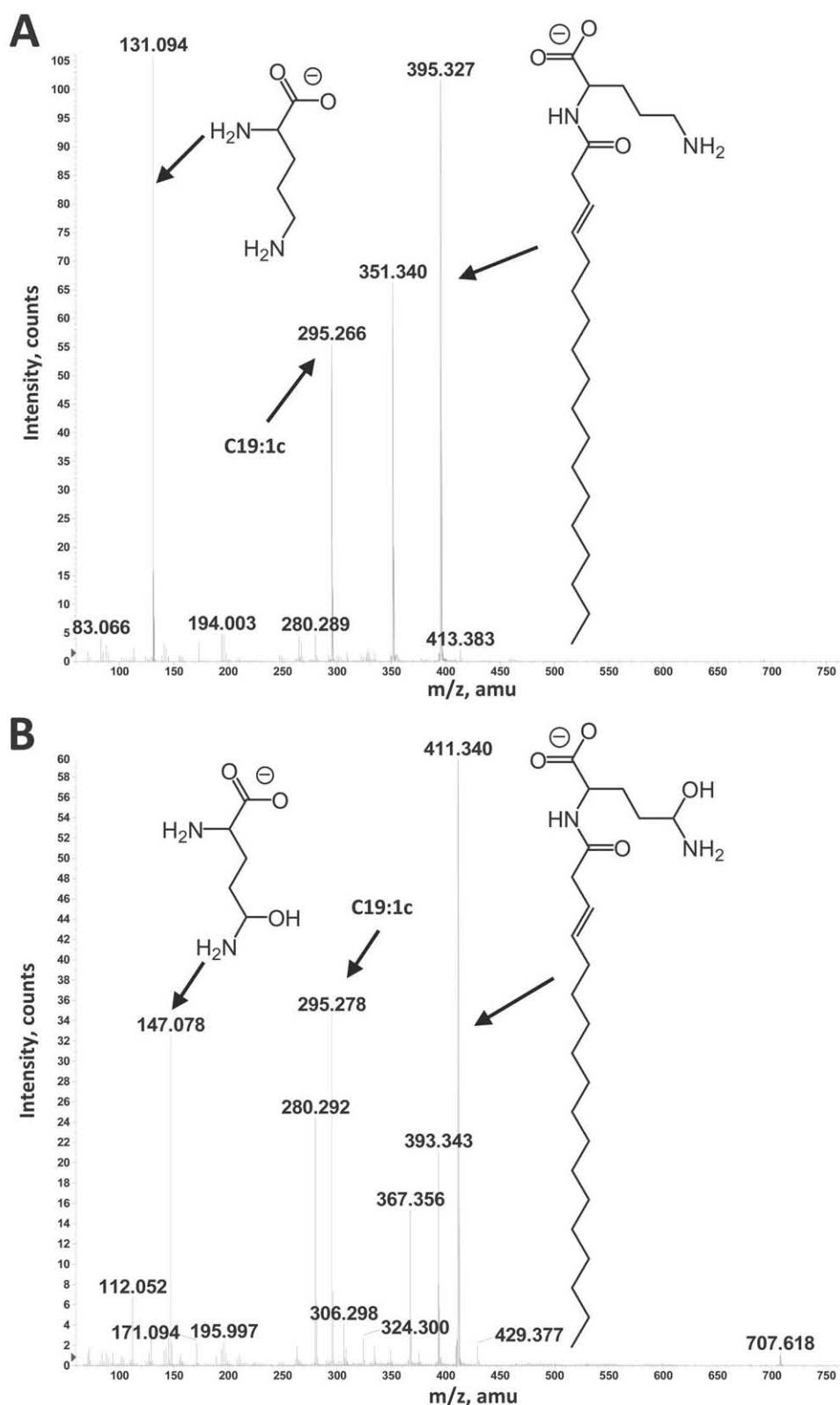
Fig. 5. Collision-induced dissociation mass spectra of ornithine lipids S1 and S2 detected in lipid extract of *R. tropici* mutant 899-*olsCΔ1*. Negative ion collision induced dissociation mass spectra of [M-H]⁻ ions at *m/z* 671 (A) obtained from OL S1 and *m/z* 707 (B) obtained from OL S2. The structures 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.
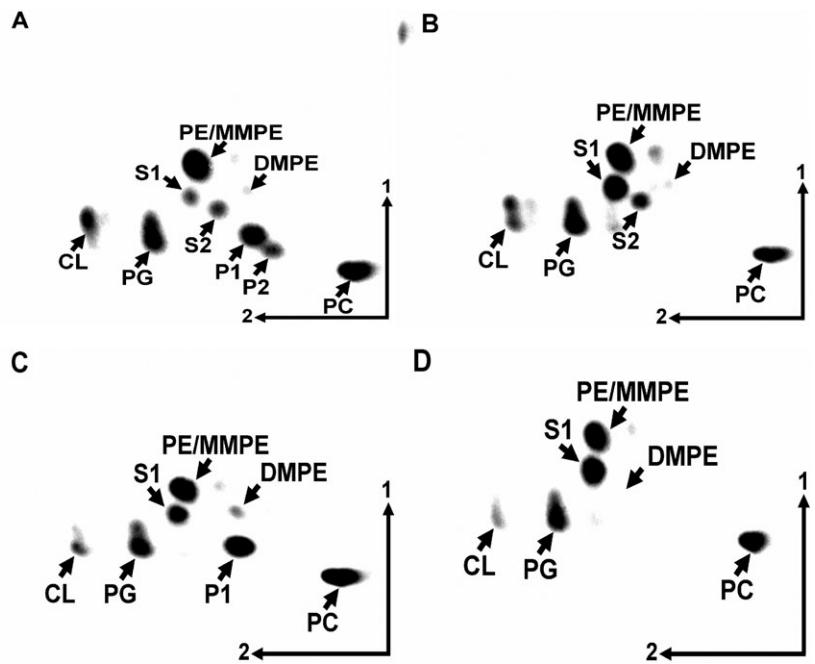
Fig. 6. Analysis of membrane lipid composition of *R. tropici* wild-type CIAT899 (A), *olsC*-deficient mutant 899-*olsCΔ1* (B), *olsE*-deficient mutant MAV04 (C) and *olsC/olsE*-deficient double mutant MAV05 (D). Lipids were labelled with [14C]acetate during growth in complex TY medium at 30°C and separated 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-*olsCΔ1* (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-*olsCΔ1*, 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 (*ΔolsE*) 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 (*ΔolsE*) 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-*olsCΔ1* and MAV05 (*ΔolsCΔolsE*) 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-*olsCΔ1* 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 (*ΔolsE*) the major lipid accumulated is P1, whereas in the mutants 899-*olsCΔ1* and MAV05 (*ΔolsCΔolsE*) 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-*olsCΔ1* and MAV05) seem to grow slightly slower than the other two strains (Fig. 7E). At 42°C
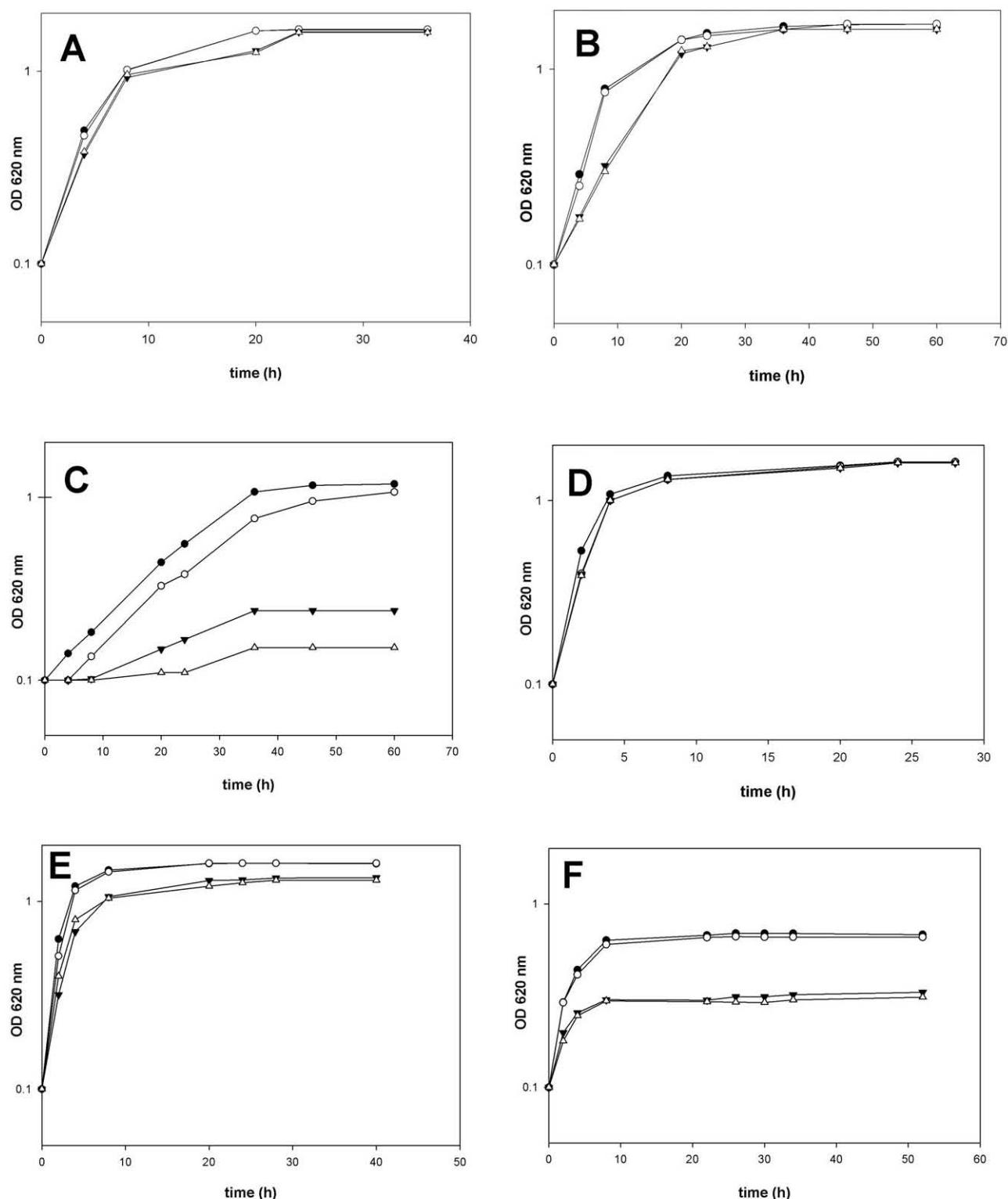
**Fig. 7.** Growth of *R. tropici* mutants lacking *olsC* is affected under stress conditions. *R. tropici* wild-type CIAT899 and mutants were grown in complex TY medium adjusted to pH 7.0 (A), pH 4.5 (B) or pH 4.0 (C) at 30°C or in complex TY medium at 30°C (D), 37°C (E) or 42°C (F). The result of a 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-*olsC*Δ*1* 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-*olsC*Δ*1*) 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-*olsC*Δ*1* 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-*olsC*Δ*1* 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
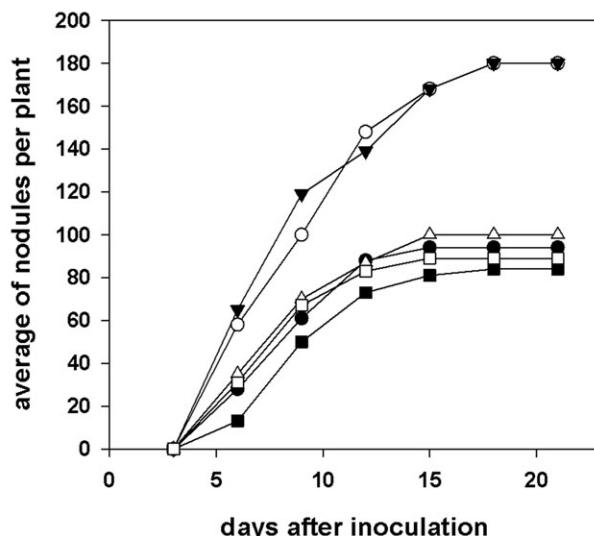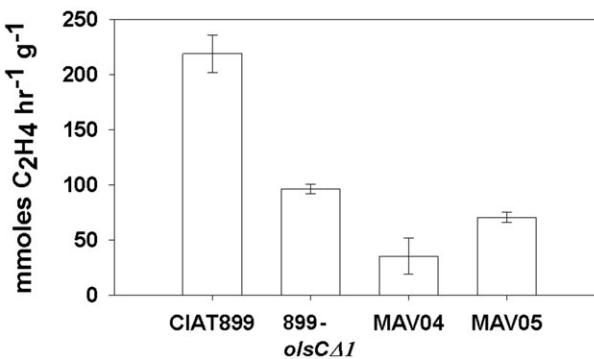
Fig. 8. Symbiotic phenotypes of *R. tropici* wild-type CIAT899 and strains deficient in OL modification on bean plants.
A. Nodulation assay. Nodules were counted every second or third day. Plants were harvested 21 days post inoculation; nodules were assayed for nitrogen fixation activity. The experiment was repeated three times with five plants for each strain. The result of a typical experiment is shown. CIAT899 (closed circles), 899-*olsC*Δ*1* (open circles), MAV04 (closed squares), MAV05 (open squares), 899-*olsC*Δ*1*.pERMAV05 (closed triangles), 899-*olsC*Δ*1*.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-*olsC*Δ*1*, MAV04 and MAV05. Values are the mean ± SD of three repetitions.

interferes with the development of functional nodules during *R. tropici*–bean symbiosis.

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-*olsCΔ1*	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 *lacZΔM15*; cloning strain	Hanahan (1983)
S17-1	*thi pro recA hsdR⁻ hsdM⁺* RP4 integrated in the chromosome, 2-Tc::Mu, Km::Tn7(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-*olsCΔ1* 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 m-mol^{-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 treat-
ment 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 quanti-
fied 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 fraction-
ated in 500 µl aliquots. For each fraction the protein concen-
tration 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 mea-
surements 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 respec-
tively. 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 accord-
ing 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 ninhy-
drin 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 spectrom-
eter, 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 pre-
parative 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.

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➤ **La segunda sección es acerca de un artículo de revisión, donde se incluyeron los resultados de las posibles funciones de las hidroxilasas de OLs, "Ornithine lipids and their structural modifications: from A to E and beyond". Publicado en FEMS Microbiology Letters 2012; 355 (1): 1-10. <u>Vences-Guzman, M. A.</u>, Geiger, O., and Sohlenkamp, C.**

Ornithine lipids and their structural modifications: from A to E and beyond

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Abstract

Ornithine lipids (OLs) are phosphorus-free membrane lipids that are widespread in eubacteria, but absent from archaea and eukaryotes. They contain a 3-hydroxy fatty acyl group attached in amide linkage to the α-amino group of the amino acid ornithine. A second fatty acyl group is ester-linked to the 3-hydroxy position of the first fatty acid. About 25% of the bacterial species whose genomes have been sequenced are predicted to have the capacity to form OLs. Distinct OL hydroxylations have been described in the ester-linked fatty acid, the amide-linked fatty acid, and the ornithine moiety. These modifications often seem to form part of a bacterial stress response to changing environmental conditions, allowing the bacteria to adjust membrane properties by simply modifying already existing membrane lipids without the need to synthesize new lipids.

Introduction

The permeability barrier of cells is formed by amphipathic lipids, which consist of a hydrophobic and a hydrophilic portion. The hydrophobic moieties have the propensity to self-associate, and the hydrophilic moieties have the tendency to interact with each other and the aqueous environment, leading to the formation of membrane structures. In general, glycerophospholipids such as phosphatidylglycerol, phosphatidylethanolamine, cardiolipin, phosphatidylcholine, phosphatidylserine, and phosphatidylinositol are the primary building blocks of membranes, but several other lipid classes can be also important and essential membrane components. Almost all Gram-negative bacteria have the lipid-A-containing lipopolysaccharide in the outer layer of the outer membrane (Raetz *et al.*, 2007), but several other lipid classes such as hopanoid and steroid lipids, sphingolipids, glycosylated diacylglycerols, sulfolipids, betaine lipids, and ornithine lipids (OLs) have been described that can be formed only by certain bacterial groups or under specific stress conditions. For example, under phosphorus-limiting growth conditions, some bacteria replace the majority of their glycerophospholipids with phosphorus-free membrane lipids such as sulfolipids, betaine lipids, glycolipids, and OLs (Benning *et al.*, 1995; Geiger *et al.*, 1999; Weissenmayer *et al.*, 2002; Gao *et al.*, 2004). Challenging of some bacteria with low pH conditions can cause the modification of already existing membrane lipids, such as the formation of lysyl-phosphatidylglycerol from phosphatidylglycerol or the hydroxylation of OLs (Rojas-Jiménez *et al.*, 2005; Sohlenkamp *et al.*, 2007; González-Silva *et al.*, 2011; Vences-Guzmán *et al.*, 2011).

Distribution and structure of OLs

The capacity to form OLs is apparently widely distributed in eubacteria, but so far, OLs have not been detected in archaea and eukaryotes (López-Lara *et al.*, 2003; Geiger *et al.*, 2010). They 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, the so-called piggyback fatty acid, is ester-linked to the 3-hydroxy position of the first fatty acid (Knoche & Shively, 1972; Geiger *et al.*, 1999). In some bacteria, OLs can be modified by hydroxylation in one or more positions. In recent years,

several genes coding for OL hydroxylases have been identified. OLs can be hydroxylated in the ester-linked fatty acid, the amide-linked fatty acid, and the ornithine moiety (Rojas-Jiménez *et al.*, 2005; González-Silva *et al.*, 2011; Vences-Guzmán *et al.*, 2011). In *Gluconobacter cerinus*, OLs hydroxylated in the C-2 position of the ester-linked fatty acid can be modified with a taurine residue that is amide-linked to the α-carboxy group of ornithine. This tauro-OL is also called cerilipin after the bacterial species from which it was isolated (Tahara *et al.*, 1976a, b). Although OLs are present in both membranes of Gram-negative bacteria, they are more abundant in the outer membrane (Dees & Shively, 1982; Palacios-Chaves *et al.*, 2011; Vences-Guzmán *et al.*, 2011).

Structurally similar lipids in which other amino acids are present instead of ornithine have been described. A lysine lipid has been described in an *Agrobacterium tumefaciens* strain (Tahara *et al.*, 1976a, b), glycine lipids were detected in *Cytophaga johnsonae* and *Cyclobacterium marinus* (Kawazoe *et al.*, 1991; Batrakov *et al.*, 1999), glutamine lipids were described in *Rhodobacter sphaeroides* (Zhang *et al.*, 2009, 2011), and serineglycine lipids (SGLs) were isolated from the opportunistic pathogen *Flavobacterium meningosepticum* (Kawai *et al.*, 1988; Shiozaki *et al.*, 1998a, b).

Biosynthesis of OLs

The biosynthesis of the unmodified OL (sometimes also called S1 (Rojas-Jiménez *et al.*, 2005)) occurs in two steps. The genes coding for the acyltransferase activities OlsB and OlsA required for OL biosynthesis were first discovered in the α-proteobacterium *Sinorhizobium meliloti* (Weissenmayer *et al.*, 2002; Gao *et al.*, 2004). In the first step, the *N*-acyltransferase OlsB is responsible for the transfer of a 3-hydroxy fatty acyl group from 3-hydroxy fatty acyl-acyl carrier protein (ACP) to the α-amino group of ornithine, thereby forming lyso-ornithine lipid (LOL) (Gao *et al.*, 2004). In the second step, the *O*-acyltransferase OlsA catalyzes the transfer of an acyl group from acyl-ACP to the 3-hydroxyl group of LOL group forming OL (Weissenmayer *et al.*, 2002) (Fig. 1).

The *N*-acyltransferase OlsB

OlsB-deficient mutants have been isolated in *S. meliloti*, *Rhodobacter capsulatus*, *Brucella abortus*, and *Burkholderia cenocepacia*, and they are in all cases unable to form OLs (Gao *et al.*, 2004; Aygun-Sunar *et al.*, 2006; González-Silva *et al.*, 2011; Palacios-Chaves *et al.*, 2011). The analysis of molecular species of OLs present in different organisms suggests that the distinct OlsB proteins apparently present strong substrate specificity for specific fatty
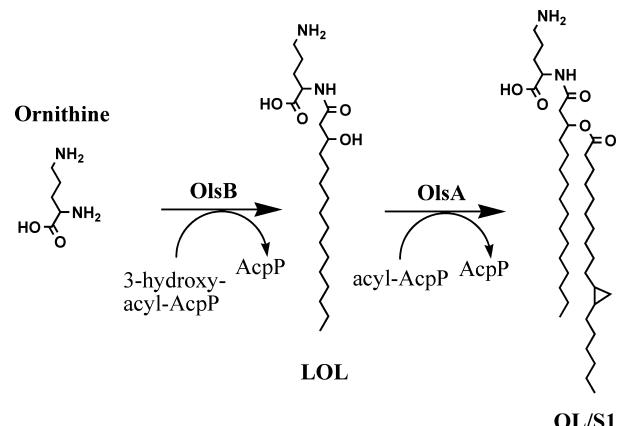

Fig. 1. OlsBA-dependent biosynthesis of OLs. AcpP, constitutive acyl carrier protein.

acid chain lengths. Apparently, OlsB enzymes from *Rhizobium tropici* and *S. meliloti* almost exclusively attach a 3-hydroxylated C18 fatty acid to ornithine (Geiger *et al.*, 1999; Vences-Guzmán *et al.*, 2011), whereas OlsB from *B. cenocepacia* almost exclusively transfers a 3-hydroxylated C16 fatty acid (González-Silva *et al.*, 2011). In contrast, OLs from *Pseudomonas aeruginosa* present a variety of chain lengths in the amide-linked fatty acid (Lewenza *et al.*, 2011), indicating that OlsB from *P. aeruginosa* shows laxer substrate specificity and can transfer a variety of 3-hydroxy fatty acids to ornithine.

The *O*-acyltransferase OlsA

OlsA-deficient mutants of *S. meliloti*, *R. capsulatus*, *B. abortus*, and *P. aeruginosa* are unable to form OLs (Weissenmayer *et al.*, 2002; Aygun-Sunar *et al.*, 2006; Lewenza *et al.*, 2011; Palacios-Chaves *et al.*, 2011). In some cases, an accumulation of LOL has been observed in OlsA-deficient mutants that can be exacerbated by OlsB overexpression (Gao *et al.*, 2004). In contrast to what has been observed for OlsB, OlsA seems to be less selective for specific fatty acids. More details relating to OlsA and OlsB can be found in Geiger *et al.* (2010).

OL-modifying activities

Once the unmodified OL S1 has been synthesized by the acyltransferases OlsB and OlsA, it can be modified in some organisms by introducing hydroxyl groups in the different moieties of the OL structure or by transfer of taurine to the α-carboxy group of ornithine (Tahara *et al.*, 1978). So far, three different OL hydroxylases have been described: OlsC, OlsD, and OlsE (Rojas-Jiménez *et al.*, 2005; González-Silva *et al.*, 2011; Vences-Guzmán *et al.*, 2011) (Fig. 2). The gene/enzyme responsible for the
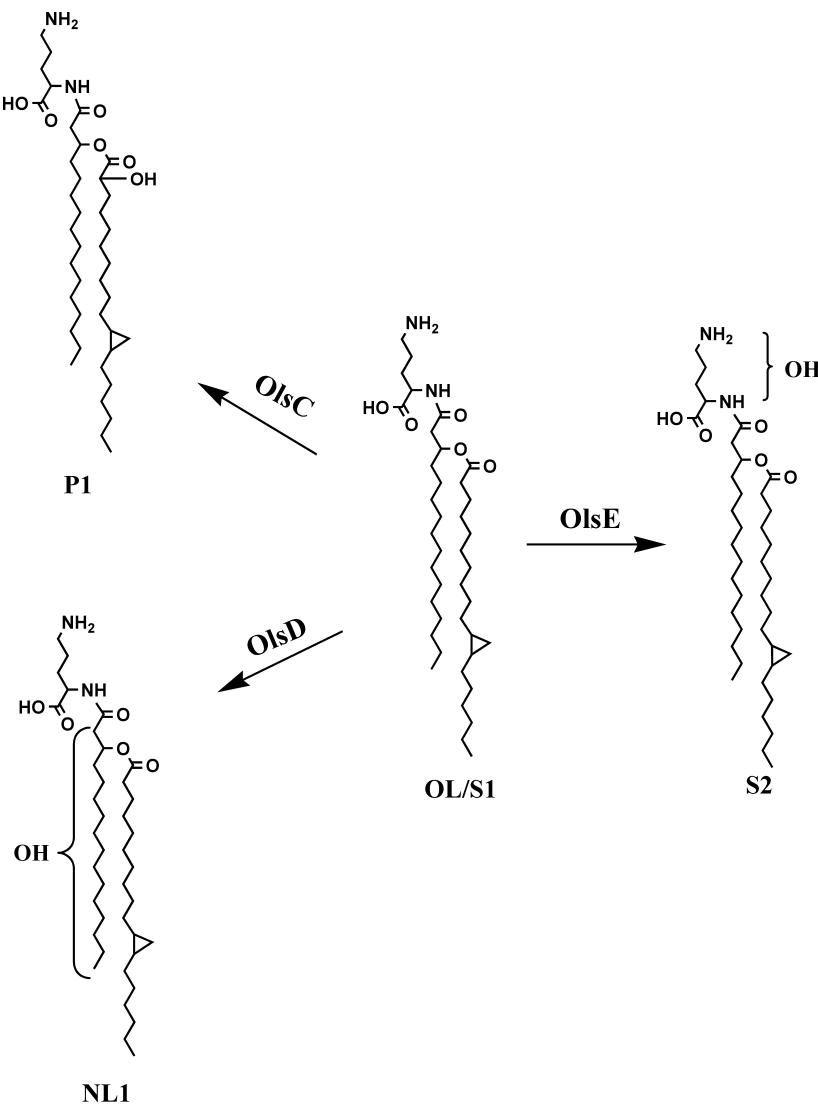
Fig. 2. Hydroxylation of OLs. Three different OL hydroxylases have been described so far. The unmodified OL S1 can be hydroxylated by OlsC, leading to the formation of the OL P1; it can be hydroxylated by OlsD, leading to the formation of OL NL1; or it can be hydroxylated by OlsE, leading to the formation of OL S2. Also, hydroxylated OLs can be subject to a second hydroxylation: for example, OL P2 is a double-hydroxylated OL hydroxylated by OlsC and OlsE (structure of P2 is not shown in figure). The brackets indicate that the exact position of the introduced hydroxyl group is not known.

taurine modification of OLs in *G. cerinus* has not been identified.

Mutants lacking OlsB activity and thereby deficient in the first step of OL biosynthesis have been shown to lack modified OLs also, indicating that there is no alternative to the OlsBA pathway in the organisms studied so far.

The OL hydroxylase OlsC

In some species of the genus *Burkholderia* (González-Silva *et al.*, 2011), *Flavobacterium* (Kawai *et al.*, 1988; Asselineau, 1991), *Thiobacillus* (Knoche & Shively, 1972),

Gluconobacter (Tahara *et al.*, 1976b), *Streptomyces* (Asselineau, 1991), *Ralstonia* (Galbraith *et al.*, 1999), and *Rhizobium* (Vences-Guzmán *et al.*, 2011), OLs hydroxylated in C-2 position of the ester-linked fatty acid have been described.

These 2-hydroxylated fatty acids are not formed during normal fatty acid biosynthesis, and specific enzyme activities are therefore necessary to introduce the hydroxyl group at this position. Gibbons *et al.* (2000) had isolated a gene from *Salmonella* responsible for the introduction of a 2-hydroxyl group into a lipid-A-bound myristic acid residue. The hydroxylation reaction is catalyzed by the

$Fe^{2+}/O_2/\alpha$-ketoglutarate-dependent LpxO dioxygenase. Rojas-Jiménez et al. (2005) had identified a gene called *olsC* in *R. tropici* encoding an LpxO homolog responsible for the synthesis of hydroxylated OLs. Later, it was shown that OlsC is responsible for the introduction of a hydroxyl group in the C-2 position of the piggy-back fatty acid of OLs (Vences-Guzmán et al., 2011).

A prediction indicates that OlsC of *R. tropici* CIAT899 is a water-soluble protein of 281 amino acids (Rojas-Jiménez et al., 2005). Owing to its homology to LpxO from *Salmonella*, it can be expected that OlsC-dependent hydroxylation of the ester-linked fatty acid will also be $Fe^{2+}/O_2/\alpha$-ketoglutarate dependent.

Genes encoding OlsC homologs can be found in *Agrobacterium vitis*, *Agrobacterium radiobacter*, *Ochrobactrum anthropi*, *Ochrobactrum intermedium*, *Aurantimonas manganoxydans*, *Fulvimarina pelagi*, *Roseomonas cervicalis*, *Chelativorans* sp., *Mycobacterium rhodesiae*, and several *Brucella* species (Supporting Information, Table S1). Interestingly, in the so-called classical *Brucella* such as *Brucella ovis*, *Brucella suis*, *Brucella melitensis*, or *B. abortus*, which are intracellular pathogens, the *olsC* gene is present only as pseudogene containing a frameshift mutation. As a consequence, the *olsC* gene is translated into two ORFs, making the gene *olsC* nonfunctional (Palacios-Chaves et al., 2011). In the genomes of several atypical *Brucella* strains such as *Brucella microti*, *Brucella* sp. BO1, or *Brucella* sp. BO2 which share several characteristics with the opportunistic soil pathogen *Ochrobactrum*, *olsC* genes lacking the frameshift can be detected that are probably functional. This observation implies that organisms like *Ochrobactrum*, *R. tropici*, and nonclassical *Brucella* such as *Brucella* isolated from soil that present both (De et al., 2008; Scholz et al., 2008a, b, 2009, 2010) an intracellular and a free-living lifestyle have preserved a functional copy of *olsC*, whereas the classical *Brucella* strains that are strictly intracellular pathogens present only a nonfunctional copy of *olsC* (Palacios-Chaves et al., 2011). A functional OlsC might confer a selective advantage in adverse abiotic stress conditions, but might not be of use or even have a negative impact when the bacteria are inside a host.

Recently, Vences-Guzmán et al. (2011) reported a more detailed study of an *olsC*-deficient *R. tropici* mutant. Strains lacking the OL hydroxylase OlsC showed a growth defect at increased temperatures (37 and 42 °C) and under acid pH conditions (4.5 and 4.0). Strain 899-*olsCΔ1* lacking OlsC formed underdeveloped nodules on bean plants 21 days after inoculation with the bacteria. The nodules lacked lenticels and fixed two times less nitrogen (Rojas-Jiménez et al., 2005). The three *R. tropici* mutants (*ΔolsC*, *ΔolsE*, and *ΔolsCΔolsE*) lacking OL hydroxylases established their symbiosis only poorly (Vences-Guzmán et al., 2011). As *R. tropici* is challenged by low pH conditions inside its host plant (Udvardi et al., 1991; Udvardi & Day, 1997), it can be speculated that the observed symbiotic phenotype is a consequence of the mutants' increased acid sensitivity.

The OL hydroxylase OlsD

The OL hydroxylase OlsD was first isolated from *B. cenocepacia* J2315, a β-proteobacterium known as an opportunistic pathogen of humans. González-Silva et al. (2011) originally suggested that 2-hydroxylation of OLs in *B. cenocepacia* might be performed by an LpxO homolog called OlsD (BCAM2401). OlsD indeed hydroxylated OL, but the hydroxylation did not occur on the ester-linked fatty acid. Surprisingly, data obtained by mass spectrometry suggested that OlsD modifies the amide-linked fatty acid of OLs with a hydroxyl group (Fig. 2), a modification that was previously unknown. Unfortunately, their analysis did not allow for the determination of the exact position of the hydroxyl group. OlsD from *B. cenocepacia* is a 249-amino-acid protein, apparently lacking transmembrane helices (González-Silva et al., 2011). It is widely distributed within the genus *Burkholderia*, but homologs are also present in three *Serratia* strains. The gene coding for the 2-hydroxylase activity hydroxylating the ester-linked fatty acyl residue in the C-2 position in *B. cenocepacia* has not been identified yet.

The OL hydroxylase OlsE

Rojas-Jiménez et al. (2005) had described the presence of four different OLs in *R. tropici* CIAT899. The presence of OlsC alone could not explain this number of distinct structures. Using a functional expression screen conjugating a cosmid bank from *R. tropici* into *S. meliloti*, Vences-Guzmán et al. (2011) identified the gene *olsE* coding for the hydroxylase OlsE. Mass spectrometry analysis showed that OlsE introduced a hydroxyl group in the ornithine moiety. So far, the exact position of the hydroxylation could not be determined, but ninhydrin staining of the different OLs shows that the hydroxyl group affects the reactivity of the lipid to ninhydrin.

Bioinformatic predictions indicate that the OlsE protein (331 amino acids) from *R. tropici* CIAT899 is highly hydrophobic and might form between 4 and 6 transmembrane helices. 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, and C-4 sterol methyl oxidase (Arthington et al., 1991; Bard et al., 1996; Mitchell & Martin, 1997; Kennedy et al., 2000). A similar motif can be found in membrane-bound fatty acid desaturases such as *OLE1* from *Saccharomyces cerevisiae* and in bacterial alkane

hydroxylase and xylene monooxygenase (Kok *et al.*, 1989; Suzuki *et al.*, 1991). In these proteins, the conserved histidine residues act to co-ordinate an oxo-bridged di-iron cluster (Fe-O-Fe) that functions as part of the reaction center (Fox *et al.*, 1993; Shanklin *et al.*, 1994). The closest OlsE homologs are present in all the sequenced *Agrobacterium* strains, *Rhodospirillum centenum*, *Parvibaculum lamentivorans*, *Verrucomicrobium spinosum*, *Micavibrio aeruginosavorus*, and *Azospirillum amazonense*. More distant homologs are present in several actinomycetes, a few *Gammaproteobacteria*, and a few other *Alphaproteobacteria* (Table S1).

No growth phenotype was observed for the OlsE–deficient mutant at increased temperatures or under pH stress conditions. Bean plants infected with OlsE-deficient mutants presented less red nodules and more white nodules than plants infected with the wild type. Nitrogen fixation of nodules from OlsE mutant-infected plants was clearly reduced (Vences-Guzmán *et al.*, 2011).

Taurine transfer to OLs

In *G. cerinus*, a taurine residue can be amide-linked to the α-amino group of the ornithine moiety of OL (Tahara *et al.*, 1976a, b). It has been shown that a cell-free protein crude extract from *G. cerinus* contains an enzymatic activity responsible for the transfer of taurine to OL hydroxylated in the 2-position of the piggy-back fatty acid. This taurine transfer activity depends on the presence of ATP and bivalent cations (Tahara *et al.*, 1976a, b, 1978). As no *G. cerinus* strain has been sequenced so far, a bioinformatic search for candidate genes/proteins has not been possible.

Prediction of OL distribution

The wealth of genome sequence information that has been produced in recent years allows for an accurate analysis of the distribution of OL biosynthesis genes. Genes coding for OlsB have a high predictive value, and it should be possible to predict the capacity of an organism to synthesize OL from the presence of the *olsB* gene. In many cases, where the *olsB* gene is phylogenetically less well conserved, the fact that *olsB* often occurs in an operon with *olsA* is of help. For the purpose of predicting the distribution of OLs, we analyzed all sequenced bacterial genomes for the presence of a gene encoding an OlsB homolog. BLAST searches with OlsB sequences from *S. meliloti* and *B. cenocepacia* pick up OlsB homologs in about 25% of the sequenced bacterial species which belong to the *Alpha-*, *Beta-*, *Gamma-*, *Deltaproteobacteria*, *Actinomycetales*, spirochetes, green nonsulfur bacteria, verrucomicrobia, firmicutes, *Aquificales*, and cyanobacteria (Table S1). Within the class *Alphaproteobacteria*, OlsB homologs can be detected in most sequenced species belonging to the orders *Rhizobiales*, *Rhodobacterales*, and *Rhodospirillales*, but are generally absent from species belonging to the orders *Caulobacterales*, *Rickettsiales*, and *Sphingomonadales*. OlsB can also be detected in the majority of sequenced *Betaproteobacteria*, including most *Burkholderiales* and many *Neisseriales*, but are absent from the *Nitrosomonales*. In the *Gammaproteobacteria*, OlsB homologs are absent from *Enterobacterales*, *Vibrionales*, *Pasteurellales*, *Legionellales*, and *Aeromonadales*. Many organisms presenting OlsB homologs belong to the orders *Acidithiobacillales*, *Chromatiales*, *Pseudomonadales*, *Methylococcales*, and *Thiotrichales*. In this context, it has to be mentioned that OLs have been described in *Serratia marcescens*, which belongs to the *Enterobacteriaceae* (Miyazaki *et al.*, 1993). Unfortunately, no complete genome sequence of *S. marcescens* has been published so far. Within the *Deltaproteobacteria*, OlsB homologs are encoded in the genomes of *Stigmatella aurantiaca*, *Bacteriovorax marinus*, and *Bdellovibrio bacteriovorus*. Interestingly, OLs have been detected in the Deltaproteobacterium *Sorangium cellulosum* So ce56 (Keck *et al.*, 2011), but no gene encoding an OlsB homolog is present in the genome. The best hit when searching the *S. cellulosum* genome with OlsB from *B. cenocepacia* is the gene *rimI1*, which is predicted to encode a ribosomal protein alanine acetyltransferase (*sce1382*). This suggests that a second unrelated family of *N*-acyl transferases might be responsible for LOL formation in *S. cellulosum* and possibly in other bacteria. Among the actinomycetes are several species encoding OlsB homologs. Most of them can be classified into the families *Gordoniaceae*, *Micromonosporaceae*, *Mycobacteriaceae*, *Nocardiaceae*, *Pseudonocardiaceae*, and *Streptomyceteae*. Among the spirochetes, several species from the genus *Leptospira* present a gene encoding an OlsB homolog. Only very few species belonging to other taxonomical groups present a gene encoding an OlsB homolog in their genomes. Compared to the large number of bacterial species that have been shown to form OL or that are predicted to be able to form OL, only few bacterial species have the now known OL-modifying enzymes. The identified OL hydroxylases belong either to the $Fe^{2+}/O_2/α$-ketoglutarate-dependent superfamily of hydroxylases (OlsC and OlsD) or to the di-iron fatty acid hydroxylase superfamily (OlsE) (Table S1). The phylogenetic distribution of these OL hydroxylases is described in the sections The OL hydroxylase OlsC, The OL hydroxylase OlsD and The OL hydroxylase OlsE and in Table S1. The 2-hydroxylase from *Burkholderia* species has not been isolated yet, so it is not known whether it belongs to the already mentioned superfamilies or to yet another superfamily such as the cytochrome P450-dependent enzymes (Matsunaga

et al., 2000; Lee *et al.*, 2003; Girhard *et al.*, 2007; Fujishi-ro *et al.*, 2011). As possible OL modifications might occur only under specific stress conditions, it is possible that additional modifications with their respective responsible enzymatic activities and genes will be found in the future in other organisms.

Regulation of OL biosynthesis and modification

It has been observed that the biosynthesis of OLs is regulated by the presence of certain nutrients in the growth medium. Some organisms such as *S. meliloti* synthesize only very minor amounts of OLs under phosphate-replete conditions (Geiger *et al.*, 1999). If the same organism is cultivated in a medium with limiting phosphate concentrations, then *olsB* gene transcription, which is regulated by the transcriptional regulator PhoB (Geiger *et al.*, 1999; Krol & Becker, 2004), is increased. It seems that at least in *S. meliloti* OlsB is the limiting factor for OL formation because constitutive expression of OlsB in *S. meliloti* 1021 causes the accumulation of OLs whether the bacteria are grown in high or low concentrations of phosphate (Gao *et al.*, 2004). However, many other bacteria such as *Brucella* species, *Burkholderia* species, *Agrobacterium* species, *Mesorhizobium loti* (Devers *et al.*, 2011), and *R. tropici* synthesize OLs constitutively in relatively high amounts even when grown in rich culture media containing high phosphate concentrations (González-Silva *et al.*, 2011; Palacios-Chaves *et al.*, 2011; Vences-Guzmán *et al.*, 2011). The reason for this difference occurring even in closely related bacterial species is not understood.

The OL biosynthesis genes *olsA* and *olsB* are separated by more than ten genes in *S. meliloti*, whereas in *P. aeruginosa* and many other organisms, they form an operon. These differences in gene organization might indicate differences in the regulation of gene expression. This is consistent with the observation that phosphate starvation induces *olsB* expression, but not *olsA* expression in *S. meliloti* (Gao *et al.*, 2004; Krol & Becker, 2004), whereas in *P. aeruginosa* also *olsA* is induced by phosphate limitation (Lewenza *et al.*, 2011).

A different nutritional condition, low magnesium ion concentration, has been shown to repress OL biosynthesis in *Pseudomonas fluorescens* (Minnikin & Abdolrahim-zadeh, 1974).

The frequency of OL hydroxylation seems to correlate in some cases with abiotic stress conditions. In *B. cenocepacia* and *R. tropici*, increased temperatures (42 °C) caused the accumulation of OL species hydroxylated in the C-2 position of the piggy-back fatty acid (Taylor *et al.*, 1998; Vences-Guzmán *et al.*, 2011). Under acidic growth conditions, both the OlsD-dependent hydroxyl-

ation and the OlsC-dependent hydroxylation seem to be induced in *B. cenocepacia* and *R. tropici*, respectively (González-Silva *et al.*, 2011; Vences-Guzmán *et al.*, 2011).

OL functions

Although several mutants deficient in OL biosynthesis have been constructed and characterized, the roles that OLs play are still not clear. In Gram-negative bacteria, OLs are enriched in the outer membrane (Dees & Shively, 1982; Lewenza *et al.*, 2011; Vences-Guzmán *et al.*, 2011), and owing to their zwitterionic nature, it had been proposed that they play an important role in the stabilization of negative charges of LPS and therefore in outer membrane stability (Freer *et al.*, 1996). One common observation seems to be that OLs are involved in stress response. For the case of 2-hydroxylated lipid A, it has been suggested that the additional hydroxyl groups might increase the extent of hydrogen bonding between the lipid molecules, thereby decreasing the outer membrane fluidity while at the same time making it less permeable (Gibbons *et al.*, 2000). These changes should be of advantage under abiotic stress conditions such as increased temperature or low pH. The introduction of a 2-hydroxyl group into OLs should have similar consequences as described above for lipid A hydroxylation. Interestingly, both *B. cenocepacia* and *R. tropici* show an increase in OL 2-hydroxylation under thermal stress conditions (Taylor *et al.*, 1998; Vences-Guzmán *et al.*, 2011), and *R. tropici* mutants deficient in the OL hydroxylase OlsC show a severe growth defect under this condition.

Earlier studies have reported an increase in resistance to antimicrobial peptides correlating with OL accumulation in some bacteria (Minnikin & Abdolrahimzadeh, 1974; Dorrer & Teuber, 1977). Recently, however, it has been demonstrated that OLs are not required to increase the resistance to antimicrobial peptides in *B. abortus* and *P. aeruginosa* (Lewenza *et al.*, 2011; Palacios-Chaves *et al.*, 2011).

During the last year, two more OL hydroxylations have been described (González-Silva *et al.*, 2011; Vences-Guzmán *et al.*, 2011). As OLs from some bacteria can present multiple hydroxylations within the same molecule, it probably can be assumed that different modifications affect membrane properties in different ways. Accordingly, the responsible hydroxylase activities should be regulated differentially. At high temperature or in acid pH, conditions under which the OlsC-modified OL P1 accumulated in *R. tropici* CIAT899 (Vences-Guzmán *et al.*, 2011), the OlsE-hydroxylated OLs S2 and P2 could not be detected. Consistent with this idea, we have observed in *A. tumefaciens* that the relative amount of the OlsE-hydroxylated OL S2 increases at lower growth temperature

(Vences-Guzmán et al., preparation). This indicates that the OlsE-dependent hydroxylation might increase, for example, membrane fluidity, which would be opposite to the predicted effect of the OlsC-dependent hydroxylation.

In the purple nonsulfur facultative phototroph R. capsulatus, it has been shown that OL biosynthesis and the steady-state amounts of some extracytoplasmic proteins, including various c-type cytochromes, are interrelated. In the absence of OLs, R. capsulatus does not contain a full complement of c-type cytochromes under certain physiological conditions (Aygun-Sunar et al., 2006). One possible explanation is that protein–lipid interactions between OLs and certain membrane proteins are required for the localization, folding, stability, assembly, and/or enzymatic activity of certain integral membrane proteins (Aygun-Sunar et al., 2006).

Interestingly, OLs also serve functions outside the membrane in some organisms. It has been reported that OLs are used as emulsifiers for crude oil in the marine bacterium Myroides sp. (Maneerat et al., 2006).

OLs as bioactive lipids

The lipid A moiety of LPS is detected by the TLR4/MD2 receptor of the mammalian innate immune response, causing macrophages to synthesize potent mediators of inflammation, such as TNF-α and IL-1β (Beutler & Cerami, 1988; Dinarello, 1991); especially, its phosphate and acyl-oxyacyl groups are needed to trigger full TLR4/MD2 activation in human cells (Rietschel et al., 1994). OLs and SGLs also contain the acyloxyacyl structure present in lipid A.

It has been shown that OLs and SGLs can be used as adjuvants (Kato & Goto, 1997; Kawai et al., 1999, 2002) and when injected into mice before lipid A can prevent the lethal effects of the latter. It was speculated that the OLs and SGLs might function as antagonistic blockers of events triggered by lipid A (Kawai et al., 1991). The components involved in the translation of the signal induced by OLs have not yet been identified. The structural similarity of the OLs with lipid A and the SGLs suggests that OLs will probably use the same components as the lipid A and SGLs.

A recent study showed that B. abortus OLs do not stimulate cytokine secretion in murine macrophages, whereas OLs from Bordetella pertussis notably stimulated TNF-α and IL-6 secretion (Palacios-Chaves et al., 2011). At first glance, the only difference between OLs from B. abortus and OLs from B. pertussis seems to be with respect to fatty acyl chain lengths (Palacios-Chaves et al., 2011). An alternative explanation might be that B. pertussis presents hydroxylated OLs under specific growth conditions. Most studies failed to detect hydroxylated OL in B. pertussis, but Thiele & Schwinn (1973) clearly detected the presence of a ninhydrin-positive lipid migrating

similarly as a hydroxylated OLs from B. cenocepacia or R. tropici (Taylor et al., 1998; Rojas-Jiménez et al., 2005; González-Silva et al., 2011; Vences-Guzmán et al., 2011).

Perspectives

The recent decade has brought many advances in our knowledge about OL biosynthesis and function. In 2002 and 2004, Geiger and coworkers identified two acyltransferases required for OL biosynthesis. The general idea is that both proteins are sufficient for OL biosynthesis. However, the expression of sinorhizobial OlsBA in Escherichia coli is not sufficient to convert this host into an OL producer (O. Geiger and I.M. López-Lara, unpublished data). Our combined analysis of the scientific literature with respect to OLs and the presence of OlsB-encoding genes in bacterial genome sequences indicates that in addition to the OlsBA-dependent pathway, other pathways for OL biosynthesis must exist at least in S. cellulosum and Flavobacterium sp. More recently, three OL hydroxylases have been discovered, two of which catalyzing modifications that were not known previously. Still, the gene encoding the 2-hydroxylase from Burkholderia, one of the first organisms where the 2-hydroxylation of the piggyback fatty acid has been described, is still unknown.

The exact functions of the different OL modifications have not been defined yet, although several OL biosynthesis genes are now known and mutants deficient in these activities have been constructed and partially characterized. The analysis of the mutants should continue, especially with respect to changes in membrane properties caused by the presence and absence of the distinct modifications. Hand in hand should go a structural determination of the products of the OlsD- and OlsE-catalyzed reactions. The exact structure of both modifications is required to understand the function/properties of the different lipids on a biophysical level.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of OlsB, OlsC, OlsD and OlsE homologs present in eubacteria.

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➢ **La tercera sección trata sobre el artículo "Agrobacteria lacking ornithine lipids induce more rapid tumour formation Environmental Microbiology". 2013; 15(3):895-906. <u>Vences-Guzmán, M. A.</u>, Guan, Z., Bermudez-Barrientos, J. R., Geiger O., and Sohlenkamp, C.**

Agrobacteria lacking ornithine lipids induce more rapid tumour formation

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Summary

Ornithine lipids (OLs) are phosphorus-free membrane lipids that 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. It has been shown that OLs can be hydroxylated within the amide-linked fatty acyl moiety, the secondary fatty acyl moiety or within the ornithine moiety. These modifications have been related to increased stress tolerance and symbiotic proficiency in different organisms such as *Rhizobium tropici* or *Burkholderia cenocepacia*. Analysing the membrane lipid composition of the plant pathogen *Agrobacterium tumefaciens* we noticed that it forms two different OLs. In the present work we studied if OLs play a role in stress tolerance and pathogenicity in *A. tumefaciens*. Mutants deficient in the OLs biosynthesis genes *olsB* or *olsE* were constructed and characterized. They either completely lack OLs (ΔolsB) or only form the unmodified OL (ΔolsE). Here we present a characterization of both OL mutants under stress conditions and in a plant transformation assay using potato tuber discs. Surprisingly, the lack of agrobacterial OLs promotes earlier tumour formation on the plant host.

Introduction

Ornithine lipids (OLs) are phosphorus-free membrane lipids that are widespread among Gram-negative bacteria and also present in some Gram-positive bacteria, especially in actinomycetes species, but seem to be absent from Archaea and Eukarya (López-Lara *et al.*, 2003; Geiger *et al.*, 2010; Vences-Guzmán *et al.*, 2012). They 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. The genes *olsB* and *olsA* encoding the two enzymes essential for OL biosynthesis from ornithine and acyl-ACPs have been first described in *Sinorhizobium meliloti* 1021 (Fig. S1) (Weissenmayer *et al.*, 2002; Gao *et al.*, 2004; Geiger *et al.*, 2010). For a long time it has been known that the ester-linked fatty acid of OLs can be hydroxylated at the C-2 position in some organisms (Asselineau, 1991). Recently, two more OL modifications have been described. In *Burkholderia cenocepacia* the amide-linked fatty acid can be hydroxylated in an unknown position by the hydroxylase OlsD (González-Silva *et al.*, 2011) and in *Rhizobium tropici* the ornithine headgroup can be hydroxylated in a still unknown position (Vences-Guzmán *et al.*, 2011). All three modifications have been related to increased stress tolerance or symbiotic proficiency (Taylor *et al.*, 1998; Rojas-Jiménez *et al.*, 2005; González-Silva *et al.*, 2011; Vences-Guzmán *et al.*, 2011).

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* and *R. tropici* (Dees and Shively, 1982; Vences-Guzmán *et al.*, 2011). It has been speculated that OL hydroxylation may increase hydrogen bonding between neighbouring OL molecules similarly as has been suggested for LpxO-hydroxylated lipid A in *Salmonella* and hydroxylated sphingolipids (Gibbons *et al.*, 2000; Nikaido, 2003; Murata *et al.*, 2007). These additional hydrogen bonds should result in bilayer stabilization and a decrease in membrane permeability which could help to explain the observed stress resistance phenotype related to hydroxylated OLs.

Ornithine lipids together with other phosphorus-free membrane lipids such as diacylglycerol-*N,N,N*-trimethylhomoserine (DGTS) or sulfolipids (SLs) accumu-

late under phosphate-limiting conditions in several bacterial species while at the same time phospholipids are actively degraded (Benning *et al.*, 1995; López-Lara *et al.*, 2003; Geiger *et al.*, 2010; Zavaleta-Pastor *et al.*, 2010). In *S. meliloti* it has been shown that this remodelling is under the control of the response regulator PhoB (Geiger *et al.*, 1999; Zavaleta-Pastor *et al.*, 2010). As OLs and phosphatidylethanolamine (PE) both are zwitterionic lipids, it has been speculated that OLs might replace PE under these conditions (Benning, 1998), although clear experimental evidence is lacking so far. Some other bacteria such as *Brucella abortus* (Thiele and Schwinn, 1973; Comerci *et al.*, 2006; Bukata *et al.*, 2008), *Mesorhizobium loti* (Devers *et al.*, 2011), *B. cenocepacia* (González-Silva *et al.*, 2011) and *R. tropici* (Rojas-Jiménez *et al.*, 2005; Sohlenkamp *et al.*, 2007; Vences-Guzmán *et al.*, 2011) also form significant amounts of OLs during growth in standard laboratory media such as Luria–Bertani (LB) which contain phosphate in concentrations that are not growth-limiting. It is not clear if OLs biosynthesis is regulated by PhoB in these organisms. We had observed that OLs apparently play an important role in *R. tropici* both under abiotic stress conditions such as heat stress and acid stress but also under symbiotic conditions (Vences-Guzmán *et al.*, 2011).

Agrobacterium tumefaciens is α-proteobacterium able to form tumours on many dicotyledonous plant species. Analysing the membrane lipid composition of the plant pathogen *A. tumefaciens* we noticed that it forms two different OLs, corresponding to the unmodified OL S1 and the hydroxylated OL S2 (Fig. S1). In the present work we wanted to study if OLs play a role in stress tolerance and pathogenicity in *A. tumefaciens*.

Agrobacterium tumefaciens mutants deficient in the OLs biosynthesis genes *olsB* or *olsE* were constructed and characterized. They either completely lack OLs (Δ*olsB*) or only form the unmodified OL (Δ*olsE*). Here we present a characterization of both OL mutants under different abiotic stress conditions and in a plant transformation assay using potato tuber discs.

Results

Agrobacterium tumefaciens *forms two different ornithine lipids*

When analysing the lipid composition of [^{14}C]acetate-labelled *A. tumefaciens* C58 by two-dimensional thin layer chromatography (TLC), in addition to the phospholipids phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), dimethyl PE, cardiolipin (CL), and the unmodified OL S1, a lipid migrating like the OL S2 from *R. tropici* CIAT899 (Fig. S1) (Rojas-Jiménez *et al.*, 2005; Vences-Guzmán *et al.*, 2011) can be observed
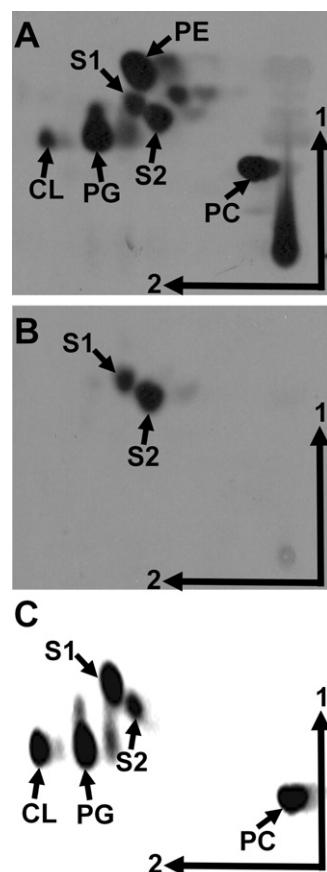

Fig. 1. *Agrobacterium tumefaciens* forms an OL migrating similarly as S2 from *R. tropici*. [^{14}C]acetate- (A) and [^{14}C]ornithine- (B) labelled lipids from *A. tumefaciens* wild-type C58 grown at 30°C in 20E medium and [^{14}C]acetate-labelled lipids from *S. meliloti* CS111.pNG25.pERMAV14 grown in TY medium (C) were separated by two-dimensional TLC. The phospholipids PE, monomethyl PE (MMPE), PC, phosphatidylglycerol (PG), cardiolipin (CL), and the OLs S1 and S2 are indicated.

(Fig. 1A). In order to confirm that this unknown lipid is biosynthetically derived from the amino acid ornithine, *A. tumefaciens* C58 cells were labelled with [^{14}C]ornithine and the lipids were extracted and analysed by TLC. Apparently two ornithine-derived lipids are present in *A. tumefaciens* C58 (Fig. 1B). An identical result was obtained with *A. tumefaciens* strain A208 (data not shown).

The plant pathogen Agrobacterium tumefaciens *also forms the ornithine lipid S2*

Three different hydroxyl group modifications of OLs have been described in bacteria. To identify in which part of the second agrobacterial OL the modification is encountered, lipids were extracted according to Bligh and Dyer from a 1 l culture of *A. tumefaciens* wild-type A208. Both putative OLs were purified from the total lipid extract and analysed by normal phase LC/ESI-MS/MS in the negative ion
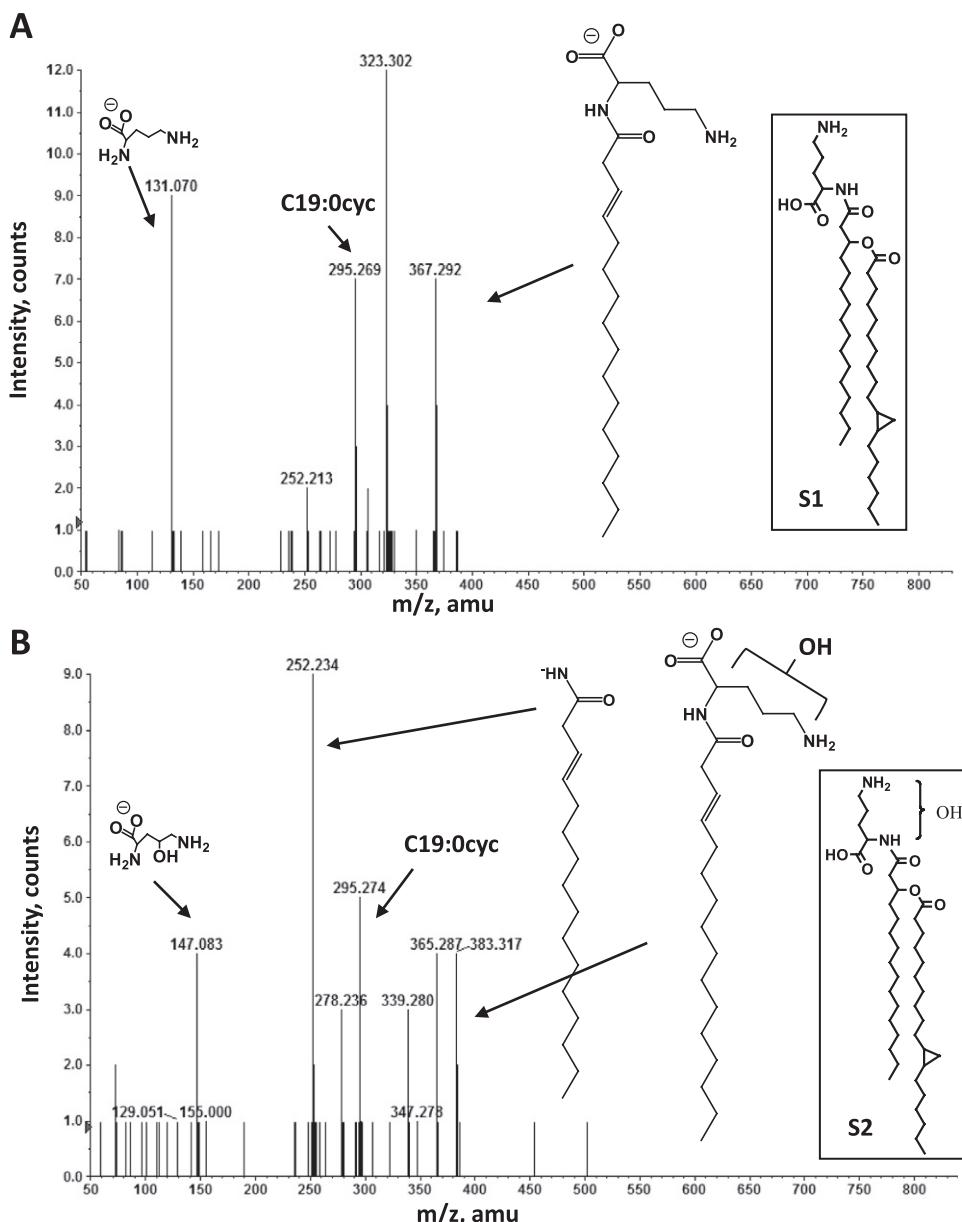
Fig. 2. Collision-induced dissociation mass spectra of OL S1 and S2 detected in lipid extract of *A. tumefaciens*. Negative ion collision induced dissociation mass spectra of [M-H]⁻ ions at *m/z* 663.6 (A) obtained from OL S1 and *m/z* 679.6 (B) obtained from OL S2. The structures of major fragment ions are indicated. The structures of the parental lipids are shown in the boxes.

mode. The [M-H]⁻ molecular ions of OL S1 and S2 were detected at *m/z* 663.6 and 679.6 respectively (Fig. S2). The mass difference of 16 amu suggests the presence of an additional oxygen atom indicating the presence of an additional hydroxyl group in S2. Comparing the fragmentation patterns of both lipids it was determined that a hydroxyl group was present within the ornithine moiety in the case of the unknown OL but not in the fatty acyl chains (Fig. 2). *Agrobacterium tumefaciens* therefore forms the OLs S1 and S2 (Fig. S1). The fragment ion spectra of

the [M-H]⁻ ions of S1 and S2 at *m/z* 663.6 and 679.6 respectively, revealed that the amide-linked fatty acid is almost exclusively a C16 (3-OH) fatty acid indicating a substrate specificity of the agrobacterial OlsB for this fatty acid.

Ninhydrin staining of the *A. tumefaciens* membrane lipids separated by two-dimensional TLC confirmed our earlier observation of the OLs from *R. tropici*. S2 reacts with a delay in comparison to S1 and the developed colour by S2 is different. While S1 upon reaction with
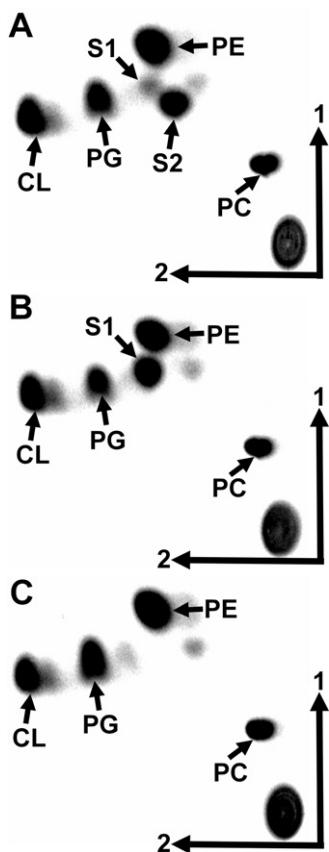
Fig. 3. Analysis of membrane lipid composition of *A. tumefaciens* wild-type A208 (A), *olsE*-deficient mutant MAV07 (B) and *olsB*-deficient mutant MAV08 (C). Lipids were labelled with [^{14}C]acetate during growth in 20E medium at 30°C and separated using two-dimensional TLC. The phospholipids phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL) and the OLs S1 and S2 are indicated.

ninhydrin develops a red-to-purple colour, the reaction of S2 causes the formation of an orange colour (Fig. S3).

Atu0318 is responsible for the synthesis of the second ornithine lipid

The presence of the OL S2 indicates that the *A. tumefaciens* genome should encode an OlsE homologue. One of the closest homologues of OlsE from *R. tropici* CIAT899 is indeed Atu0318 which presents 61% identity and 71% similarity on amino acid level. BLAST searches show (Altschul *et al.*, 1997) that other close OlsE homologues are present in all sequenced *Agrobacterium* strains, *Rhodospirillum centenum* SE, *Azospirillum amazonense*, *Verrucomicrobium spinosum* and *Parvibaculum lavamentivorans* DS-1. Further homologues are present in several actinobacteria, a few γ-proteobacteria and a few other α-proteobacteria (Vences-Guzmán *et al.*, 2012). To

confirm that Atu0318 serves the same function as OlsE from *R. tropici* it was cloned into the broad host range plasmid pRK404 and subsequently conjugated into *S. meliloti* CS111.pNG25. In this strain a lipid migrating similarly as S2 from *R. tropici* was present that was absent in the negative control strain CS111.pNG25 (Fig. 1C and data not shown).

Lipid composition analysis of olsE and olsB mutants

We had observed a role for OLs in *R. tropici* stress resistance and symbiosis (Vences-Guzmán *et al.*, 2011) and we therefore considered it a possibility that OLs might play a role in stress resistance and pathogenesis in *A. tumefaciens*. To study the role of OLs in *A. tumefaciens* in more detail, mutants deficient in *olsE* and mutants deficient in *olsB* were constructed. Their lipid compositions were compared with the wild-type strain *A. tumefaciens* A208 (Fig. 3 and Table 1). As expected the *olsE*-deficient mutant MAV07 lacked the OL S2, and the *olsB*-deficient mutant MAV08 lacked S1 and S2. The total amount of OLs, being the sum of S1 and S2, is around 15% in both, the *A. tumefaciens* wild-type and the *olsE*-deficient mutant MAV07 when grown in complex LB medium at 30°C (Table 1). In the absence of OLs in the mutant MAV08, more PE is formed, whereas no major differences are observed in the relative amounts of the phospholipids, PC, PG and CL between the different strains (Table 1). This suggests that the amounts of PE and OLs are inversely proportional.

Both mutants, MAV07 ($\Delta olsE$) and MAV08 ($\Delta olsB$), were also complemented. When introducing a functional copy of *olsB in trans* into the mutant MAV08 again formation of S1 and S2 was observed, while when introducing a functional copy of *olsE in trans* into the mutant MAV07 again formation of S2 was observed (data not shown).

Table 1. Membrane lipid composition of *A. tumefaciens* wild-type A208, *olsE*-deficient mutant MAV07 and *olsB*-deficient mutant MAV08 after growth on complex LB medium at 30°C.

Lipid	Composition (% of total ^{14}C)		
	A208	MAV07 ($\Delta olsE$)	MAV08 ($\Delta olsB$)
PC	21.3 ± 0.5	26.0 ± 1.2	24.2 ± 0.5
S1	5.8 ± 0.2	15.9 ± 0.3	n.d.
S2	9.5 ± 0.2	n.d.	n.d.
PE	41.5 ± 0.4	35.7 ± 1.3	53.2 ± 0.2
PG	18.8 ± 0.4	20.5 ± 0.2	20.2 ± 0.9
CL	3.1 ± 0.5	1.9 ± 0.1	2.4 ± 0.2

The values shown are mean values ± standard deviation derived from at least three independent experiments.
S1: unmodified ornithine lipid; S2: hydroxylated ornithine lipid; n.d.: not detected.

Table 2. Membrane lipid composition of *A. tumefaciens* wild-type, *olsE*-deficient mutant MAV07 and *olsB*-deficient mutant MAV08 after labelling for 48 h with [^{14}C]acetate in Sherwood minimal medium at 30°C or 15°C.

	Composition (% of total ^{14}C)					
	30°C			15°C		
Lipid	A208	MAV07	MAV08	A208	MAV07	MAV08
PC	13.6	14.7	19.3	28.4	34.1	33.3
S1	6.8	20.2	n.d.	n.d.	16.0	n.d.
S2	14.3	n.d.	n.d.	16.8	n.d.	n.d.
PE	29.7	30.8	43.0	39.0	31.3	52.6
PG	12.2	11.5	18.9	11.6	15.0	11.7
CL	23.4	22.8	18.8	4.2	3.6	2.4

The values shown are mean values derived from at least two independent experiments.
S1: unmodified ornithine lipid; S2: hydroxylated ornithine lipid; n.d.: not detected.

Hydroxylated OL S2 accumulates when A. tumefaciens *is grown at 15°C*

We had reported earlier that in *R. tropici* no OlsE-dependent hydroxylation could be observed at 42°C (Vences-Guzmán *et al.*, 2011). We therefore wondered if OlsE might be more active at lower temperatures than at higher temperatures. When comparing the membrane lipid composition of cells grown at 15°C to cells grown at 30°C several differences can be observed (Table 2). The relative amounts of the phospholipids PE and PC seem to increase, whereas CL is drastically reduced and the amount of PG does not change when going from 30°C to 15°C. Interestingly, virtually all OL seems to be in the hydroxylated form at 15°C indicating that OlsE is sufficiently active to convert all OL S1 into hydroxylated S2. When studying the growth of both mutants and the wild-type at 15°C, all strains grow with similar generation times

indicating that the presence or absence of the OLs does not affect growth of *A. tumefaciens* under these conditions (data not shown).

Absence of OLs does not affect growth of A. tumefaciens *under conditions of high osmolarity*

Agrobacterium tumefaciens wild-type A208 and both mutants deficient in OL biosynthesis were cultivated in complex 20E medium supplemented with up to 1 M NaCl. Up to 0.8 M NaCl no significant differences in generation time could be observed between the three strains. At 0.9 M NaCl only residual growth was observed for all strains and in the presence of 1.0 M NaCl none of the strains was able to grow. Under all conditions studied both mutants behaved very similar to the wild-type indicating that OLs do not play an important role in protecting the bacteria against osmotic stress (data not shown).

Growth of A. tumefaciens *under phosphate limitation*

Agrobacterium tumefaciens wild-type A208 and mutants deficient in OL biosynthesis were grown in Sherwood minimal medium with high (1.3 mM) or low phosphate (20 μM) concentrations. Under high phosphate conditions the phospholipids PC, PG, PE, dimethyl PE and CL were detected in the wild-type (Table 3). In addition, as observed earlier in cells grown on complex medium the two OLs S1 and S2 were detected. Under low phosphate conditions all phospholipids were reduced and both OLs increased to a total of about 45–50% in the wild-type. In addition, several new lipids were observed in the phosphate-deplete grown cells. In addition to DGTS several unknown lipids were detected. As Benning and colleagues (1995) and Devers and colleagues (2011) had observed the formation of glycolipids in *R. sphaeroides*

Table 3. Analysis of *A. tumefaciens* strains grown in Sherwood medium containing 20 μM or 1.3 mM phosphate.

	Composition (% of total ^{14}C)					
	High phosphate			Low phosphate		
	A208	MAV07	MAV08	A208	MAV07	MAV08
PC	13.6 ± 0.5	14.7 ± 0.5	19.3 ± 0.7	7.3 ± 0.6	9.5 ± 0.5	10.0 ± 1.0
PG	12.2 ± 0.2	11.5 ± 0.5	18.9 ± 0.7	3.6 ± 0.6	4.5 ± 0.1	5.3 ± 0.6
PE	29.7 ± 0.6	30.8 ± 1.4	43.0 ± 1.2	2.7 ± 0.4	2.0 ± 0.6	6.6 ± 0.5
CL	23.5 ± 1.0	22.8 ± 1.1	18.8 ± 0.9	3.9 ± 0.9	5.9 ± 0.1	7.0 ± 0.1
S1 + Gl4	6.8 ± 1.0	20.2 ± 0.3	n.d.	21.8 ± 0.8	42.9 ± 0.9	6.4 ± 0.5
S2	14.2 ± 0.3	n.d.	n.d.	30.0 ± 0.1	n.d.	n.d.
GL1	n.d	n.d.	n.d.	12.9 ± 0.9	20.9 ± 0.5	17.1 ± 0.3
GL2	n.d	n.d.	n.d.	2.6 ± 0.5	2.9 ± 0.3	11.3 ± 0.8
GL3	n.d.	n.d.	n.d.	1.5 ± 0.4	2.5 ± 0.1	2.6 ± 0.5
DGTS	n.d.	n.d.	n.d.	13.7 ± 0.5	8.9 ± 0.5	33.7 ± 1.4

A208 – wild-type, MAV07 – Δ*olsE* mutant, MAV08 – Δ*olsB* mutant. The values shown are mean values ± standard deviation derived from at least three independent experiments. The bacterial strains were labelled for 48 h with [^{14}C]acetate.
S1: unmodified ornithine lipid; S2: hydroxylated ornithine lipid; GL1, GL2, GL3, GL4: unknown glycolipids; n.d.: not detected.

and *M. loti* under low phosphate conditions we considered it a possibility that the unknown lipids were glycolipids. Naphthol staining of membrane lipids separated by TLC confirmed the presence of at least four different glycolipids. In contrast to *S. meliloti* and *R. sphaeroides* no sulfolipid was detected under phosphate starvation conditions which is consistent with the absence of genes coding for homologues involved in their biosynthesis in the *A. tumefaciens* genome. In the *olsB*-deficient mutant unable to form OLs increased amounts of DGTS can be detected in addition to increased amounts of the unknown lipids (Table 3).

Bioinformatic prediction of the PhoB regulon

A bioinformatic search of the *A. tumefaciens* C58 genome was performed for the presence of Pho boxes to find out if the OL biosynthesis genes are under the control of PhoB and at the same time understand the phosphate starvation response in *A. tumefaciens*. A position-specific scoring matrix (PSSM) was constructed using Pho box sequences from *S.meliloti* 1021 identified in an earlier study (Yuan *et al.*, 2006). The presence of a Pho box was predicted for the *olsB*, but not for the *olsE* promoter from *A. tumefaciens* (data not shown). Additionally, our bioinformatic analysis predicted PhoB-dependent regulation for DGTS biosynthesis as had been shown for *S. meliloti*. Putative Pho boxes were also predicted to precede the genes encoding the putative phosphate transport regulator Atu4634, the putative phospholipase C PlpC (Atu1649), and the putative phosphohydrolase Atu0877. Interestingly, four genes (*Atu0842*, *Atu1808*, *Atu2222*, *Atu2297*) are preceded by putative Pho boxes that might encode glycosyltransferases involved in the biosynthesis of the several unknown and putative glycolipids that accumulate under conditions of phosphate starvation in *A. tumefaciens*.

Phenocopying the OL composition of R. tropici *in* A. tumefaciens

We had observed earlier that the presence of the hydroxylase OlsC responsible for the synthesis of P1 was important to confer acid resistance to *R. tropici* (Vences-Guzmán *et al.*, 2011). *Agrobacterium tumefaciens* forms the OLs S1 and S2 and by introducing the gene *olsC* we would phenocopy or mimic the OL composition of *R. tropici*. We speculated that *A. tumefaciens* harbouring *olsC* might show an increase in acid resistance. Wild-type strain A208, mutants deficient in *olsB* or *olsE* and the wild-type expressing *olsC* from *R. tropici* were grown at pH 5.0, pH 5.5 and pH 7.0. Although OLs P1 and P2 could be detected in the strain expressing *olsC*, the presence of
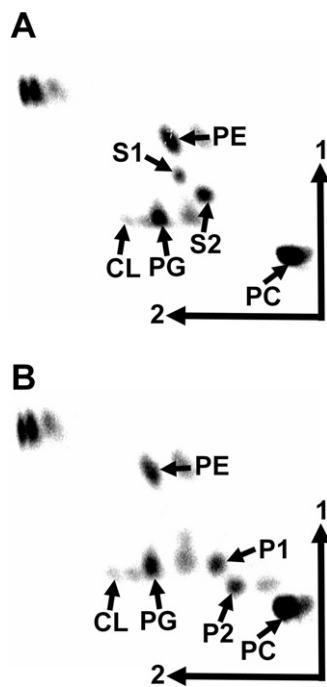

Fig. 4. Heterologous expression of *olsC* from *Rhizobium tropici* in *Agrobacterium tumefaciens* A208. Lipids of the wild-type *A. tumefaciens* A208 harbouring the empty plasmid pERMAV06 (A) or the *olsC*-containing plasmid pERMAV15 (B) were labelled with [^{14}C]acetate for 48 h during growth in LB medium at 30°C and separated using two-dimensional TLC. The phospholipids phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL) and the OLs S1, and S2 are indicated. OLs P1 and P2 derived from OlsC-dependent hydroxylation of S1 and S2 are also indicated.

OlsC did not improve the growth of *A. tumefaciens* at low pH (Fig. 4, data not shown).

Plant assays of tumours in A. tumefaciens

The virulence of *A. tumefaciens* mutants deficient in the formation of OLs was assayed using a potato tuber disc system (Shurvinton and Ream, 1991; Tsai *et al.*, 2009). All strains caused the formation of tumours on potato tuber discs. Interestingly, the mutants deficient in the formation of OLs behaved differently from the wild-type. Tumours appeared within 18 days on discs inoculated with MAV08 or MAV07 while tumours appeared 6 days later on discs inoculated with the wild-type *A. tumefaciens* A208 (Fig. 5 and Table 4). Complementation of both mutants with the respective gene *in trans* restored the wild-type phenotype. The tumours on potato tuber discs inoculated with the mutant strains are also larger than the tumours on tuber discs inoculated with the wild-type. This size difference is probably due to the longer development of the tumours on tuber discs inoculated with the OL-deficient mutant strains.
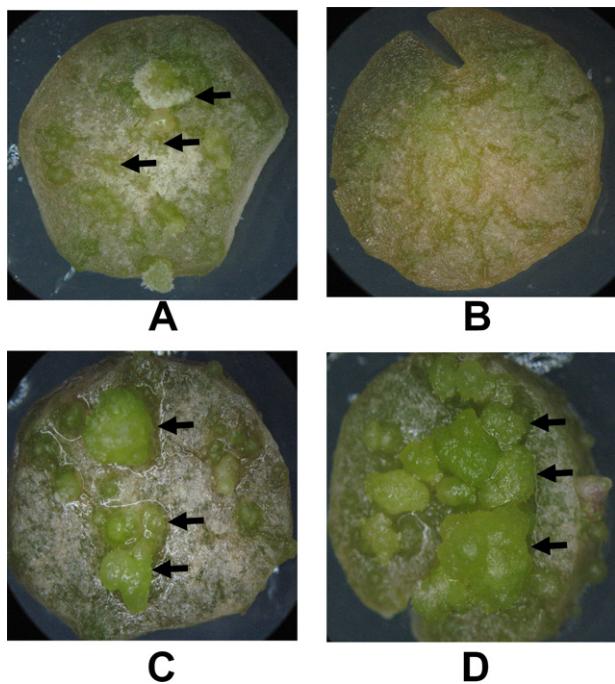
Fig. 5. Assay of tumour formation by *Agrobacterium tumefaciens* using potato tuber discs. Potato discs were inoculated with (A) *A. tumefaciens* A208, (B) no bacteria, (C) OlsE-deficient mutant MAV07, (D) OlsB-deficient mutant MAV08. Potato tuber discs were scored every 3 days for tumour formation. The tuber discs have a diameter of 1 cm. Images were taken 48 days after inoculation. Tuber discs were incubated at 28°C. The arrows highlight some tumours on the potato discs.

Discussion

Ornithine lipids are widespread in eubacteria. In addition to the unmodified OL consisting of a 3-hydroxy fatty acid linked by an amide bond to the α-amino group of ornithine and a second fatty acid bound in ester linkage to the first, several hydroxylated forms of OL have been described. The exact function of these modifications is not known yet, but several studies now hint at functions related to abiotic stress resistance and during interactions with

eukaryotic hosts (Dees and Shively, 1982; Asselineau, 1991; Taylor *et al.*, 1998; Rojas-Jiménez *et al.*, 2005; González-Silva *et al.*, 2011; Vences-Guzmán *et al.*, 2011).

Here, we wanted to study the role of OLs in the plant pathogen *A. tumefaciens*. We characterized *A. tumefaciens* mutants deficient in OL biosynthesis under a set of abiotic stress conditions: low growth temperatures, high salt concentrations, low phosphate concentration in the growth medium, and acid stress.

It has been speculated that the 2-hydroxylation of the ester-linked fatty acid in OL may increase hydrogen bonding between neighbouring OL molecules similarly as has been suggested for LpxO-hydroxylated lipid A in *Salmonella* and hydroxylated sphingolipids (Gibbons *et al.*, 2000; Nikaido, 2003; Murata *et al.*, 2007). These additional hydrogen bonds should result in bilayer stabilization and a decrease in membrane permeability. The fact that the OL S2 with OlsE-dependent modification accumulates at low growth temperatures in *A. tumefaciens* indicates that introducing a hydroxyl group into the OL headgroup might have the opposite effect than introducing it into the ester-linked fatty acid.

Agrobacterium tumefaciens forms two OLs even under high phosphate conditions. Together both OLs make up about 15% of the membrane lipids. Under low phosphate conditions we observed an accumulation of OLs to up to 45–50% of total lipids. This is consistent with our bioinformatic analysis indicating that agrobacterial *olsB* is preceded by a Pho box. Apparently although *olsB* is constitutively expressed in *A. tumefaciens*, the response regulator PhoB can further induce the levels of *olsB* transcription under conditions of phosphate limitation. No Pho box was predicted for the *olsE* promoter.

The presence of the OL hydroxylase OlsC has been shown to be important in *R. tropici* to resist conditions of acid stress and high temperature. *Agrobacterium tumefaciens* wild-type grows in complex media at pH 5.2 whereas *R. tropici* is distinctively more acid-resistant and grows at pH 4.0. Expressing *olsC* from *R. tropici* in

Table 4. Kinetics of tumour formation by *A. tumefaciens* on potato tuber discs.

Strain	Genotype	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
							Days after inoculation										
No bacteria		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MAV07	*olsE⁻*	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+
MAV07.pERMAV05	*olsE⁻*	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+
MAV07.pERMAV28	*olsE⁺*	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
MAV08	*olsB⁻*	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+
MAV08.pERMAV06	*olsB⁻*	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+
MAV08.pERMAV30	*olsB⁺*	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
A. tumefaciens A208	Wild-type	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+

Discs were inculated with 10^8 bacteria on day 0 and bacteria were killed by timentin treatment after 48 h. Discs were scored for tumour formation every 3–4 days. The experiment was repeated independently three times. Each experiment contained 30 replicates of each strain.

A. tumefaciens would enable *A. tumefaciens* to synthesize the OL P1 which has an important role under these conditions in *R. tropici* (Vences-Guzmán *et al.*, 2011). Although OlsC is clearly active in the *Agrobacterium* strain harbouring the *olsC*-containing plasmid, no effect on acid stress resistance can be observed. This indicates that the stress resistance observed in *R. tropici* and that depended on the presence of OlsC might not be a direct effect of the presence of hydroxylated OL, but rather an indirect effect. The hydroxylated OL might somehow influence the enzyme activity of a protein important in stress resistance present in *R. tropici* but absent in *A. tumefaciens*. An alternative explanation is that OlsC might be able to hydroxylate a second substrate in addition to OLs and that this hydroxylation is responsible for the observed stress resistance.

Potato tuber discs inoculated with the mutant MAV08 (*ΔolsB*) deficient in OL formation or with the mutant MAV07 (*ΔolsE*) deficient in formation of the OL S2, show tumour formation about 1 week earlier than plants inoculated with wild-type bacteria. Complementation of both mutants (MAV07 and MAV08) with the respective genes *in trans* restores the slow tumour growth phenotype. A consequence of the earlier onset of tumour formation is the increased size of the tumours that is observed. Crown gall tumour formation occurs because *A. tumefaciens* can transfer an oncogenic T-DNA from its tumour-inducing (Ti) plasmid into plant cells. After integration of the T-DNA into the plant host genome, T-DNA-encoded proteins cause cytokine and auxin synthesis eventually leading to crown gall formation. T-DNA transfer from *A. tumefaciens* to the plant cell requires activation of the *vir* regulon present on the Ti plasmid (Gelvin, 2006). Salicylic acid and ethylene, two plant signal molecules involved in the regulation of defence-regulated gene expression during plant–microbe interaction (Ecker and Davis, 1987; Prithiviraj *et al.*, 2005) have been shown to inhibit expression of the *vir* regulon (Yuan *et al.*, 2007; Nonaka *et al.*, 2008). One possible hypothesis for the earlier appearance of tumours is that (hydroxylated) OLs can be detected by the plant and that the presence of (hydroxylated) OLs causes some kind of plant defence leading to the production of ethylene or salicylic acid. In the absence of OLs this response is not as strong or absent and the infection process is accelerated. Interestingly, OLs share a 3-acyl-oxyacylamide structure with lipid A from Gram-negative bacteria, which has been shown to work as an elicitor in plant–microbe interactions (Scheidle *et al.*, 2005; Silipo *et al.*, 2008; 2010; Madala *et al.*, 2011). Both, OLs and lipid A are OM lipids. Several studies have shown that plants can sense lipid A but owing to the limited number of lipid A structures determined from plant-associated bacteria, no clear structure–activity relationship exists as for lipid A effects in animals (Madala *et al.*, 2011). Our observation that the

absence of an agrobacterial membrane lipid affects tumour formation is not unprecedented. Wessel and colleagues (2006) had observed that *A. tumefaciens* mutants not able to form PC are deficient in tumour formation because they completely lack the type IV secretion machinery (Wessel *et al.*, 2006).

The exact functions of OLs are still unknown but the present study adds more evidence to an important role of these lipids in bacteria–host interactions.

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 S1. *Agrobacterium tumefaciens* strains were grown in complex LB medium at 30°C, in complex 20E medium at 30°C (Werner *et al.*, 1975), or in Sherwood minimal medium (Sherwood, 1970) at 30°C or 15°C. *Escherichia coli* strains were grown in LB medium at 37°C. *Sinorhizobium meliloti* CS111.pNG25 was grown in complex tryptone-yeast extract medium (TY) (Beringer, 1974) supplemented with 10 mM $CaCl_2$. When needed, antibiotics were added at the following final concentrations (mg ml^{-1}): kanamycin (Km) 50; carbenicillin (Cb) 100; tetracycline (Tc) 8; timentin (Tim) 100, and rifampicin (Rif) 10.

DNA manipulations

Recombinant DNA techniques were performed according to standard protocols (Russell and Sambrook, 2001). Oligonucleotide primer sequences are listed in Table S2.

Expression of the candidate ORF Atu0318 from Agrobacterium tumefaciens

The candidate ORF for OlsE (*Atu0318*) was amplified using genomic DNA from *A. tumefaciens* A208 as a template and XL-PCR polymerase (Applied Biosystems). Specific oligonucleotide primers oC58OlsEN and oC58OlsEH incorporating NdeI and HindIII sites into the PCR product were used. After digestion with the respective enzymes, the PCR product was cloned as NdeI/HindIII fragment into pET17b yielding pEMAV27. Subsequently, the plasmid was linearized with HindIII and cloned into the HindIII site of pRK404 yielding pERMAV28. As a negative control pET17b was linearized with HindIII and cloned into the HindIII site of pRK404 yielding pERMAV05.

In vivo *labelling of* S. meliloti *and* A. tumefaciens *with* [¹⁴C]acetate *or* [¹⁴C]ornithine *and quantitative analysis of lipid extracts*

The lipid compositions of bacterial strains were determined following labelling with [1-¹⁴C]acetate (Amersham Biosciences). In the case of *A. tumefaciens*, cells were also labelled with [¹⁴C]ornithine to observe specific incorporation into OLs. Cultures (1 ml) of wild-type and mutant strains were

inoculated from precultures grown in the same medium. After addition of 0.5 μCi [^{14}C]acetate (60 mCi mmol^{-1}) or 1 μCi [^{14}C]ornithine (56 mCi mmol^{-1}) to each culture, the cultures were incubated for 4 h if not indicated otherwise. Under phosphate starvation conditions cells were labelled for 48 h in Sherwood minimal medium (Sherwood, 1970) containing 20 μM phosphate. Lipid extraction and analysis are described in detail in Vences-Guzmán and colleagues (2011).

Construction of the olsB and olsE deletion mutants

Oligonucleotide primers oC58OlsEar1 and oC58OlsEar2 were used in a PCR (XL-PCR kit; Applied Biosystems) to amplify about 1.0 kb of genomic DNA upstream of the putative *olsE* (*Atu0318*) gene from *A. tumefaciens*, introducing EcoRI and BamHI sites into the PCR product. Similarly, primers oC58OlsEab1 and oC58OlsEab2 were used to amplify about 1.0 kb of genomic DNA downstream of the putative *olsE* gene from *A. tumefaciens*, introducing BamHI and XbaI sites into the PCR product.

After digestion with the respective enzymes, PCR products were cloned as EcoRI/BamHI or BamHI/XbaI fragments into pUC18 to yield the plasmids pUMAV18 and pUMAV19 respectively. Then, the BamHI/XbaI fragment from pUMAV19 was subcloned into pUMAV18 to yield pUMAV20. Plasmid pUMAV20 was digested with EcoRI and XbaI to subclone the regions usually flanking the *olsE* gene into the suicide vector pK18*mobsacB* (Schäfer *et al.*, 1994) to yield pPMAV21. Via diparental mating using *E. coli* S17-1 as a mobilizing strain, pPMAV21 was introduced into the wild-type strain *A. tumefaciens* A208. Transconjugants were selected on LB medium containing neomycin and rifampicin 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 LB medium containing 12% (w/v) sucrose. Several large and small colonies grew after 4 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). Southern blot analysis confirmed that the S2-deficient strains were indeed double recombinants in which the gene *olsE* was deleted (data not shown).

Similarly, an *olsB* deletion mutant was constructed. Oligonucleotide primers oC58OlsBar1 and oC58OlsBar2 were used in a PCR (XL-PCR kit; Applied Biosystems) to amplify about 1.0 kb of genomic DNA upstream of the putative *olsB* gene from *A. tumefaciens*, introducing SmaI and BamHI sites into the PCR product. Similarly, primers oC58OlsBab1 and oC58OlsBab2 were used to amplify about 1.0 kb of genomic DNA downstream of the putative *olsB* gene from *A. tumefaciens*, 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 pUMAV23 and pUMAV24 respectively. The *A. tumefaciens* BamHI/HindIII fragment from pUMAV24 was subcloned into pUMAV23 to

yield pUMAV25. Plasmid pUMAV25 was digested with SmaI and HindIII to subclone the regions usually flanking the *olsB* gene into the suicide vector pK18*mobsacB* (Schäfer *et al.*, 1994) to yield pPMAV26. Via diparental mating using *E. coli* S17-1 (Simon *et al.*, 1983) as a mobilizing strain, pPMAV26 was introduced into the wild-type strain *A. tumefaciens*. Subsequent steps were performed as described above for the construction of the *olsE* mutant. Four clones lacking OLs S1 and S2 were identified. Southern blot analysis confirmed that the S1- and S2-deficient strains were indeed double recombinants in which the gene *olsB* was deleted (data not shown).

Complementation of the A. tumefaciens *mutants MAV07 and MAV08*

The *olsE* (*Atu0318*) gene was amplified using genomic DNA from *A. tumefaciens* A208 as template and XL-PCR polymerase (Applied Biosystems). Specific oligonucleotide primers incorporating NdeI and HindIII sites into the final PCR product were used (oC58OlsXN and oC58OlsXH). The digested PCR product was cloned into pET17b to yield the plasmid pEMAV27. Plasmid pEMAV27 was linearized with HindIII and cloned into HindIII-digested pRK404 to yield pERMAV28. As a negative control pET17b was linearized with HindIII and cloned into the HindIII site of pRK404 yielding pERMAV05. In this construct *olsE* is expressed under control of the T7 promoter. In earlier work we had observed that this promoter causes the constitutive expression of the genes under control in different *Rhizobiaceae* (Gao *et al.*, 2004; Sohlenkamp *et al.*, 2007; Vences-Guzmán *et al.*, 2011).

Similarly, the *olsB*-deficient mutant MAV08 was complemented with an intact copy of *olsB in trans*. The gene *olsB* (*Atu0344*) was amplified using genomic DNA from *A. tumefaciens* A208 as template and XL-PCR polymerase (Applied Biosystems). Specific oligonucleotide primers incorporating NdeI and BamHI sites into the final PCR product were used (oC58OlsBN and oC58OlsBB). The digested PCR product was cloned into pET9a to yield the plasmid pEMAV29. Plasmid pEMAV29 was linearized with BamHI and cloned into BamHI-digested pRK404 to yield pERMAV30. As a negative control pET9a was linearized with BamHI and cloned into the BamHI site of pRK404 yielding pERMAV06. In this construct *olsB* is expressed under control of the T7 promoter.

ESI-MS/MS analysis of ornithine lipids

In order to identify in which part of the second OL the modification is encountered, a 1 l culture *A. tumefaciens* was grown to an optical density of 1.2 at 620 nm in LB medium, and lipids were extracted according to Bligh and Dyer (1959). The lipids were separated by one-dimensional TLC and the area of the TLC plate corresponding to the OLs was identified by iodine and ninhydrin staining as described above. Silica containing OLs was scraped from the TLC plates and the OLs were extracted from the silica. OL-containing fractions were dried under N$_2$ stream and redissolved in methanol/chloroform (1:1, v/v). Normal phase LC-MS/MS of lipids was made as described in detail in Vences-Guzmán and colleagues (2011).

Identification of potential Pho boxes in the
Agrobacterium tumefaciens *genome*

To study if OL biosynthesis and/or modification are under control of PhoB the genome sequence of *A. tumefaciens* C58 was searched for the presence of putative Pho boxes. A PSSM was constructed using Pho box sequences reported previously for *S. meliloti* 1021 (Yuan *et al.*, 2006). *Escherichia coli* sequences were excluded for matrix construction. Sequences used for matrix construction are listed in Table S3. The program Info-Gibbs (Defrance and van Helden, 2009) was used for matrix construction. One motif per sequence was expected and the desired matrix size was fixed to 18 bp. As background model the regions upstream of *A. tumefaciens* C58 genes were employed. The resulting matrix has a maximum score for putative Pho boxes of 12.4, and the threshold used for the search was set to 7. The program Matrix-scan (Turatsinze *et al.*, 2008) was used to search the *A. tumefaciens* C58 genome for the presence of Pho boxes. The Markov order used was 0. Again the upstream regions of genes from *A. tumefaciens* C58 were employed as the background model. The search was performed scanning the upstream regions of predicted genes in 18 bp windows and comparing them with the PSSM. This search was performed for both strands. The list of genes presenting putative upstream Pho boxes was analysed for the presence of OL biosynthesis genes *olsB*, *olsE* and genes coding for glycosyltransferases (Cantarel *et al.*, 2009).

Tumour assays using potato tuber discs

The tumour assays were performed according to Tsai and colleagues (2009) (Shurvinton and Ream, 1991; Tsai *et al.*, 2009). Potato tubers were peeled and then immersed for 5 min in 1.05% sodium hypochlorite (17.5% bleach) solution. Tubers were rinsed three times with sterile water. A sterile cork borer was used to cut 1.0 cm diameter cylinders of potato tubers. About 1 cm pieces were removed from each end of a cylinder and the remaining cores were cut into 2–3 mm thick discs and placed on water agar plates. Each disc was inoculated on one side with 10^6 cells suspended in 100 μl PBS. Discs were placed on 1% water agar plates and incubated for 2 days in a controlled growth chamber at 28°C with a 15 h day/9 h night cycle. Potato discs were treated with timentin to kill all bacteria and then transferred to 1% (w/v) water agar plates containing timentin. Potato discs were incubated for 48 days under the conditions described above. Discs were scored every 3 days for the presence of tumours. After 48 days images were taken of the potato discs using a stereomicroscope equipped with a digital camera.

Acknowledgements

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Biosynthesis of OLs in *Agrobacterium tumefaciens*. The genes coding for OlsB and OlsA have been first identified in *Sinorhizobium meliloti*. Here we describe the identification of the gene encoding the OL hydroxylase OlsE introducing a hydroxyl group in the ornithine moiety of OL S1 leading to the formation of OL S2. LOL, lyso-ornithine lipid.

Fig. S2. Negative ion mode mass spectra of OL S1 (A) and OL S2 (B) purified from *A. tumefaciens* A208. The major OL [M-H]⁻ ion species are labelled with arrows.

Fig. S3. Ornithine lipid S2 shows a different ninhydrin staining compared with OL S1. Unlabelled lipids were extracted according to Bligh and Dyer from 50 ml cultures of *A. tumefaciens* A208 (A) and *R. tropici* CIAT899 (B) grown in LB medium and separated in duplicate by two-dimensional TLC. Lipids were subjected to ninhydrin staining. The phospholipids PE, monomethyl PE (MMPE), and the OLs S1, S2, P1 and P2 are indicated.

Table S1. Bacterial strains and plasmids used in this study.

Table S2. Oligonucleotides used in this study. Introduced restriction sites are underlined.

Table S3. Sequences used for PSSM construction using the program Info-Gibbs. Sequences were from taken from Yuan and colleagues (2006).