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ACTIVIDADES EN *Sinorhizobium meliloti* QUE DEGRADAN A LOS LÍPIDOS DE MEMBRANA Y LIBERAN ÁCIDOS GRASOS

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Resumen

Los fosfolípidos son bien conocidos por su función principal de formar membranas y delimitar a la célula del mundo exterior. Las bacterias poseen bicapas lipídicas compuestas de diversas especies de lípidos. En diferentes etapas de crecimiento o condiciones fisiológicas, los lípidos membranales pueden ser sintetizados, degradados o parte de ellos transferidos para formar nuevas moléculas o también como precursores de señales. Algunas de las enzimas responsables o procesos que *Sinorhizobium meliloti* utiliza para contender con los cambios a la accesibilidad a fosfato (Zavaleta-Pastor *et al.*, 2010) y fase de crecimiento (Pech-Canul *et al.*, 2011) han sido recientemente descritos. *S. meliloti* es un microorganismo capaz de realizar simbiosis con plantas de alfalfa, dando origen a nuevos órganos llamados nódulos, donde ocurre la fijación del nitrógeno. El reconocimiento específico para la formación y el desempeño de la simbiosis es de gran importancia y está dado por diferentes señales efectuadas por ambos simbiontes. Una mutante deficiente en FadD de *S. meliloti* incapaz de convertir los ácidos grasos libres a sus derivados de CoA, acumula ácidos grasos libres durante la fase estacionaria de crecimiento. Las actividades enzimáticas responsables de la generación de estos ácidos grasos libres eran desconocidas en los rizobios. Una búsqueda en el genoma de *S. meliloti* identificó una posible lisofosfolipasa (SMc04041) y dos fosfolipasas A del tipo patatina (SMc00930, SMc01003). Tanto SMc00930 como SMc01003 contribuyen a la liberación de ácidos grasos libres en *S. meliloti* pero ninguna utiliza fosfolípidos como sustrato. En este estudio demostramos que SMc01003 puede convertir diacilglicerol a monoacilglicerol y ácido graso libre, y a continuación el monoacilglicerol puede ser degradado en otro ácido graso libre y glicerol. Una mutante deficiente en SMc01003 acumula transitoriamente diacilglicerol (DAG), sugiriendo que SMc01003 actúa como una DAG lipasa (DglA) en su propio fondo genético. La expresión de DglA en *Escherichia coli* genera lisis en células que se encuentran en la fase estacionaria. El estudio de la actividad en SMc00930 demostró que es capaz de liberar ácidos grasos insaturados de cadena larga al sobre-expresarse en su propio fondo genético. Aunado a esto, los lisofosfolípidos formados a partir de los lípidos de la membrana de *S. meliloti,* pueden ser consumidos por esta actividad, confirmando que SMc00930 codifica para una lisofosfolipasa. Sorprendentemente, la ausencia de SMc00930 o una doble mutante

deficiente en SMc00930 y DglA deriva en cambios en la morfología de los nódulos, donde la tasa de nódulos multilobulados respecto a nódulos sencillos aumenta. Actualmente los mecanismos involucrados en la adaptación de las bacterias a diferentes situaciones ambientales están siendo caracterizados y permitiendo incrementar nuestro conocimiento actual en los ciclos de lípidos.

Abstract

Phospholipids are well-known for their membrane-forming properties and thereby delimit any cell from the exterior world. Bacteria contain bilayers composed of diverse lipid species. At different growth stages or physiological conditions, membrane lipids can be synthesized, turned over or parts of them can be transferred to form new structural features, precursors for signals or consumed into the bacterial metabolism. Some of the responsible enzymes and processes *Sinorhizobium meliloti* utilizes to cope with changes in phosphate availability (Zavaleta-Pastor *et al.*, 2010) and stationary phase of growth (Pech-Canul *et al.*, 2011) were recently discovered. *S.meliloti* is a microorganism able to form symbiosis with alfalfa plant, leading in consequence to the establishment of the nitrogen-fixing root nodule symbiosis. For establishing a successful symbiosis, specific recognition is given by different signals from both symbionts.

A FadD-deficient mutant of *Sinorhizobium meliloti*, unable to convert free fatty acids to their coenzyme A derivatives, accumulates free fatty acids during the stationary phase of growth. Enzymatic activities that release free fatty acids were unknown in rhizobia. Searching the genome of *S. meliloti*, we identified a potential lysophospholipase (SMc04041) and two predicted patatin-like phospholipases A (SMc00930, SMc01003). Although SMc00930 as well as SMc01003 contribute to the release of free fatty acids in *S. meliloti*, neither can use phospholipids as substrates. Here we show that SMc01003 converts diacylglycerol (DAG) to monoacylglycerol (MAG) and a fatty acid, and that MAG can be further degraded by SMc01003 to another fatty acid and glycerol. A SMc01003-deficient mutant of *S. meliloti* transiently accumulates DAG, suggesting that SMc01003 also acts as diacylglycerol lipase (DglA) in its native background. Expression of the DglA lipase in *Escherichia coli* causes lysis of cells in stationary phase of growth. In addition, the SMc00930 PLP enzymatic activity is able to liberate unsaturated long chain fatty acids in its native *S. meliloti* background. Lysophospholipids (LPL) formed from *S. meliloti* membrane phospholipids are consumed by SMc00930, confirming that it is a lysophospholipase. Surprisingly, the lack of SMc00930 or of SMc00930 and SMc01003, provokes a change of the nodulation phenotype caused by *S. meliloti*. Presently, mechanisms involved in adaptation to different environmental conditions in

bacteria are being characterized and enrich our knowledge on physiologically relevant lipid cycles.

Abreviaturas

AA: ácido araquidónico

ACC: acetil co-A carboxilasa

ACP: proteína acarreadora de grupos acilo

AG: ácidos grasos

ATP: adenosina trifosfato

CDP: citidina difosfato

CDP-DAG: difosfato de citidina-diacilglicerol

CdsA: CDP-diglicerido sintetasa

CL: cardiolipina

CMP: citidina monofosfato

CoA: Coenzima A

DAG: diacilglicerol

DAHP: dihidroxiacetona fosfato

DGHS: diacilgliceril homoserina

DglA: diacilglicerol lipasa

DGTS: diacilgliceril trimetilhomoserina

FAS: sintasa de ácidos grasos

G3P: glicerol 3 fosfato

GL: glicolípido

LOL: lisolípido de ornitina

LPL: lisofosfolípido

LPS: lipopolisacárido

MAG: monoacilglicerol

MDO: oligosacárido derivado de la membrana

ME: membrana externa

MI: membrana interna

NADH: dinucleótido de nicotinamida y adenina

OL:lípido de ornitina

OMPLA: fosfolipasa A de la membrana externa

PA: ácido fosfatídico

PC: fosfatidilcolina

PE: fosfatidiletanolamina

PG: fosfatidilglicerol

PI: fosfatidilinositol

PL: fosfolípido

$PLA_{1/2}$: fosfolipasa A_1 o A_2

PLB: fosfolipasa B

PLC: fosfolipasa C

PLD: fosfolipasa D

PS: fosfatidilserina

SAH: *S*-adenosil-L-homocisteina

SAM: *S*-adenosil-L-metionina

SL: sulfolípido

TM: transmembranal

1 Introducción

1.1 La composición de la membrana y sus elementos estructurales

La membrana citoplasmática separa el contenido celular del medio ambiente (Madigan *et al.,* 2014). Para mantener la integridad celular, son requeridos diversos factores bioquímicos y genéticos. La membrana citoplasmática está compuesta principalmente de una bicapa de fosfolípidos (PL), que a su vez existe en varias formas y variedades. Los lípidos de membrana son esenciales para la estabilidad e integridad de las membranas (Raetz & Dowhan, 1990).

Conocer la composición del contenido de fosfolípidos (Kanfer & Kennedy, 1964), así como las rutas de biosíntesis tanto en la bacteria modelo *Escherichia coli,* como en otras especies se ha logrado utilizando distintos métodos bioquímicos y microbiológicos.

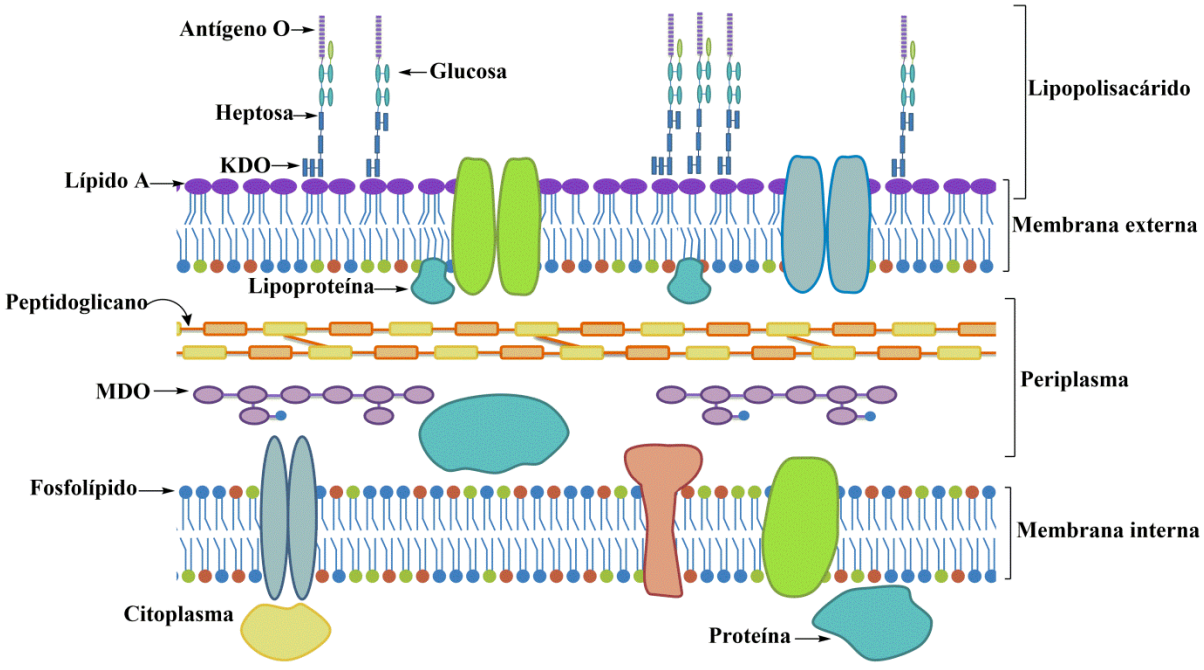

Figura 1. Representación esquemática de la envoltura celular de *E. coli*.

Las bacterias Gram-negativas presentan las siguientes características: la membrana citoplasmática o membrana interna (MI), compuesta principalmente de fosfolípidos y proteínas integrales de membrana o parcialmente ancladas a la membrana. Entre 3 a 5 capas de peptidoglicano entre la membrana interna y la membrana externa (ME), situada en el espacio periplásmico, que también posee oligosacáridos derivados de la membrana (MDO). La ME posee el lípido A del lipopolisacárido (LPS) 3-desoxi-D-*mano*-oct-2-ácido ulosónico (KDO) (Tomado y modificado de Raetz & Dowhan, 1990).

1.1.1 Membranas en bacterias Gram-negativas

En bacterias Gram-negativas, los fosfolípidos forman parte de las membranas interna y externa. La membrana interna consiste principalmente en una bicapa de fosfolípidos, mientras que la membrana externa posee una monocapa interna compuesta principalmente por fosfolípidos y una monocapa externa compuesta por el lípido A que a su vez forma parte del lipopolisacárido (LPS) (Figura 1). Entre la membrana externa y la membrana interna, se encuentra el espacio del periplasma. En *E. coli,* este espacio posee varias proteínas, la pared celular (peptidoglicano) y oligosacáridos derivados de la membrana (MDO).

1.2 Biosíntesis de lípidos de membrana con fósforo (fosfolípidos)

A continuación, se describe la biosíntesis de fosfolípidos en bacterias esquematizada en la figura 2.

El principal intermediario para la biosíntesis de los glicerofosfolípidos es el glicerol-3-fosfato (G3P). Existen dos maneras distintas de formar este intermediario; a partir de glicerol, o mediante el intermediario glicolítico dihidroxiacetona fosfato (DHAP). Cuando el glicerol es utilizado como fuente de carbono, las enzimas del operón del catabolismo de glicerol (glp) son inducidas. A continuación, mediante la actividad de la glicerol cinasa (GlpK) el glicerol es fosforilado hacia glicerol-3-fosfato. En contraste, si el crecimiento ocurre en fuentes de carbono distintas al glicerol, la reducción de DHAP con NADH formará el G3P. Este último paso es realizado por la enzima glicerol-3-fosfato deshidrogenasa (GpsA)(Rock, 2008).

En *E. coli,* la glicerol-3-fosfato aciltransferasa PlsB utiliza tanto acil-CoA como acil-ACP como el donador de grupos acilo. Esta enzima cataliza la primera acilación en la posición *sn*-1 del G3P, formando como producto 1-acil-glicerol-3-fosfato. Por contraparte, la ruta de biosíntesis más distribuida para la acilación de la primera posición del G3P incluye la actividad de las enzimas PlsX y PlsY (Lu *et al.,* 2006). A continuación, la acilación del ácido graso en la segunda posición de la molécula de glicerol es catalizada por la 1-acil-glicerol-fosfato-aciltransferasa PlsC que forma como producto el ácido fosfatídico (PA).

Posteriormente la conversión de PA para formar CDP-diacilglicerol (DAG) es catalizada por la actividad de la enzima CDP-diglicérido sintetasa (CdsA) (Rock, 2008).

En la biosíntesis de fosfatidilglicerol (PG), participa la enzima PG fosfato sintasa (PgsA). PgsA transfiere G-3-P a CDP-DAG, dando como resultado la liberación de CMP y PG-fosfato (PGP). Finalmente, para la formación de PG, es necesaria la actividad de una fosfatasa. En *E. coli,* se han descrito 3 enzimas diferentes con esta misma actividad, que dan lugar a PG a partir de PGP (PgpA, PgpB y PgpC)(Rock, 2008).

En la mayoría de las bacterias, la cardiolipina sintasa (ClsB), mediante una reacción de trans-esterificación, condensa dos moléculas de PG para formar cardiolipina (CL) y glicerol libre. En actinobacterias, existe otra ruta de biosíntesis que involucra una cardiolipina sintasa de tipo eucarionte (ClsE) (Sandoval-Calderón *et al.,* 2009). En este caso, ClsE condensa CDP-DAG con PG para formar CL y CMP.

La biosíntesis de fosfatidilserina (PS) consiste en la condensación de CDP-DAG con serina. Este paso es catalizado por PS sintasa (Pss). Para formar PE, la descarboxilación de PS es catalizada por una PS descarboxilasa (Psd). Algunas bacterias, además de PG, PE y CL también poseen fosfatidilcolina (PC) como componente de sus membranas. En bacterias, la biosíntesis de PC, ocurre mediante 3 metilaciones secuenciales de PE mediante la actividad de una fosfolípido-metiltransferasa (PmtA) que utiliza *S*-adenosil-L-metionina (SAM) como el donador de grupos metilo. Además de esta ruta de biosíntesis de PC, existe una segunda ruta donde colina se condensa con CDP-DAG, formando PC y CMP como producto final (Sohlenkamp *et al.,* 2003*)*. Este último paso es catalizado por la actividad de la PC sintasa (Pcs).

La formación de fosfatidilinositol (PI), un fosfolípido que se encuentra en los Actinomicetales y algunos *Myxococcus*, requiere la actividad de una fosfatidilinositolfosfato sintasa (Pips). Las Pips catalizan la condensación entre inositol-1-fosfato y CDP-DAG. A continuación, PIP es defosforilado mediante la actividad de una fosfatasa de PIP (Pipp) hacia PI defosforilado (Morii *et al*., 2010 y 2014).

En algunas α-proteobacterias y esfingobacterias aparentemente existe una vía alterna para la formación de fosfolípidos con inositol (Jorge *et al*., 2015). Una CTP: L-*myo*-inositol-1-

fosfatocitidiltransferasa convierte inositol-1-fosfato a CDP-inositol. Subsecuentemente, una actividad todavía desconocida condensa CDP-inositol con dialquilesteres de glicerol formando fosfolípidos de inositol (Jorge *et al*., 2015).

1. 3 Biosíntesis de lípidos de membrana sin fósforo

El fósforo constituye un elemento esencial para todos los organismos, se encuentra en moléculas como el ATP, ácidos nucleicos, fosfoproteínas y fosfolípidos. Debido a su baja disponibilidad y movilidad en los suelos así como su alta capacidad de absorción (Bieleski, 1973) puede encontrarse en cantidades limitantes para los organismos del suelo.

En algunas bacterias, en condiciones de limitación de fosfato, los fosfolípidos de membrana pueden ser reemplazados por lípidos sin fósforo (Minnikin & Abdolrahimzadeh, 1974). En la bacteria simbionte *S. meliloti* también se forman sulfolípidos como el sulfoquinovosil-DAG (SL), lípidos derivados de ornitina (OL) y diacilgliceril-*N,N,N*-trimetilhomoserina (DGTS) (Geiger *et al.,* 1999). El DGTS, SL y los glicolípidos (GL) son formados a partir de DAG como precursor común (Geiger *et al*., 2010).

La biosíntesis de los lípidos de ornitina (OLs) ocurre en dos pasos. En el primer paso, una *N*-aciltransferasa (OlsB), cataliza la transferencia de un grupo acilo 3-hidroxilado a partir de una ACP hacia el grupo α-amino de la ornitina, formando como producto el lisolípido de ornitina (LOL) (Gao *et al*., 2004). En el segundo paso, una *O*-aciltransferasa (OlsA) cataliza la transferencia de otro grupo acilo a partir de ACP al grupo 3-hidroxilo del LOL formando como resultado el lípido de ornitina (OL) (Weissenmayer *et al*., 2002).

Algunas especies bacterianas capaces de formar OLs, no poseen homólogos cercanos a los genes *olsBA*. En contraste, poseen la ruta de biosíntesis que utiliza la aciltransferasa bifuncional, OlsF (Vences-Guzmán *et al.,* 2014). OlsF realiza los dos pasos de acilación requeridos para la biosíntesis del LO. Homólogos a OlsF están presentes en γ-, δ- y ε-proteobacterias, así como en el grupo de Cytophaga-Flavobacterium-Bacteroidetes, sugiriendo que la distribución del LO es mucho más amplia de lo que se conocía.

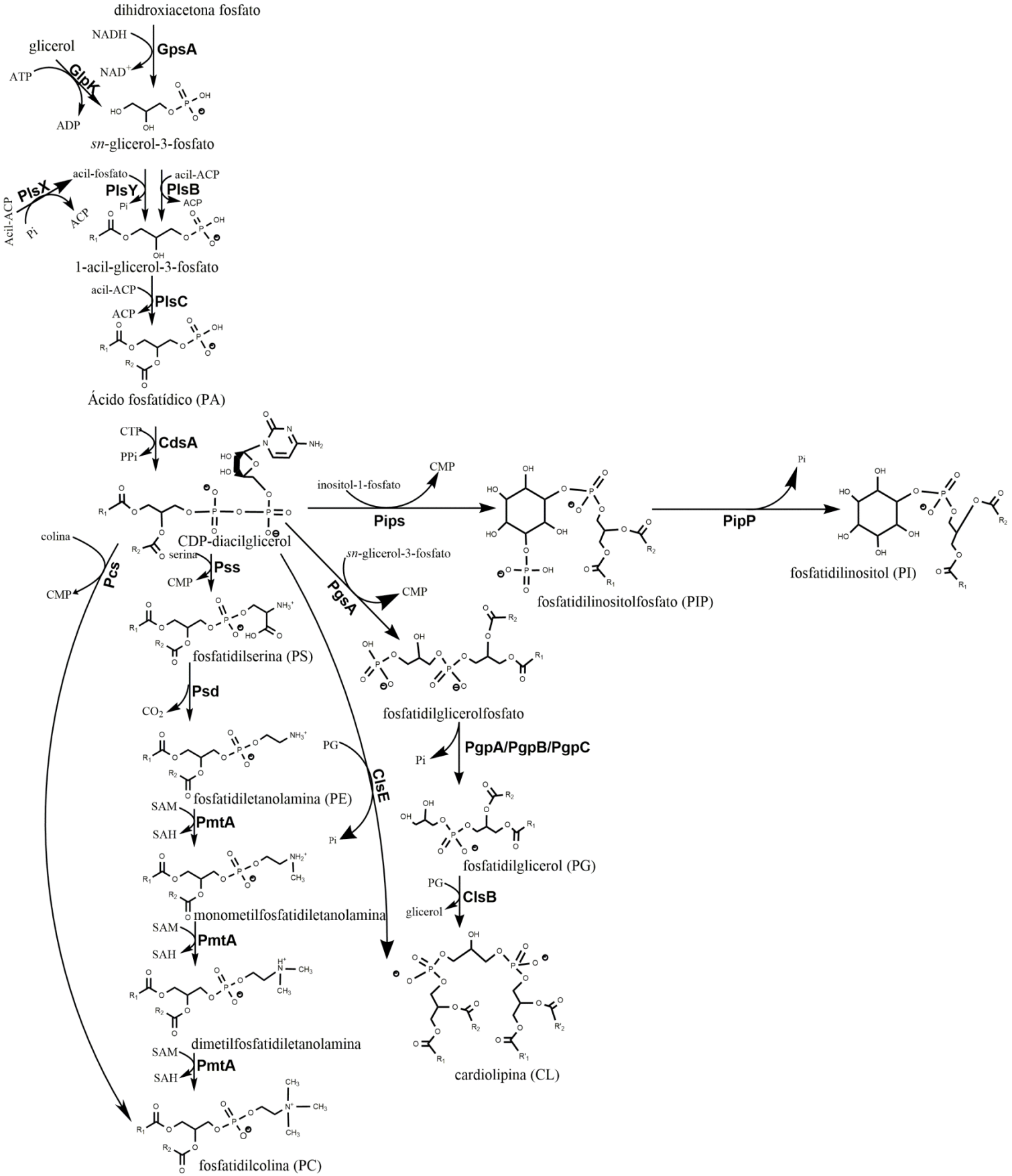

Figura 2. Biosíntesis de fosfolípidos en bacterias.

Los SLs como el sulfoquinovosil-DAG, se encuentran ampliamente distribuidos en organismos fotosintéticos (Benning, 1998). Para algunas bacterias, como *Rhodobacter sphaeroides* (Benning *et al*., 1993) la ausencia de SL en condiciones limitantes de fosfato afectan el crecimiento de la bacteria. Se conocen 4 genes involucrados en la biosíntesis de sulfolípidos de *R. sphaeroides* (*sqdA, sqdB, sqdC y sqdD*). La UDP-sulfoquinovosa se forma a partir de UDP-glucosa y sulfito posiblemente mediante la actividad de una enzima codificada por el gen *sqdB*. Se propone que los productos de los genes *sqdC* y *sqdD* catalizan la transferencia de sulfoquinovosa de UDP-sulfoquinovosa a DAG (Benning, 2007). En contraste, la función bioquímica de la proteína codificada por *sqd*A permanece aún desconocida.

El lípido de betaína diacilgliceril-*N*,*N*,*N*-trimetilhomoserina (DGTS) forma parte de las membranas de algas verdes, musgos, helechos y hongos (Künzler & Eichenberger, 1997) (Furlong *et al*., 1986). En algunas α-proteobacterias, DGTS constituye un lípido membranal sin fósforo que reemplaza a PC en condiciones de limitación de fosfato (Geiger *et al*., 1999).

En *R. sphaeroides*, están presentes los genes estructurales *btaAB* que codifican para dos enzimas involucradas en la biosíntesis de DGTS. BtaA posee actividad de *S*-adenosilmetionina/DAG 3-amino-3-carboxipropil transferasa, y convierte el DAG en diacilgliceril-homoserina (DGHS). Durante la formación del enlace éter, la *S*-adenosilmetionina funciona como el donador del grupo homoserina. Posteriormente, BtaB que es una *S*-adenosilmetionina-diacilglicerilhomoserina-*N*-metiltransferasa, cataliza tres metilaciones consecutivas para formar DGTS (Riekhof *et al*., 2005).

Los glicosil-DAG (GDAG) son glicolípidos membranales que se encuentran comúnmente en plantas, animales y bacterias. En *B. subtilis, ypfP* codifica para una UDP-glicosiltransferasa. Durante la biosíntesis, *ypfP* transfiere hasta cuatro residuos de glucosa de UDP-glucosa a DAG (Jorasch *et al.,* 1998) y como resultado, se forman distintas especies de glicolípidos.

1.4 Síntesis de ácidos grasos

Los ácidos grasos son esenciales para la sobrevivencia de la célula, tanto como una fuente de carbono, así como para la biosíntesis de otras moléculas estructurales (López-Lara & Geiger, 2010). El sistema de síntesis de ácidos grasos tipo II (FAS II), está presente en la mayoría de las bacterias así como en los organelos celulares (cloroplastos, mitocondria, apicoplastos). Los componentes de este sistema son proteínas independientes codificadas por genes separados (Rock, 2008). En contraste, el sistema tipo I de síntesis de ácidos grasos (FAS I), es un complejo multienzimático que se encuentra comúnmente en el citoplasma de las células eucariotas y solamente en algunas bacterias (López-Lara & Geiger, 2010). Otra característica del sistema FASII consiste en que los intermediarios de la biosíntesis de las moléculas aciladas, poseen un enlace tioéster entre los grupo acilos y ACP. La síntesis de ácidos grasos consta de tres etapas; la de inicio, de elongación y la de transferencia.

En la etapa de inicio, la acetil-CoA carboxilasa (ACC) cataliza el primer paso de la biosíntesis de ácidos grasos. El producto de esta reacción es el malonil-CoA. El grupo malonilo es transferido a ACP mediante una malonil-CoA:ACP transacilasa (FabD), formando como producto malonil-ACP. A continuación, la condensación de malonil-ACP con acetil-CoA mediante 3-oxoacil-ACP sintasa III (FabH) da como resultado la formación de acetoacetil-ACP y CO_2 (Rock, 2008). La elongación de la molécula de ácido graso (AG) mediante la adición de dos unidades de carbono sucede mediante un ciclo de reacciones que involucran condensación, reducción, deshidratación y un segundo paso de reducción. Cada nuevo ciclo se inicia mediante la actividad de la enzima FabF o FabB que condensan el acil-ACP con malonil-ACP para la elongación de la cadena hidrocarbonada. Posteriormente, la reducción es catalizada por FabG, una 3-oxoacil-ACP reductasa. El siguiente paso, la deshidratación del 3 oxoacil-ACP hacia *trans*-2-enoil-ACP es catalizado por FabA o FabZ (Rock, 2008). Cada ciclo se completa mediante la enoil-ACP reductasa (FabI) que forma acil-ACP como producto. El producto acil-ACP formado después de la segunda reducción será condensado con malonil-ACP para un nuevo ciclo de elongación de la cadena de acilo. Después de esta etapa se formarán generalmente, cadenas de acilo de número par.

La tercera etapa es la de transferencia. El producto acil-ACP será substrato de diferentes acil-transferasas para la transferencia de los grupos acilo a otras biomoléculas como ocurre por ejemplo durante la síntesis de fosfolípidos, de lípido A o de las lipoproteínas.

Después de la síntesis de los lípidos de membrana, los ácidos grasos ligados al glicerol todavía pueden ser alterados. Dentro de las modificaciones más estudiadas se encuentran la introducción de nuevas insaturaciones, la ciclopropanación y la *cis-trans* isomerización de los enlaces dobles (Cronan, 2002). Estas modificaciones ya han sido estudiadas detalladamente en algunos organismos modelo (Zhang & Rock, 2008).

1.5 Degradación de ácidos grasos mediante *β*-oxidación

Los ácidos grasos libres exógenos pueden ser utilizados por *E. coli* incorporándolos a los fosfolípidos de la membrana mediante el sistema de aciltransferasas PlsB/PlsC. Alternativamente, al ser degradados mediante β-oxidación pueden ser utilizados como fuente de carbono. Cualquiera de las dos vías (PlsB/PlsC) puede utilizar derivados de coenzima A. Para utilizar los ácidos grasos de cadena larga como fuente de carbono, es necesario un transportador de ácidos grasos de cadena larga (FadL) y FadD, una acil-CoA sintetasa que esterifica los ácidos grasos libres a coenzima A (Figura 3 y 11):

La oxidación de los ácidos grasos es realizada en 4 pasos. El primer paso consiste en la deshidrogenación del derivado acil-CoA mediante la actividad de una acil-CoA deshidrogenasa (FadE). A continuación el enoil-CoA resultante es hidratado por la actividad de una enoil-CoA hidratasa (FadB) y consecutivamente oxidado por la actividad de β-hidroxiacil-CoA deshidrogenasa de la misma FadB. Finalmente, la β-cetoacil-CoA tiolasa (FadA) libera acetil-CoA y el acil-CoA con dos átomos de carbono menos que al inicio del ciclo. Este ciclo se repite hasta que el ácido graso es totalmente oxidado a unidades de acetil-CoA (Nelson & Cox, 2013).

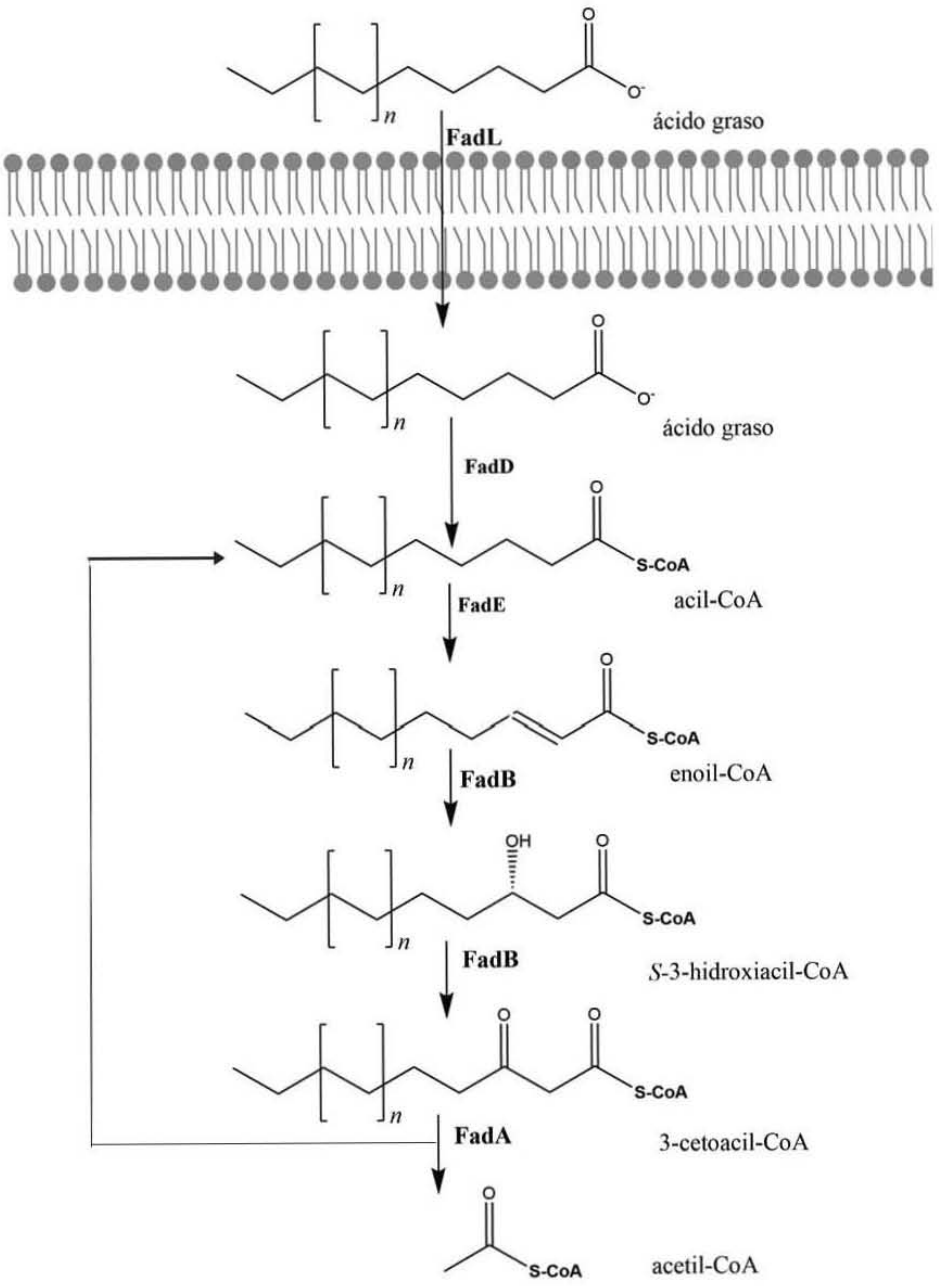

Figura 3. Esquema de la degradación de ácidos grasos mediante β-oxidación. FadL es un transportador de membrana de los ácidos grasos de cadena larga. FadD se asocia con la membrana plasmática y forma tioésteres de CoA. La β-oxidación implica la actividad de cuatro enzimas: la acil-CoA deshidrogenasa (FadE), la 2-enoil-CoA hidratasa, la β-hidroxiacil-CoA deshidrogenasa (ambas catalizadas por FadB) y la β-cetoacil-CoA tiolasa (FadA). El ciclo se repite hasta que el ácido graso es metabolizado completamente.

2 Enzimas esenciales que participan en el reciclaje de lípidos

Mediante diferentes actividades enzimáticas que incluyen hidrólisis así como la transferencia de diversos grupos funcionales, las bacterias son capaces de modificar su contenido lipídico. El estudio de las enzimas involucradas en la transferencia, hidrólisis y modificación de los componentes de la membrana permiten entender las distintas estrategias e intermediarios que forman parte de los ciclos de lípidos, así como su contribución para adaptarse al ambiente.

2.1 Hidrolasas que actúan sobre los lípidos

2.1.1 Lipasas

Las lipasas pueden ser producidas por bacterias y catalizan la hidrólisis y síntesis de esteres de glicerol y ácidos grasos de cadena larga. Las lipasas forman parte de la familia de las serina hidrolasas y actúan en la interfase de lípido-agua. La triada catalítica está compuesta por Ser-Asp/Glu-His y usualmente posee la secuencia consenso (Gly-x-Ser-x-Gly) alrededor del centro activo de la serina (Nardini *&* Dijkstra, 1999). Las lipasas son en general de secreción y se ha reportado muy poco sobre lipasas intrínsecas o intracelulares, así como su papel en la fisiología de la célula. Recientemente, se describió una monoacilglicerol (MAG) lipasa en *Mycobacterium smegmatis* que genera cambios en la morfología de las colonias y en la interacción celular (Dhouib *et al*., 2010). En este contexto, además de degradar lípidos exógenos, la MAG lipasa podría estar involucrada en la propia fisiología bacteriana. Las lipasas además, poseen una importancia económica destacada, ya que pueden ser utilizadas como recursos biotecnológicos (Jaeger *et al*., 1997).

2.1.2 Fosfolipasas

Las enzimas que rompen los enlaces éster en diferentes secciones de la molécula del fosfolípido son llamadas fosfolipasas. Su clasificación se basa principalmente en la posición donde es hidrolizado el enlace éster. Pueden ser asignadas en alguno de los 4 grupos principales (A, B, C y D) (Figura 4) (Nelson & Cox, 2013). Dependiendo de su estructura, función o por su actividad específica de hidrolizar el enlace éster, pueden ser

llamadas acil-hidrolasas (en caso de hidrolizar el enlace éster entre el ácido graso y el glicerol), o fosfodiesterasas (si la hidrólisis ocurre en el enlace fosfodiéster).

El estudio de las fosfolipasas se centra en la hidrólisis de los fosfolípidos de las membranas del huésped, que a su vez se encuentra correlacionado con la destrucción de las células. La clasificación y las propiedades generales de virulencia de las lipasas bacterianas han sido estudiadas en bacterias (Bender & Flieger, 2010).

Las bacterias poseen al menos un gen que codifica para una fosfolipasa dentro de su genoma (Moraleda-Muñoz & Shimkets, 2007). Aunque la mayoría de las enzimas lipolíticas estudiadas en bacterias degradan las membranas del hospedero, se espera que algunas de las enzimas con estas propiedades, estén involucradas en ciclos de lípidos y/o en la degradación intrínseca de la membrana bacteriana.

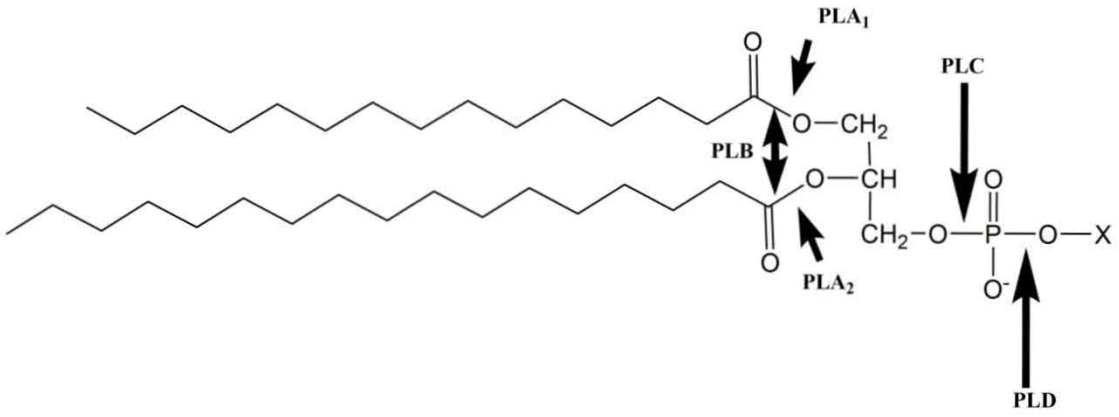

Figura 4. Clasificación general de las fosfolipasas. Las fosfolipasas están divididas en cuatro grupos dependiendo de su especificidad. Las fosfolipasas A (PLA) están subdivididas en dos grupos dependiendo la posición del ácido graso que liberan; A_1 y A_2. Las fosfolipasas B, poseen actividad de fosfolipasa A_1 y A_2, y lisofosfolipasa A_1 y A_2, que puede resultar en la liberación completa de todos los ácidos grasos de la molécula del fosfolípido. Las fosfolipasas C, rompen el enlace entre el grupo fosfato y el DAG, mientras que las fosfolipasas D cortan entre el grupo cabeza del fosfolípido y el PA.

2.1.2.1 Acil hidrolasas

Las fosfolipasas A_1, A_2 y B, así como las lisofosfolipasas A_1 o A_2, constituyen las actividades conocidas de acil-hidrolasa. La PLA_1 cataliza la hidrólisis de los ácidos grasos exclusivamente en la posición *sn*-1 de los fosfolípidos, en contraste la PLA_2, hidroliza el ácido graso en la posición *sn*-2 del fosfolípido. El resultado de cualquiera de estas dos

actividades, consiste en un ácido graso libre y una molécula de lisofosfolípido (Richmond *et al*., 2011). Las enzimas bacterianas con actividades PLA_1 se encuentran tanto en la membrana externa como en el compartimiento citoplasmático. Una representante de este grupo, también es una de las más estudiadas, la fosfolipasa A de la membrana externa (OMPLA). Esta proteína de la membrana externa se encuentra ampliamente distribuida en bacterias Gram-negativas (Snijder y Dijkstra, 2000) y posee una amplia variedad de actividades enzimáticas (PLA_2, liso-PLA_1 y liso-PLA_2)(Nishijima *et al*., 1977).

2.1.2.2 Fosfodiesterasas

Las fosfodiesterasas son representadas por la fosfolipasa C (PLC) y la fosfolipasa D. (PLD). La PLC hidroliza el enlace entre el glicerol y el grupo fosfato en el fosfolípido. La distribución de PLC se encuentra representada en un gran número de bacterias. La función de PLC, consiste en asegurar el abastecimiento de fosfato a la célula. La regulación de algunos genes que codifican para PLC por niveles exógenos de fosfato soportan esta hipótesis (Titball, 1993). En *S. meliloti* PlcP es inducida en condiciones de limitación de fosfato, y degrada a los fosfolípidos zwiteriónicos. El resultado de la degradación de estos fosfolípidos puede ser reutilizado para la biosíntesis de lípidos sin fósforo (Zavaleta-Pastor *et al*., 2010)(Figura 11).

La familia de las PLD incluye enzimas que están involucradas en el metabolismo de fosfolípidos. La actividad de fosfolipasa D consiste en la hidrólisis del fosfolípido en ácido fosfatídico y el alcohol del grupo cabeza como resultado. PLD funge también como un factor de virulencia en *Corynebacterium pseudotuberculosis, Corynebacterium ulcerans,* y *Arcanobacterium haemolyticum* (McNamara *et al.*, 1995). Aunado a esto, la actividad de PLD puede ser un factor de virulencia crítico para la vida intracelular de algunos miembros de las Rickettsias (Renesto *et al*., 2003).

En resumen, las fosfolipasas generan una amplia variedad de moléculas que están involucrados en el control de la señalización celular, la adapatación de la célula a su ambiente o como precursores de nuevas moléculas. Aunque la importancia de estas enzimas no es subestimada en la patogenicidad, aún existe mucho por ser estudiado respecto a la regulación así como su papel fisiológico en el reciclaje de lípidos.

2.2 Transferasas

Mientras que las hidrolasas transfieren los grupos funcionales a agua, las transferasas catalizan la transferencia de grupos funcionales a otros grupos funcionales de biomoléculas. Las transferasas de lípidos transfieren una parte del fosfolípido hacia otra molécula, lípido o lipoproteína. Las actividades que involucran la transferencia de un ácido graso específico, o de un grupo cabeza, se han descrito en detalle anteriormente. Un ejemplo de esta familia de enzimas es la PlsC que utiliza como donador de grupos acil-ACP durante la biosíntesis de fosfolípidos bacterianos.

3 Ciclos de lípidos

3.1 El ciclo de Lands

Los fosfolípidos celulares transitan por distintos procesos de deacilación y reacilación, este conjunto de procesos es llamado el ciclo de Lands (Lands *et al*., 1965). El ciclo de Lands consiste en un proceso de remodelado de fosfolípidos estudiado en profundidad en células eucariotas. Durante el proceso, una fosfolipasa celular cPLA, genera lisofosfolípidos (LPL) y ácidos grasos libres que luego serán reciclados para formar nuevos patrones de ácidos grasos dentro de la mólecula existente. La regulación de este ciclo es muy importante debido a que la acumulación de LPL puede llegar a ser tóxica para la célula. El ciclo de Lands constituye una de las rutas principales para la incorporación y liberación de ácido araquidónico (AA). La liberación del AA mediante la actividad de una fosfolipasa A_2 es un importante recurso para la síntesis de la familia de los eicosanoides, que son los mediadores de la inflamación como las prostaglandinas, tromboxanos, leucotrienos y lipoxinas (Das *et al*., 2011)(Figura 5). Para comprender la importancia funcional de la acilación y reacilación de los fosfolípidos bacterianos, es importante el estudio de fosfolipasas intrínsecas que puedan acudir a mecanismos similares para poder adaptarse y señalizar en diferentes condiciones fisiológicas.

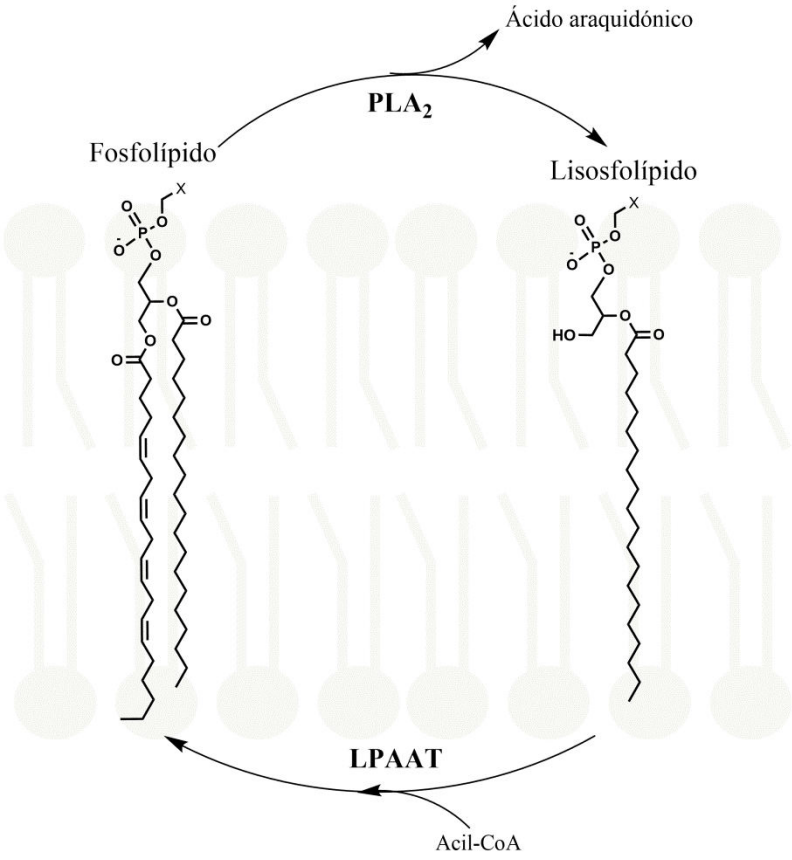

Figura 5. Ciclo de Lands. PLA_2 hidroliza la cadena acilada de un fosfolípido de membrana, generando como producto un ácido graso libre y un lisofosfolípido. La re-acilación del lisofosfolípido a fosfolípido (ahora con una nueva cadena acilada en la posición *sn*-2) es catalizada por LPAAT (ácido lisofosfatídico aciltransferasa) que utiliza un ácido graso unido a CoA como sustrato. El ácido graso liberado es el ácido araquidónico, que sirve de precursor para la biosíntesis de eicosanoides, prostaglandinas y leucotrienos

3.2 Ciclos de lípidos en bacterias

Además de los organismos eucariotas, las bacterias tienen la habilidad de alterar las estructuras de lípidos preexistentes, para reciclar y utilizar estos elementos para la síntesis y formación de nuevos lípidos de membrana u otras biomoléculas.

3.2.1 El ciclo clásico de diacilglicerol (DAG)

El espacio periplásmico de *E.* coli está formado principalmente por peptidoglicano y por oligosacáridos derivados de la membrana (MDO). Los MDO´s poseen de 8 a 10 residuos de glucosa unidos por enlaces β-1-2 y β-1-6. Generalmente poseen substituyentes como *sn*-1-fosfoglicerol (derivado de PG), fosfoetanolamina o residuos ester *O*-succinilo, que dan como resultado una carga neta negativa a la molécula (Rock, 2008).

Durante la biosíntesis de MDO, el grupo cabeza de PG se transfiere para formar MDO cargado negativamente y DAG. El DAG puede volver a formar PG al reinsertarse a la ruta de biosíntesis de lípidos mencionada anteriormente (Rock, 2008) (Figura 6).

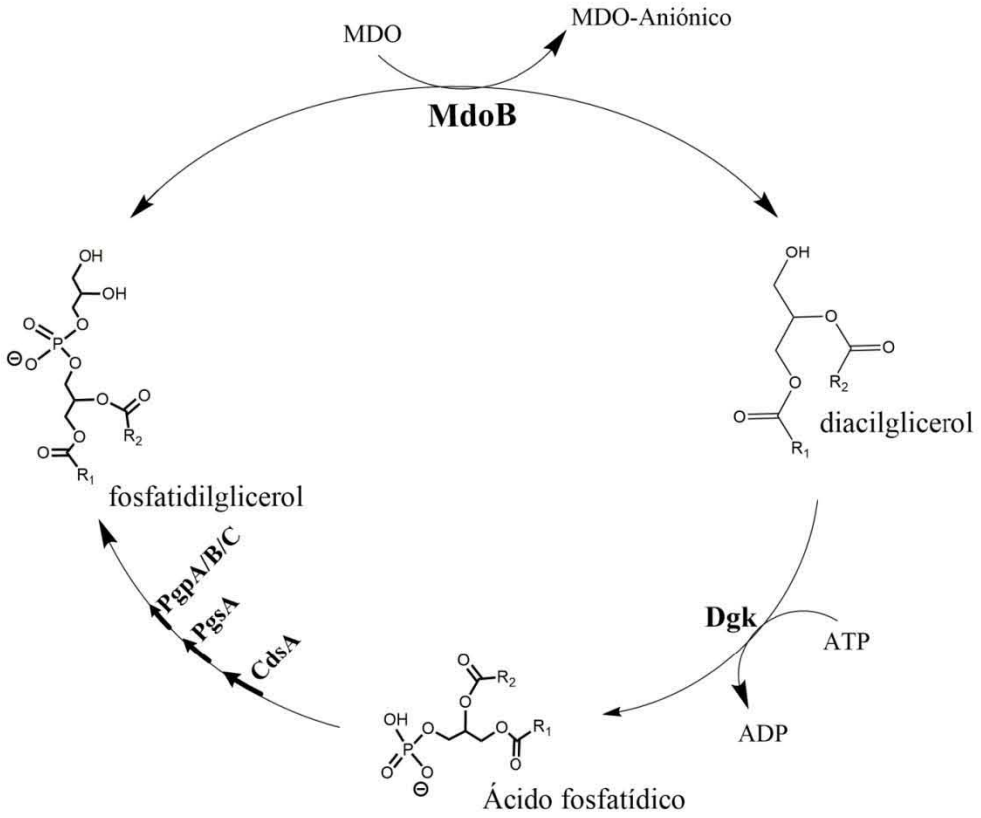

Figura 6. El ciclo clásico de diacilglicerol (DAG). El reciclaje de los fosfolípidos en el ciclo de DAG involucra la transferencia de *sn*-1-glicerol fosfato a partir de PG hacia MDO, que es catalizado por MdoB. DAG puede ser convertido a PA mediante la actividad de Dgk. Finalmente, PA puede regenerar PG ingresando nuevamente en la ruta de biosíntesis de fosfolípidos (Rock, 2008).

3.2.2 El ciclo de 2-acilglicerofosfoetanolamina (2-acil-GPE)

2-acil-GPE se forma a partir de PE mediante la transferencia de una molécula de ácido graso de la posición *sn*-1 de PE hasta el N-término de la proteína de la membrana externa (Lpp), este proceso es catalizado por la fosfolípido acil transferasa Lnt. Como resultado de esta síntesis se forma 2-acil-GPE que puede ser transportado al lado citosólico de la membrana mediante la actividad de la flipasa LpIT (Harvat *et al*., 2005). Posteriormente, 2-acil-GPE es reacilado por la actividad de 2-acil-GPE aciltransferasa/acil-ACP sintetasa

(Aas) que utiliza acil-ACP como el donador de grupos acilos y forma PE nuevamente. A continuación, el PE formado puede ser transportado de la capa interna de la membrana a la externa de la MI mediante MsbA (Doerrler *et al*., 2004). Alternativamente, el ácido graso de 2-acil-GPE puede ser hidrolizado a GPE y ácido graso libre mediante la actividad de una lisofosolipasa *pldB* (Hsu *et al.,* 1991) (Figura 7).

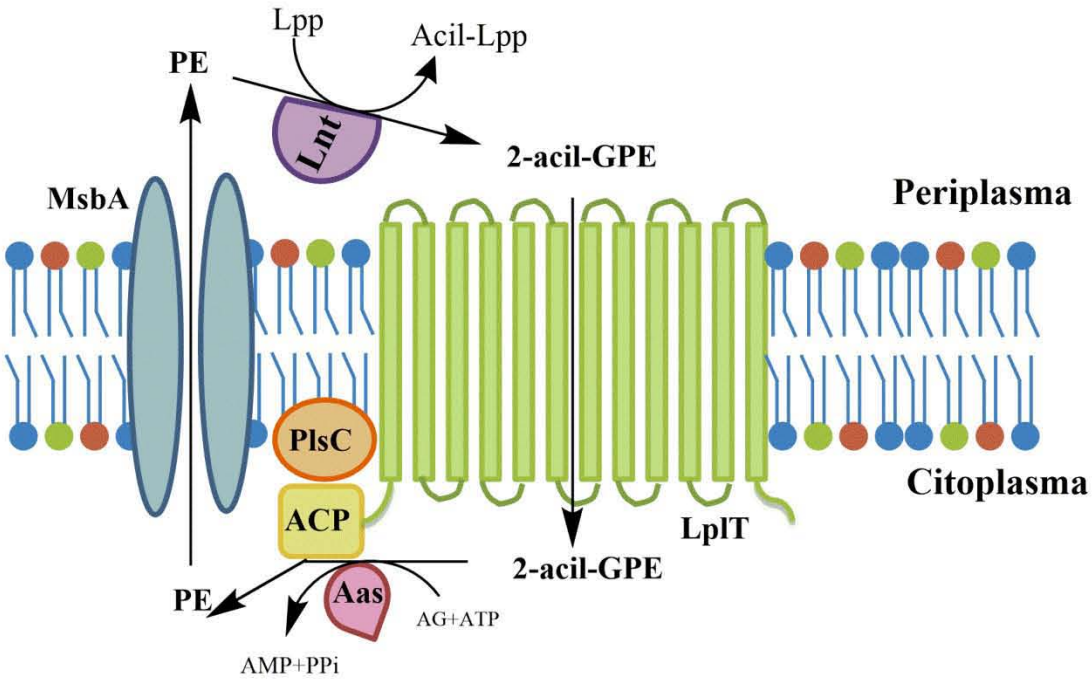

Figura 7. El ciclo de 2-acil-GPE. Diagrama topológico de las proteínas involucradas en el ciclo de 2-acil-GPE en *E. coli*. El 2-acil-GPE externo es generado por una reacción de transacilación de la posición *sn*-1 *de* ácido graso de PE hacia el N-término de la proteína de la membrana externa (Lpp). El 2-acil-GPE resultante, es transportado al lado citosólico de la membrana mediante LplT, donde posteriormente será acilado por la enzima bifuncional Aas. Aas utiliza acil-ACP como el donador de grupos acilos (Adaptado de Zhang & Rock 2008). MsbA transporta el PE formado a la capa externa de la MI.

3.2.3 Ciclos de lípidos en *S. meliloti*

Los fosfolípidos principales que componen la membrana de *S. meliloti* son PE, PC, PG y CL, que se forman por las rutas de biosíntesis ya conocidas (Figura 2).

En condiciones de limitación de fosfato, los lípidos zwiteriónicos son degradados a DAG (Zavaleta-Pastor *et al.*, 2010), mientras que en condiciones de baja osmolaridad el grupo cabeza de PG se transfiere para formar glucanos cíclicos aniónicos (Geiger *et al.,* 1991) y

DAG. El DAG posteriormente puede ser convertido a PA o puede ser utilizado como la base de construcción de lípidos de membrana sin fósforo como SL, DGTS (Weissenmayer *et al.*, 2000).

3.2.4 Acumulación de ácidos grasos libres en mutantes deficientes de FadD

Durante la fase estacionaria de crecimiento, en *S. meliloti,* una mutante deficiente en FadD acumula ácidos grasos libres (Pech-Canul *et al.,* 2011). Estos ácidos grasos se acumulan porque no pueden ser activados a derivados de coenzima A y por ende no pueden ser degradados por β-oxidación (Rock, 2008). La composición de los ácidos grasos acumulados en la mutante en FadD refleja la composición encontrada usualmente en los lípidos de membrana que indica que estos son liberados a partir de los lípidos de membrana. Los genes y los procesos responsables de la liberación de ácidos grasos en rizobias son desconocidos. No se ha encontrado ninguna fosfolipasa A de la membrana externa (OMPLA) en el genoma secuenciado de *S. meliloti*, sin embargo se encontró un homólogo a la lisofosfolipasa PldB de *E. coli* (SMc04041) y dos fosfolipasas tipo A de la familia "patatin-like" (PLP) potenciales (SMc01003 y SMc00930) que pueden ser responsables de la liberación de ácidos grasos a partir de los lípidos de membrana (Figura 11).

Al identificar los componentes y las actividades que consumen, degradan, transfieren partes de los componentes membranales, los ciclos de lípidos en bacterias pueden volverse mucho más comprensibles.

4 Hipótesis

La liberación de ácidos grasos en *S. meliloti* es causada por lipasas novedosas

5 Objetivos

5.1 Objetivo general

Conocer las enzimas responsables de la liberación de ácidos grasos en *S. meliloti.*

5.2 Objetivos específicos

1. Identificar genes candidatos para posibles lipasas en el genoma de *S. meliloti* y evaluar su contribución para liberar ácidos grasos.

2. Determinar la actividad enzimática así como la especificidad por los sustratos lipídicos de las posibles lipasas.

3. Proponer un papel fisiológico para los genes que codifican para posibles lipasas en el genoma de *S. meliloti.*

6 Resultados

Los resultados principales de este trabajo fueron publicados en un artículo, y otro manuscrito que se encuentra en preparación.

6.1 Artículo publicado

'Fatty acid-releasing activities in *Sinorhizobium meliloti* include unusual diacylglycerol lipase'

Diana X. Sahonero-Canavesi, Christian Sohlenkamp, Mario Sandoval-Calderón, Anne Lamsa, Kit Pogliano, Isabel M. López-Lara and Otto Geiger (2015) Environmental Microbiology 17: 3391-3406.

6.2 Artículo en preparación

'Lysophospholipase from *Sinorhizobium meliloti* affects nodule morphology during symbiosis with alfalfa*'*

Diana X. Sahonero-Canavesi, Kalpana Nanjareddy, Miguel Lara, Christian Sohlenkamp, Isabel M. López-Lara and Otto Geiger

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Fatty acid-releasing activities in *Sinorhizobium meliloti* include unusual diacylglycerol lipase

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Summary

Phospholipids are well known for their membrane-forming properties and thereby delimit any cell from the exterior world. In addition, membrane phospholipids can act as precursors for signals and other biomolecules during their turnover. Little is known about phospholipid signalling, turnover and remodelling in bacteria. Recently, we showed that a FadD-deficient mutant of *Sinorhizobium meliloti*, unable to convert free fatty acids to their coenzyme A derivatives, accumulates free fatty acids during the stationary phase of growth. Enzymatic activities responsible for the generation of these free fatty acids were unknown in rhizobia. Searching the genome of *S. meliloti*, we identified a potential lysophospholipase (SMc04041) and two predicted patatin-like phospholipases A (SMc00930, SMc01003). Although SMc00930 as well as SMc01003 contribute to the release of free fatty acids in *S. meliloti*, neither one can use phospholipids as substrates. Here we show that SMc01003 converts diacylglycerol to monoacylglycerol and a fatty acid, and that monoacylglycerol can be further degraded by SMc01003 to another fatty acid and glycerol. A SMc01003-deficient mutant of *S. meliloti* transiently accumulates diacylglycerol, suggesting that SMc01003 also acts as diacylglycerol lipase (DglA) in its native background. Expression of the DglA lipase in *Escherichia coli* causes lysis of cells in stationary phase of growth.

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Introduction

Upon cultivation on most culture media, *Escherichia coli* forms phosphatidylglycerol (PG), cardiolipin (CL) and phosphatidylethanolamine (PE) as major membrane lipids (Rock, 2008), whereas rhizobial bacteria additionally make substantial amounts of phosphatidylcholine (PC) (Geiger *et al*., 2013). Pathways for the biosyntheses of these lipids are well understood. Although it is known from eukaryotic systems that membrane phospholipids are subject to turnover (Nelson and Cox, 2013), this area of research has been little explored in bacteria. In *E. coli*, two cycles that involve membrane lipid remodelling are known (Rock, 2008), the 2-acylglycerophosphoethanolamine cycle and the diacylglycerol (DAG) cycle. In the 2-acylglycerophosphoethanolamine cycle, the acyl moiety of the 1-position of PE is transferred to the outer membrane lipoprotein forming 2-acylglycerophosphoethanolamine. Subsequently, 2-acylglycerophosphoethanolamine can be re-acylated using acyl-acyl carrier protein as acyl donor (Rock, 2008). In the classic version of the DAG cycle, phosphoglycerol moieties are transferred from PG to an oligosaccharide backbone leading to the formation of the so-called membrane-derived oligosaccharides and of DAG as the second product. DAG can be phosphorylated and, as phosphatidic acid, can re-enter bacterial phospholipid biosynthesis (Rock, 2008). A similar DAG cycle is known in *Sinorhizobium meliloti*, when neutral cyclic glucan are decorated with PG-derived phosphoglycerol residues converting neutral into anionic cyclic glucans with the concurrent formation of DAG (Wang *et al*., 1999). Also in *S. meliloti*, DAG kinase completes the classic DAG cycle (Miller *et al*., 1992).

Bacteria of the rhizobial group are soil bacteria able to interact in a host-specific way with legume plants leading in consequence to the establishment of the nitrogen-fixing root nodule symbiosis (Spaink, 2000). Rhizobia therefore have to confront such distinct environments as soils or life inside the root nodule. For establishing a successful symbiosis, an adequate formation of bacterial membrane phospholipids seems to be important (De Rudder *et al*., 2000; Vences-Guzmán *et al*., 2008). *Sinorhizobium meliloti* can largely replace its phospholipids by phosphorus-free membrane lipids (sulfolipids, ornithine

lipids and diacylglyceryl trimethyl homoserine) under phosphate-limiting conditions of growth (Geiger *et al*., 1999). Although these phosphorus-free membrane lipids are important for growth efficiency in phosphate-limiting conditions, they are not required for the establishment of symbiosis (López-Lara *et al*., 2005). Upon phosphorus limitation, a phospholipase C (PlcP) is induced that degrades PC of the bacterium's own membrane to DAG (Zavaleta-Pastor *et al*., 2010). DAG in turn is thought to serve as membrane anchor during the biosynthesis of phosphorus-free membrane lipids, such as sulfolipids, and diacylglyceryl trimethyl homoserine (Zavaleta-Pastor *et al*., 2010). Alternatively, when phosphate concentrations are not growth limiting, such as in the early stages of symbiosis, the structural gene for DAG kinase is induced (Zhang and Cheng, 2006), DAG can be phosphorylated to phosphatidic acid, and as such re-enter phospholipid biosynthesis.

FadD is an acyl-coenzyme A (CoA) synthetase responsible for the activation of long-chain fatty acids (FA) converting them into acyl-CoAs. In *E. coli* as well as in *S. meliloti*, mutants deficient in *fadD* accumulate a mixture of free FA during the stationary phase of growth that seem to be derived from bacterial membrane lipids (Pech-Canul *et al*., 2011). Enzymatic activities responsible for the release of these free FA from membrane lipids were unknown in rhizobia.

The *S. meliloti* genome encodes for a potential lysophospholipase (SMc04041) and two predicted patatin-like phospholipases A (SMc00930, SMc01003). Here we show that SMc00930 as well as SMc01003 contribute to the release of free FA in *S. meliloti*, but neither one can use phospholipids as substrates. SMc01003 can degrade DAG to monoacylglycerol (MAG) and a fatty acid and can degrade MAG further to another fatty acid and glycerol. Expression of SMc01003-encoded DAG lipase in *E. coli* causes lysis of cells in stationary phase of growth.

Results

Potential phospholipase and lysophospholipase genes in S. meliloti

A *fadD*-deficient mutant of *S. meliloti* accumulates free FA in the stationary phase of growth (Pech-Canul *et al*., 2011). Although these free FA seem to be derived from membrane lipids, it was not clear by which enzymes they would be released. We therefore searched the genome of *S. meliloti* 1021 for genes that might encode phospholipases or lysophospholipases. Although widespread in Gram-negative bacteria (Istivan and Coloe, 2006), we could not detect any homologue of the outer membrane phospholipase A during a search of the *S. meliloti* genome. However, a homologue (SMc04041) of the well-characterized *E. coli* lysophospholipase L2 (PldB) (Kobayashi *et al*., 1985) was found, and SMc04041 shows 32% identity, 43% similarity and an E value of $2 \times e^{-29}$ with PldB from *E. coli*. ExoU is an important virulence factor of *Pseudomonas aeruginosa* and was the first patatin-like phospholipase (PLP) characterized in bacteria (Sato *et al*., 2003). Another member of PLPs in *P. aeruginosa* is PlpD (Salacha *et al*., 2010) and a search of the *S. meliloti* genome identifies two PlpD homologues (SMc00930 and SMc01003) that display the four motifs (Fig. 1) previously shown to be essential for phospholipase activity (Banerji and Flieger, 2004). When compared with the N-terminal domain of PlpD, SMc01003 shows 32% identity, 50% similarity and an E value of

	BLOCK I			BLOCK II			BLOCK III			BLOCK IV		
ExoU	107	LVLSGGGAKG	116	139	SGSSAGG	145	311	QAAHISGSFPGVFQKV	326	343	QDGGVMINVP	352
PlpD	27	LVLSGGAARG	36	57	AGTSMGA	63	183	QAIRASMSIPAVFAPV	198	206	VDGGMVDNIP	215
SMc01003	42	LALGGGAARG	51	72	AGTSIGA	78	165	TAIRASYALPGIFEPV	180	188	IDGALVNPVP	197
SMc00930	27	LALGGGGARG	36	57	AGSSIGA	63	164	KALAASCALPAVFMPV	179	187	IDGGIYNPIP	196
VipD	32	LVLSGGGAKG	41	64	SGASAGA	70	257	QVVQWSGAHPVLFVPG	272	281	ADGGILDNMP	290
MXAN_3852	52	LVLSGGGAKG	61	83	FGVSVGA	89	183	DAVWQSSTLPILWEPV	198	204	VDGGLRNATP	213
RT0522	51	IAFSGGGAKG	60	83	AGSSVGA	89	226	LACRASASIPIVFKPV	241	249	VDGGYRDNIP	258
YvdO	7	MTFDGGGTLG	16	41	SGNSIGS	47	149	DVILRSSGAPATQRAY	164	168	VDGYVVATNP	177

Fig. 1. Alignment of conserved blocks of *S. meliloti* PLP homologues to PlpD of *Pseudomonas aeruginosa* and other characterized PLPs in bacteria. Phospholipases of the patatin-like family have four conserved motifs (block I to block IV). Block I consists of a glycine-rich region containing a conserved basic residue, arginine or lysine (Arg35 in SMc00930 and Arg50 in SMc01003), which probably serves as an oxyanion hole. Block II comprises the typical lipase motif, G-X-S-X-G which includes the putative conserved serine active site (Ser60 in SMc00930 and Ser75 in SMc01003). Block III possesses a conserved serine (Ser169 in SMc00930 and Ser170 in SMc01003) which is considered an important structural element. Block IV comprises the putative active site aspartate (Asp188 in SMc00930 and Asp189 in SMc01003). Blocks III and IV possess highly conserved proline residues (Banerji and Flieger, 2004). The conserved Ser of block II and the conserved Asp of block IV compose the catalytic dyad of PLPs. All motifs are conserved in SMc01003 and SMc00930. Identical residues defining the four blocks are shaded in grey. Sequences used for the alignment were SMc00930 and SMc01003 (*S. meliloti*), PlpD and ExoU (*P. aeruginosa*), VipD (*Legionella pneumophila*), MXAN_3852 (*Myxococcus xanthus*), RT0522 (*Rickettsia typhi*) and YvdO (*Bacillus subtilis*).

$3 \times e^{-38}$ whereas SMc00930 displays 33% identity, 51% similarity and an E value of $1 \times e^{-30}$. In this study, we analyse one potential lysophospholipase and two potential phospholipases that may be responsible for the membrane phospholipid degradation or turnover.

Expression of potential sinorhizobial phospholipase A/lysophospholipase A genes cause increased formation of free FA

The three potential phospholipase A/lysophospholipase A genes from *S. meliloti*, *smc00930*, *smc01003* and *smc04041*, were cloned in distinct vectors to express them either in *E. coli* or in *S. meliloti*. An *E. coli* strain harbouring an empty pET17b plasmid produced the membrane phospholipids PE, PG, CL and only minor amounts of free FA (Fig. 2A, Table S1). Upon expression of *smc00930*, *smc01003* or *smc04041* in *E. coli*, in addition to the membrane phospholipids, significantly increased amounts of free FA were detected in lipidic extracts (Fig. 2A). Whereas free FA comprised about 5% of total lipids in an *E. coli* strain harbouring an empty vector

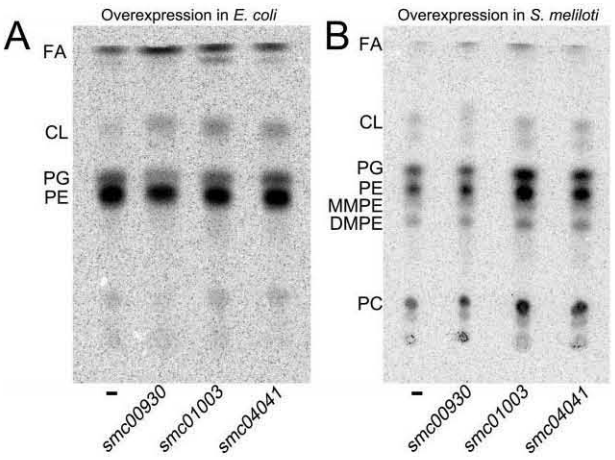

Fig. 2. Expression of *smc00930*, *smc01003* and *smc04041* in *E. coli* and of *smc00930* and *smc01003* in *S. meliloti* causes fatty acid accumulation. Lipid profile analysis of *E. coli* (A) and *S. meliloti* (B) strains that express genes encoding for potential phospholipases. *Escherichia coli* strains were induced with IPTG and labelled with ^{14}C-acetate for 4 h and *S. meliloti* strains were labelled for 4 h with ^{14}C-acetate. Lipids were extracted according to Bligh and Dyer (1959) and separated in one-dimensional (1D) thin-layer chromatography (TLC) using chloroform : methanol : acetic acid as the mobile phase. Lipids from *E. coli* BL21(DE3) x pLysS containing the empty pET17b vector (–), or the vector with cloned *smc00930* (plasmid pDS10), *smc01003* (plasmid pDS11) or *smc04041* (plasmid pDS30) genes (A). Lipids from *S. meliloti* containing the empty pNG28 vector (–), the *smc00930*-expressing plasmid pDS12, the *smc01003*-expressing plasmid pDS13 or the *smc04041*-expressing plasmid pDS31 (B). FA, fatty acids; CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; MMPE, monomethyl-PE; DMPE, dimethyl-PE; PC, phosphatidylcholine.

(Table S1), they increased to more than 8% when SMc04041 was expressed (Table S1). Expression of SMc00930 or of SMc01003 in *E. coli* increased the relative amount of free FA to more than 17% (Table S1). Studies with a *S. meliloti* strain harbouring an empty pNG28 vector showed that the membrane phospholipids PC, PE, monomethyl-PE, dimethyl-PE, PG, CL and only minor amounts of free FA (Fig. 2B, Table S2) were formed. Expression of any of the potential phospholipase A/lysophospholipase A genes in *S. meliloti*, *smc00930*, *smc01003* or *smc04041*, produced similar lipid profiles as observed for the strain harbouring the empty vector. However, the relative amount of free FA was much increased when *smc00930* (4.9-fold) or *smc01003* (2.7-fold) were expressed (Fig. 2B, Table S2). These data show that SMc00930 and SMc01003 may contribute to the formation of free FA in *S. meliloti*.

Potential phospholipases A SMc00930 and SMc01003 contribute to the formation of free FA in S. meliloti *but are not required for symbiosis with alfalfa*

Mutants of *S. meliloti* deficient in SMc00930, SMc01003 or SMc04041 were constructed, and their membrane lipid profile was indistinguishable from the wild type strain (Fig. S1). Also, alfalfa seedlings were inoculated with *S. meliloti* wild type, or mutants deficient in SMc00930, SMc01003 or SMc04041, or treated with water as a control similarly as described previously (López-Lara *et al.*, 2005). Plants inoculated with any of the four strains formed nitrogen-fixing root nodules while no nodules were formed on water-treated plants. There was no significant difference in the number of nodules developing over time on the roots of plants treated with wild type or mutant bacteria (data not shown). A *S. meliloti* mutant deficient in FadD is unable to degrade free FA and therefore accumulates them to some extent in its membranes (Pech-Canul *et al.*, 2011 and Fig. 3). Notably, a double mutant deficient in FadD and SMc01003 (Fig. 3, lane 4) accumulates much less free FA (3% of total lipids) than the FadD-deficient single mutant (14% of total lipids) (Fig. 3, lane 3; Table S3) suggesting that under the physiological conditions studied, SMc01003 contributes in a significant manner to the formation of free FA in *S. meliloti*. The FadD- and SMc01003-deficient double mutant harbouring an empty broad host range plasmid accumulates little free FA (7.6% of total lipids) (Fig. 3, lane 7) similarly as observed for the vector-free double mutant (Fig. 3, lane 4). When SMc00930 (Fig. 3, lane 5) or SMc01003 (Fig. 3, lane 6) are expressed in the FadD- and SMc01003-deficient double mutant, the relative amounts of free FA increase significantly (15% of total lipids in the case of SMc00930 and 16% of total lipids in the case of SMc01003) (Table S3). The restoration of the

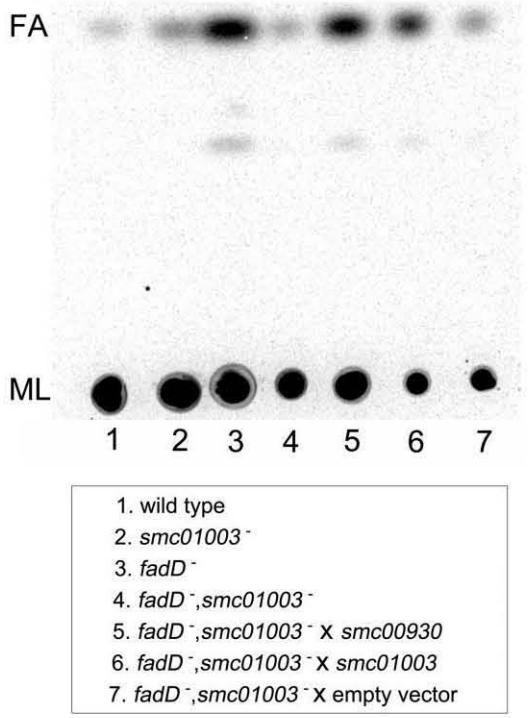

Fig. 3. *S. meliloti* double mutant deficient in *smc01003* and *fadD* accumulates less fatty acids than the single mutant lacking *fadD* and fatty acid accumulation is restored by *smc01003* or *smc00930*. After labelling for 28 h with ^{14}C-acetate, lipid profile analysis of *S. meliloti* wild type strain and distinct mutants was performed by separating lipid extracts by 1D-TLC using ethyl acetate : hexane : acetic acid as the mobile phase (Pech-Canul *et al*., 2011). Lipids of *S. meliloti* 1021 wild type, mutant DSXC2 deficient in *smc01003*, mutant FadD2 deficient in *fadD*, mutant FDXSC2 deficient in *smc01003* and *fadD*, mutant FDXSC2 containing *smc00930*-expressing plasmid pDS12, mutant FDXSC2 containing *smc01003*-expressing plasmid pDS13 and mutant FDXSC2 harbouring the empty plasmid pNG28. FA, fatty acids; ML, polar membrane lipids.

elevated formation of free FA shows that both patatin-like potential phospholipases A can contribute to the formation of free FA in their native *S. meliloti* background.

SMc01003 and SMc00930 hydrolyse p*-nitrophenyl ester substrates*

In order to obtain an easily quantifiable enzyme assay, we analysed whether cell-free extracts obtained from *E. coli* BL21(DE3) x pLysS, which had *smc00930*, *smc01003* or *smc04041* expressed, might hydrolyse *p*-nitrophenyl fatty acyl esters of various chain lengths (C10-C18) using a spectrophotometric enzymatic assay measuring the nitrophenol (NP) formed. A minor hydrolytic activity was present in cell-free extracts obtained from *E. coli* BL21(DE3) x pLysS harbouring an empty pET17b vector. Expression of *smc04041* did not increase the hydrolytic activity on any of the *p*-nitrophenyl esters assayed (data not shown). In contrast, the expression of *smc01003* generated extracts that showed an increased hydrolysis of *p*-nitrophenyl esters, especially of the medium chain *p*-nitrophenyl decanoate (specific activity 27 μmol NP min^{-1} mg protein^{-1}) but also of the long-chain *p*-nitrophenyl palmitate (specific activity 14 μmol NP min^{-1} mg protein^{-1}) and *p*-nitrophenyl stearate (specific activity 12 μmol NP min^{-1} mg protein^{-1}) (Table S4). Cell-free extracts in which *smc00930* had been expressed show much higher enzyme activities with *p*-nitrophenyl esters (Table S4). Also, SMc00930 is able to hydrolyse *p*-nitrophenylacyl esters of different chain lengths (C10, C12, C14, C16 and C18), though *p*-nitrophenyl palmitate (specific activity 5.5 mmol NP min^{-1} mg protein^{-1}) is clearly the best substrate for SMc00930.

Under tested conditions, membrane phospholipids of S. meliloti *are not hydrolysed by SMc01003 or SMc00930*

In order to confirm the lipidic substrate of SMc01003 and SMc00930, enzymatic assays, replacing the artificial substrate *p*-nitrophenyl palmitate by *S. meliloti* total ^{32}P-labelled phospholipids, were performed. Although phospholipase A_2 from *Crotalus adamanteus* degrades a mixture of sinorhizobial phospholipids (Fig. S2), treatment of the same sinorhizobial phospholipids with cell-free extracts from *E. coli* carrying an empty vector or from *E. coli*, in which SMc01003 or SMc00930 had been overexpressed, did not cause any change in the lipid profile (Fig. S2). These results are surprising as, under conditions when artificial *p*-nitrophenyl ester substrates are hydrolysed by SMc01003 or SMc00930, sinorhizobial phospholipids are not. We therefore suggest that sinorhizobial phospholipids are not substrates for the predicted phospholipases A SMc01003 and SMc00930. In the remaining part of this study, we focused our work in order to reveal the molecular function of SMc01003.

Overexpression of SMc01003 in E. coli *RZ6 leads to DAG consumption and free fatty acid formation* in vivo

Neither the major phospholipids nor lysophospholipids (data not shown) served as substrates for SMc01003 when expressed in extracts of *E. coli*. Therefore, we investigated whether other minor bacterial membrane lipids might be degraded by SMc01003. However, phosphatidic acid preparations were not consumed by SMc01003 either (data not shown). In order to see whether DAG might be a substrate for SMc01003, we used the DAG kinase (Dgk)-deficient mutant RZ6 of *E. coli* (Raetz and Newman, 1978) as a host that produces elevated levels of DAG (up to 12% of total lipid).

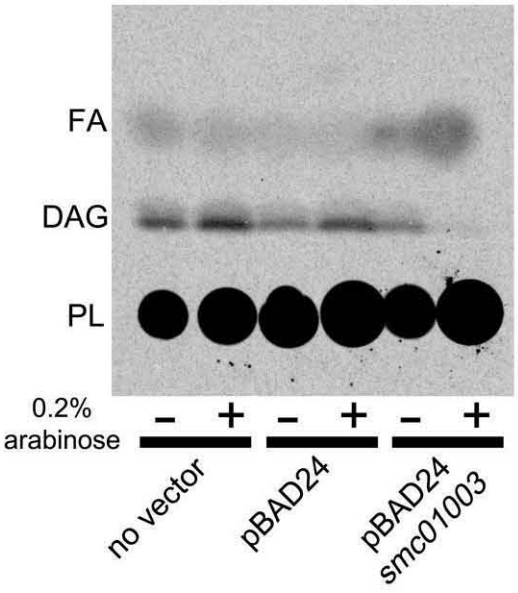

Fig. 4. Overexpression of SMc01003 in a diacylglycerol kinase (*dgk*)-deficient *E. coli* RZ6 mutant leads to diacylglycerol (DAG) consumption and free fatty acid formation. Neutral lipid profile of *E. coli* RZ6 (*dgk*-deficient) strains without vector, harbouring the empty pBAD24 vector, or the SMc01003-expressing pDS11HBAD plasmid without (–) or with (+) induction by arabinose. At an OD_{620} = 0.1 cultures were induced (or not) with 0.2% arabinose and labelled with ^{14}C-acetate until they reached OD_{620} = 0.3. From harvested cells, lipids were extracted and analysed by 1D-TLC using hexane : diethylether : acetic acid as the mobile phase. FA, fatty acids; DAG, diacylglycerol; PL, phospholipids.

The gene *smc01003* was cloned in a pBAD24 vector from which it can be expressed in the presence of arabinose (Guzman *et al*., 1995). Thin-layer chromatographic (TLC) analysis of lipid extracts (Fig. 4) shows that *E. coli* RZ6 produces similar amounts of DAG and free FA in the presence or absence of arabinose which is also true for a RZ6 strain containing the empty pBAD24 vector. When *E. coli* RZ6 contains cloned *smc01003* in the pBAD24 vector (pDS11HBAD), even in the absence of arabinose, more free FA are formed. However, when SMc01003 is expressed in RZ6 in the presence of arabinose, DAG is essentially undetectable and the amount of free FA has drastically increased (Fig. 4). Therefore, in the intact environment of a living organism, SMc01003 degrades DAG to free FA and presumably MAG and therefore seems to be a DAG lipase.

Diacylglycerol obtained from S. meliloti *is a substrate for the SMc01003 lipase*

Diacylglycerol which had been obtained by phospholipase C treatment of sinorhizobial PC was studied as a possible substrate. When radiolabelled DAG was treated with buffer or with a cell-free extract of *E. coli* harbouring an empty vector (pET17b), no disappearance of DAG was observed (Fig. 5A). In contrast, when DAG was treated with a cell-free extract of *E. coli* in which SMc01003 had been expressed from pDS11, nearly all DAG disappeared and radioactive compounds were formed that migrated similarly as free FA (Fig. 5A). A time course for DAG treatment with diluted cell-free extracts of *E. coli* x pET17b or with an extract of *E. coli* x pDS11 shows that upon incubation with cell-free extract of *E. coli* harbouring an empty vector, no disappearance of DAG was observed (Fig. 5B). In contrast, when DAG was treated with a cell-free extract of *E. coli* in which SMc01003 had been expressed, DAG is rapidly consumed, a minor compound that migrates like MAG is transiently observed at 0.5 and 2 h, and compounds that migrate like free FA are

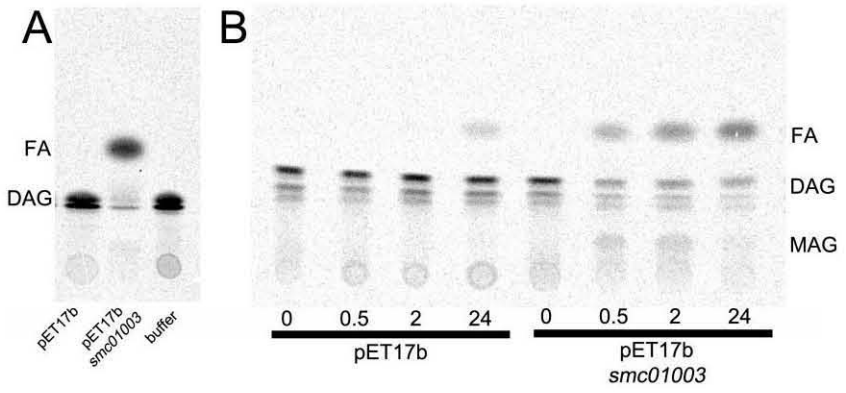

Fig. 5. SMc01003 degrades *S. meliloti*-derived diacylglycerol via monoacylglycerol to glycerol and fatty acids.
A. Conversion of diacylglycerol to free fatty acids during an extended incubation with SMc01003. Cell-free extracts of *E. coli* BL21(DE3) × pLysS expressing SMc01003 or containing the empty pET17b vector, or buffer were incubated with [^{14}C]DAG obtained from *S. meliloti* for 24 h.
B. Time course of DAG degradation by SMc01003 with the transient formation of MAG. Cell-free extracts of *E. coli* BL21(DE3) × pLysS, harbouring an empty vector (pET17b) or the SMc01003-expressing plasmid pDS11, were incubated with [^{14}C]DAG for different times (0, 0.5, 2 or 24 h). At the end of the respective incubation periods, radiolabelled lipids were extracted and separated by 1D-TLC using hexane : diethylether : acetic acid as the mobile phase. FA, fatty acids; DAG, diacylglycerol; MAG, monoacylglycerol.

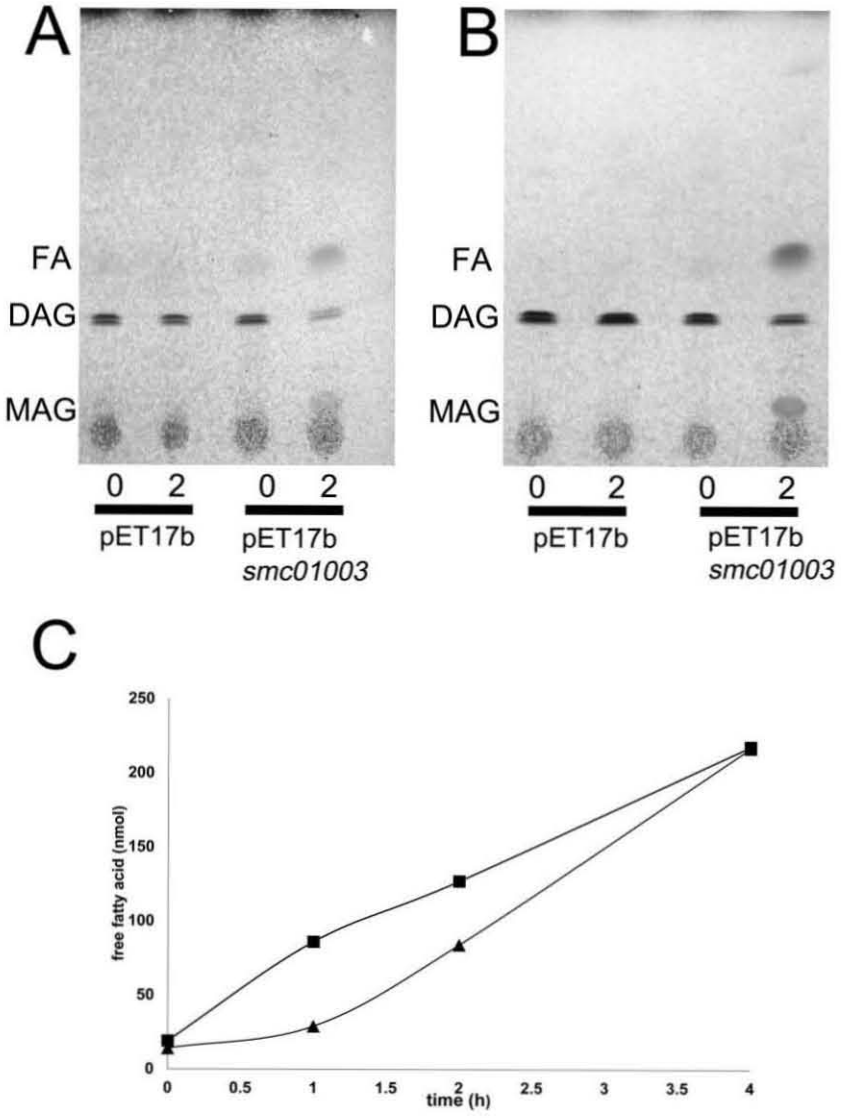

Fig. 6. SMc01003 deacylates diacylglycerols containing monounsaturated long-chain fatty acids. Cell-free extracts of *E. coli* BL21 (DE3) × pLysS, harbouring the empty vector (pET17b) or in which SMc01003 had been expressed from plasmid pDS11, were incubated with 200 nmoles of 1-palmitoyl-2-oleoyl-*sn*-glycerol (A), or 1,2 dioleoyl-*sn*-glycerol (B) for 0 or 2 h, or of 1-palmitoyl-2-oleoyl-*sn*-glycerol for 0, 1, 2, or 4 h (C). After incubation, the lipids were extracted and analysed by 1D-TLC using hexane:diethylether:acetic acid as the mobile phase and visualized by oxidative charring (A, B) or by gas chromatographic analysis of the methyl ester derivatives of the free fatty acids formed (C).
C. Time course for the hydrolysis of 1-palmitoyl-2-oleoyl-*sn*-glycerol by SMc01003. Formation of palmitic acid (■) and of oleic acid (▲) is indicated. FA, fatty acids; DAG, diacylglycerol; MAG, monoacylglycerol.

accumulating after extensive incubation (Fig. 5B). These data suggest that SMc01003 degrades the membrane lipid DAG to MAG and free FA. MAG is further consumed by SMc01003 or an *E. coli* intrinsic MAG-specific activity into FA and glycerol.

In an attempt to purify the His-tagged SMc01003 protein, elevated concentrations of imidazole were used to elute the SMc01003 protein from Ni affinity columns; however, only inactive SMc01003 protein was obtained (data not shown). Treatment of SMc01003 with 250 mM imidazole eliminates its lipase activity (data not shown).

SMc01003 lipase acts on chemically defined DAGs and MAGs

We studied whether commercially acquired DAGs or MAGs could serve as substrates for SMc01003. Cell-free extracts of *E. coli* BL21(DE3) x pLysS, in which SMc01003 had been expressed, were able to partially convert 1-palmitoyl-2-oleoyl-*sn*-glycerol (Fig. 6A) or 1,2 dioleoyl-*sn*-glycerol (Fig. 6B) to free FA and MAG which was not the case when these compounds had been incubated with cell-free extracts of *E. coli* BL21(DE3) x pLysS harbouring the empty pET17b vector (Fig. 6A and B). Other DAGs also served as substrates for SMc01003; however, for DAGs containing only saturated fatty acyl residues in the *sn*-1 and *sn*-2 positions, significant DAG-degrading activity was also present in cell-free extracts of *E. coli* BL21(DE3) x pLysS harbouring the empty pET17b vector (data not shown). A time course, performed with 1-palmitoyl-2-oleoyl-*sn*-glycerol and the cell-free extract in which SMc01003 had been expressed (Fig. 6C), shows that initially palmitate is released from the *sn*-1 position and only with delay oleate is released from the *sn*-2

position. Assays in which the MAGs DL-α-palmitin or DL-α-stearin were incubated with cell-free extracts of *E. coli* BL21(DE3) x pLysS, in which SMc01003 had been expressed, show that they can also be used as substrates by SMc01003 as they are converted to glycerol and the respective free FA (data not shown). These latter data clarify that SMc01003 also acts as MAG lipase.

SMc01003 requires active site serine75 for DAG lipase activity

SMc01003 encodes a 34.4 kD protein that comprises 321 amino acid residues and has a theoretical isoelectric point of 6.7. The lack of an N-terminal signal sequence in SMc01003 excludes it from being secreted by a type II secretion system. Ser75 and Asp189 are thought to compose the catalytic dyad of SMc01003 (Fig. 1). In an attempt to clarify whether SMc01003-related phenotypes are due to the mere presence of the SMc01003 protein or due to its enzymatic activity, we constructed a site-directed mutant replacing the supposed active site Ser75 by an alanine, giving rise to the mutant version SMc01003-S75A. Cell-free extracts from *E. coli* BL21(DE3) × pLysS harbouring the empty vector pET28a or had expressed SMc01003 with an N-terminal His tag, or the site-directed His-tagged mutant version SMc01003-S75A were incubated with radiolabelled DAG (Fig. S3). Consumption of DAG and formation of free FA was only observed with extracts in which the intact SMc01003-encoded DAG lipase had been expressed (Fig. S3). There is no significant DAG lipase activity in cell-free extracts of *E. coli* BL21(DE3) × pLysS harbouring the empty vector pET28a or when the site-directed mutant version SMc01003-S75A had been expressed (Fig. S3). Western blot analysis shows that His-tagged SMc01003 and SMc01003-S75A proteins had been expressed to similar levels and that they migrate according to a molecular weight of 36.5 kD (Fig. S3). Therefore, residue Ser75 is essential for the DAG lipase activity of SMc01003.

SMc01003-encoded DAG lipase activity is associated with S. meliloti *cells and DAG accumulates transiently in a SMc01003-deficient mutant of* S. meliloti

As many PLPs are enzymes secreted by diverse bacterial secretion systems, we studied whether the SMc01003-encoded DAG lipase activity could be detected in the spent culture medium or associated with *S. meliloti* cells. DAG lipase activity is present only in cell-free protein extracts obtained from *S. meliloti* wild type cells (Fig. S4) not however, in cell-free extracts of the *smc01003*-deficient sinorhizobial mutant. No DAG lipase activity can be detected in spent media of either strain suggesting that under normal conditions of cultivation, the SMc01003-encoded DAG lipase is not secreted by *S. meliloti* but rather remains cell associated. When *S. meliloti* is grown under low osmolarity conditions on TY medium, anionic cyclic glucans are formed (Breedveld and Miller, 1995; Wang *et al*., 1999) and in consequence DAG as a second product. In a time course of [^{14}C]acetate labelling, an increased formation of free FA during exponential growth (first 8 h) of wild type *S. meliloti* and its SMc01003-deficient mutant is observed (Fig. 7) which are consumed in stationary phase (after 24 h) in accordance with data previously reported (Pech-Canul *et al*., 2011). In the SMc01003-deficient mutant another compound migrating like DAG increased transiently during exponential growth (first 8 h) and only to a much lower extent in the wild type (Fig. 7). In wild type and the mutant, DAG disappeared after 24 h when cells had entered stationary phase (Fig. 7) maybe due to the DAG kinase reaction. The transient accumulation of DAG in the SMc01003-deficient mutant of *S. meliloti* suggests that this is due to a lack of consumption of DAG by SMc01003 and these data show

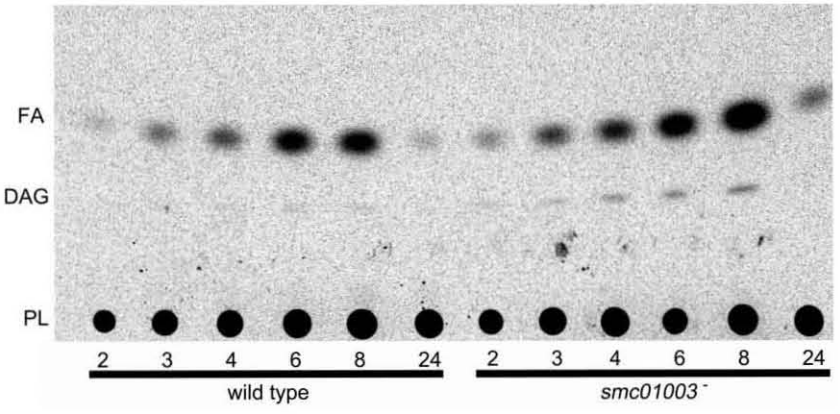

Fig. 7. Mutant of *S. meliloti* deficient in *smc01003* transiently accumulates diacylglycerol. Cultures of *S. meliloti* wild type or the *smc01003*-deficient mutant DXSC2 were grown on TY medium and labelled with ^{14}C-acetate at an OD_{620} = 0.3 for different times (2, 3, 4, 6, 8, or 24 h). At the end of the respective incubation periods, radiolabelled lipids were extracted and separated by one-dimensional thin layer chromatography using hexane:diethylether:acetic acid as the mobile phase. FA, fatty acids; DAG, diacylglycerol; PL, phospholipids.

that also in its native *S. meliloti* background, SMc01003 acts as a cell-associated, intrinsic DAG lipase.

Expression of SMc01003 in E. coli *causes a lysis phenotype in bacterial colonies*

When an *E. coli* BL21(DE3) x pLysS harbouring the SMc01003-expressing plasmid pDS11 was recultivated in isolated colonies on Luria–Bertani (LB) solid medium, we noticed an unexpected colony phenotype, which did not occur in an *E. coli* BL21(DE3) strain harbouring pLysS and an empty pET17b plasmid (Fig. 8A). Although initially normal colony morphology was observed for *E. coli* BL21(DE3) x pLysS x pDS11, after about 24 h, the colonies showed a cleared central area, suggesting cell lysis had occurred in the centre of the colony. In contrast, *E. coli* BL21(DE3) x pLysS harbouring an empty pET17b vector formed normal colonies and no clearing was observed in the oldest part of these colonies (Fig. 8A).

We used fluorescence microscopy to determine if the cleared area in the centre of the colonies expressing SMc01003 is due to lysis, which is easily visualized at the single cell level (Lamsa *et al*., 2012; Nonejuie *et al*., 2013). Samples were taken from both the expressing strain and, as a control, the strain with the empty vector (pET17b). Cells were scraped from the colony, stained with FM4–64, a fluorescent membrane stain that inserts into the outer leaflet of the bilayer, the membrane impermeable DNA stain SYTOX Green and the membrane permeable stain 4',6-diamidino-2-phenylindole (DAPI), and then visualized. Cells from the outside edge of the colony from each strain look healthy, with some membrane debris but no SYTOX Green-permeabilized cells (Fig. 8B). Cells from the centre of the colony from the strain containing the empty vector similarly showed only small amount of membrane debris, no lysed cells and no SYTOX Green staining. However, cells from the strain overexpressing SMc01003 showed lysed cells with collapsed membranes, membrane debris, increased SYTOX Green staining and SYTOX Green-stained DNA outside of the cells (Fig. 8B). This indicates that lysis has occurred in the centre of the colony overexpressing SMc01003, likely explaining the central clearing seen in these colonies.

The lysis phenotype was also observed when expression of SMc01003 was induced with arabinose from a pBAD24 vector in *E. coli* RZ6 (Fig. S5). Even in the absence of arabinose, *E. coli* RZ6 harbouring the *smc01003*-containing pBAD24 vector displayed a phenotype that looked like 'localized colony growth'. When SMc01003 was expressed in *E. coli* RZ6 in the presence of arabinose, lysis occurred at 0.05% arabinose and 0.2% arabinose. None of these lysis-related phenotypes occurred with or without arabinose when *E. coli* RZ6 harboured the empty pBAD24 vector or the pBAD24 vector expressing the site-directed mutant version SMc01003-S75A (Fig. S5). Notably, when SMc01003 was expressed from the broad host range plasmid pDS13 in *S. meliloti*, no such lysis phenotype was observed.

Discussion

Patatin-like lipases in bacteria

The patatin family of lipolytic enzymes is widespread in bacteria and members of this family are diverse with regard to substrate specificity and biological functions. The first patatin-like protein reported in bacteria has been the ExoU enzyme (Sato *et al*., 2003), a phospholipase A_2 that is rapidly cytotoxic to eukaryotic cells. Together with other effectors, ExoU is secreted by the type III secretion system of *P. aeruginosa* directly into host cells. ExoU is a

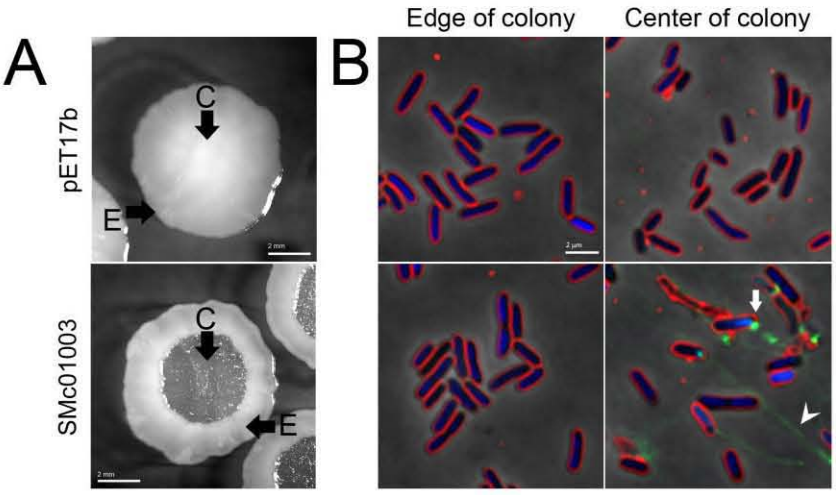

Fig. 8. Expression of SMc01003 in *E. coli* causes lysis phenotype in bacterial colonies.
A. Growth of bacterial colonies on LB solid media of *E. coli* BL21(DE3) x pLysS strains that carry the empty vector (pET17b) or the SMc01003-expressing vector pDS11. Samples were taken from the edges (E) or the centres (C) of colonies.
B. Fluorescence micrograph of cells taken from the edges or centres of colonies after 24 h of growth. Cells were stained with FM4-64 (red, membranes), DAPI (blue, DNA) and SYTOX Green (green, membrane impermeable DNA). Staining with SYTOX Green indicates lysis. The arrow indicates a lysed cell and the arrowhead external DNA strings.

large protein of 687 amino acid residues that includes a patatin-like lipase as well as other independent domains connected by bridging regions (Tyson and Hauser, 2013).

The other large patatin-like protein PlpD of *P. aeruginosa* of 728 amino acid residues displays a distinct multidomain structure (Salacha *et al*., 2010) containing a molecular system responsible for the secretion of the patatin moiety.

In the causative agent of Legionnaires' disease, *Legionella pneumophila*, there seems to be an abundance of 15 different phospholipase A-encoding genes, among them 11 different patatin-like lipases with distinct substrate specificities and modes of action (Lang and Flieger, 2011). Many patatin-like proteins of bacterial pathogens are large proteins with multidomain structures in which the patatin-like sequence composes one specific domain responsible for the lipase activity. Also in eukaryotes, patatin-like domains usually are part of multidomain structures (Kienesberger *et al*., 2009).

Another group of bacterial patatin-like proteins are much smaller and consist mainly of the patatin-like domain. One example is the lipolytic enzyme YvdO, present in dormant spores of *Bacillus subtilis*, and which can hydrolyse *p*-nitrophenyl esters of short and medium chain length (C2-C10) as well as the lysophosphatidylcholine 1-myristoyl-2-lyso-*sn*-glycero-3-phosphocholine (Kato *et al*., 2010). Other examples are the patatin-like proteins SMc00930 and SMc01003 from *S. meliloti*.

Predicted patatin-like proteins SMc00930 and SMc01003 contribute to the formation of free FA in S. meliloti *but are not phospholipases*

A *fadD*-deficient mutant of *S. meliloti* accumulates free FA in the stationary phase of growth (Pech-Canul *et al*., 2011). In this work, we wanted to identify genes and activities that contribute to the release of these FA. One candidate for a potential lysophospholipase (SMc04041) and two predicted PLPs (SMc00930 and SMc01003) were studied in more detail. Expression of each one of the candidates in *E. coli* increased the amount of free FA formed; however, the effect was much more pronounced in the cases of SMc00930 and SMc01003. Expression of the three candidates in *S. meliloti* led to increased fatty acid formation only in the cases of SMc00930 and SMc01003. Also, when cell-free extracts of *E. coli*, in which the candidate genes had been expressed, were tested for their ability to hydrolyse *p*-nitrophenylacyl esters, only SMc01003 and, to a much larger extent, SMc00930 were able to act on these artificial lipase substrates, whereas SMc04041 was not. In an activity-based screen, SMc04041 was able to hydrolyse palmitoyl-CoA, and therefore, it had been suggested that SMc04041 is a thioesterase (Chan *et al*., 2010). We therefore did not pursue to study the function of SMc04041 any further. Surprisingly, neither SMc00930 nor SMc01003 were able to degrade sinorhizobial phospholipids and therefore neither SMc00930 nor SMc01003 are phospholipases.

SMc01003 is a DAG lipase

In the present work, we focused on resolving the molecular function of SMc01003. Expression of SMc01003 in a DAG-overproducing *E. coli* strain showed that SMc01003 was responsible for the removal of DAG and the formation of increased amounts of free FA. Upon treatment of DAG with SMc01003, the temporary accumulation of a compound that migrated like MAG could be observed in TLC analyses. Using chemically defined substances, we show that SMc01003 can use different MAGs and DAGs as substrates and degrades them to the respective free FA and glycerol. Although DAGs with different acyl chain compositions are degraded by SMc01003, its action is most clear when DAGs containing unsaturated long-chain fatty acyl residues (C18:1) are employed as substrates. Notably, a mutant of *S. meliloti* deficient in SMc01003 showed a higher transient accumulation of DAG than the wild type, suggesting that also in its native *S. meliloti* background, SMc01003 acts as an internal DAG lipase. We therefore rename the *smc01003* gene *dglA* to highlight its DAG lipase function. It should not go unnoticed, however, that SMc01003 showed considerable activity with the artificial substrate *p*-nitrophenyldecanoate, suggesting SMc01003 might act as well on medium- or short-chain-containing DAGs, a possibility that will be explored in future research.

To our knowledge, SMc01003 (DglA) is the first example of a patatin-like bacterial lipase that degrades endogenous DAG. Well-supported orthologues are found in the order *Rhizobiales* of the *Alphaproteobacteria* (Fig. S6), suggesting that a patatin-like DAG lipase (DglA) might be a common feature in this group of organisms. Clear orthologues for the other sinorhizobial patatin-like protein SMc00930 are limited to members of the closely related *Rhizobiaceae* and *Phyllobacteriaceae* families of the *Rhizobiales* (Fig. S6).

Roles of DAG and MAG in living organisms

In eukaryotes, DAG is well known for its role as a second messenger produced in the hormone-sensitive phosphatidylinositol system (Nelson and Cox, 2013). When G protein-coupled receptors are activated by hormone ligands, some receptors activate a phosphatidylinositol-4,5-bisphosphate-specific phospholipase C that produces two potent second messengers,

DAG and inositol-1,4,5-trisphosphate. Whereas inositol-1,4,5-trisphosphate provokes release of Ca^{2+} from the endoplasmic reticulum to the cytosol, DAG, in cooperation with Ca^{2+}, activates protein kinase C leading to some of the cellular hormone responses (Nelson and Cox, 2013).

In higher animals, DAG lipases hydrolyse DAG to generate 2-arachidonoylglycerol, an abundant ligand for cannabinoid receptors (Reisenberg *et al*., 2012). DAG lipase-dependent endocannabinoid signalling regulates axonal growth and guidance during development and is required for the generation and migration of new neurons in the adult brain. Several MAG lipases can hydrolyse 2-arachidonoylglycerol and thereby modulate 2-arachidonoylglycerol levels and endocannabinoid signalling (Reisenberg *et al*., 2012).

Although cytolytic and membrane-perturbing properties of some lysophospholipids are well known (Weltzien, 1979), information about potential roles for DAG and MAG in bacteria are scarce. Inactivation of the gene for a MAG lipase in *Mycobacterium smegmatis* has drastic effects on colony morphology and both the MAG lipase protein as well as MAG lipids seem to affect colony morphology in this organism (Dhouib *et al*., 2010). Although some MAGs, i.e. monolaurin, are antimicrobial against many Gram-positives, they seem to be inactive on Gram-negative bacteria when applied externally (Kabara *et al*., 1977). It has been noted that increased formation of DAG in *E. coli* goes in parallel with reduced growth rates (Raetz and Newman, 1978), but such strains also form elevated levels of MAG and triacylglycerol (Rotering and Raetz, 1983) and therefore it is not clear which of the three molecules might have the damaging effect on *E. coli*. In *S. meliloti*, at least two DAG-generating pathways exist (Geiger *et al*., 2013) and one of the physiological roles of the SMc01003-encoded DAG lipase DglA might consist in degrading excessive, and potentially toxic, levels of DAG.

Expression of SMc01003 in distinct *E. coli* strains from pET- or pBAD-based vectors causes drastic phenotypes in stationary phase cells. Colonies more than 1 day old started to clear up in their centre leading to donut-shaped colonies. Analysis of cells obtained from the centre or the edge of colonies show that only cells obtained from the centre of SMc01003-expressing colonies suffered from DNA leakage to the medium due to lysis of those cells. Although we have no direct evidence, we assume that the SMc01003 (DglA)-induced lysis phenotype in *E. coli* is due to the MAG formed by the SMc01003 (DglA)-encoded DAG lipase activity. To date, we did not observe any SMc01003-provoked lysis in *S. meliloti*.

Interestingly, an extensive screening of *P. aeruginosa* mutants revealed three mutants that also displayed an autolysis phenotype in which colonies lysed at their centre (D'Argenio *et al*., 2002). Two of the autolysis mutants mapped in *pqsL* (PA4190) and one in *vfr*. PA4190 seems to be related to quorum-sensing-controlled genes and mutants deficient in PA4190 overproduce the *Pseudomonas* quinolone signal (PQS). Autolysis was suppressed by mutation of genes required for PQS biosynthesis. Vfr is a homologue of the *E. coli* cyclic AMP receptor protein CRP and acts at the top of the quorum sensing regulatory hierarchy. We are presently investigating whether SMc01003 (DglA)-induced lysis of *E. coli* requires release of catabolite repression or quorum sensing.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used and their relevant characteristics are shown in Table 1. The construction of sinorhizobial mutants deficient in putative lipases is described in Table S5. FadD-deficient mutants (FadD2 and FDXSC2) were constructed by general transduction of *fadD* gene region from 1021FDC5 (Nogales *et al*., 2010), in which the *fadD* gene had been replaced by a kanamycin resistance-conferring cassette, using the phage ΦM12 similarly to the way described previously (Finan *et al*., 1984).

Sinorhizobium meliloti strains were grown either in complex tryptone/yeast extract (TY) medium that contained 4.5 mM $CaCl_2$ (Beringer, 1974) or in minimal medium (Sherwood, 1970) with succinate (8.3 mM) replacing mannitol as the carbon source at 30°C on a gyratory shaker.

Escherichia coli strains were cultured on LB medium (Miller, 1972) at 37°C or at 30°C when the SMc00930, SMc01003 or SMc04041 proteins were expressed. Antibiotics were added to media in the following concentrations (μg ml^{-1}) when required: spectinomycin 400, gentamicin 70, nalidixic acid 40, tetracycline 10, neomycin 200, chloramphenicol 80, in the case of *S. meliloti* and spectinomycin 200, carbenicillin 100, tetracycline 20, gentamicin 10, kanamycin 50, chloramphenicol 20 in the case of *E. coli*. Plasmids were mobilized into *S. meliloti* strains by diparental mating using the *E. coli* S17-1 donor strain as described previously (Simon *et al*., 1983).

DNA manipulations

Recombinant DNA techniques were performed according to standard protocols (Sambrook *et al*., 2001). Commercial sequencing of amplified genes by Eurofins Medigenomix (Martinsried, Germany) corroborated the correct DNA sequences. The DNA regions containing *smc00930*, *smc04041* and *smc01003* were analysed using the NCBI (National Center for Biotechnology Information) BLAST network server (Altschul *et al*., 1997).

Construction of expression plasmids

Using PCR and specific oligonucleotides (oLOP190 and oLOP191 for *smc04041*, oLOP151 and oLOP152 for *smc00930*, and oLOP149 and oLOP150 for *smc01003*)

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics[a]	Reference
Sinorhizobium meliloti		
S. meliloti 1021 our	wild type used throughout this study	López-Lara *et al*., 2005
Sm 1021 derivatives		
DXSC1	*smc04041::sp*	This study
DXSC2	*smc01003::deletion*	This study
DXSC3	*smc00930::km*	This study
FadD2	*smc02162::km*	This study
FDXSC2	*smc02162::km,smc01003::deletion*	This study
E. coli		
DH5α	*recA*1, ϕ80 *lacZ*ΔM15, host for cloning	Hanahan, 1983
RZ6	*dgk-6* derivative (defective diglyceride kinase) of R4440	Raetz and Newman, 1978
S17-1	*thi*, *pro*, *recA*, *hsdR*, *hsdM+*, RP4Tc::Mu, Km::Tn*7*;Tp^R, Sm^R, Sp^R	Simon *et al*., 1983
BL21(DE3)	expression strain	Studier *et al*., 1990
Plasmids		
pUC18	cloning vector, Cb^R	Yanisch-Perron *et al*., 1985
pET17b	expression vector, Cb^R	Novagen
pET28a	expression vector, Km^R, conferring N-terminal His-tag to expressed proteins	Novagen
pLysS	production of lysozyme for repression of T7 polymerase, Cm^R	Studier *et al*., 1990
pBAD24	tightly regulated expression vector, Cb^R	Guzman *et al*., 1995
pRK404	broad host-range vector, Tc^R	Ditta *et al*., 1985
pK18*mobsacB*	suicide vector, Km^R	Schäfer *et al*., 1994
pBBR1MCS-5	cloning vector, Gm^R	Kovach *et al*., 1995
pHY109	plasmid carrying Sp^R cassette	Østerås *et al*., 1998
pTB3131	plasmid carrying Km^R cassette	Kretzschmar *et al*., 2001
pDS10	pET17b carrying *smc00930*	This study
pDS11	pET17b carrying *smc01003*	This study
pDS30	pET17b carrying *smc04041*	This study
pDS11H	pET28a carrying *smc01003*, expressed with N-terminal His tag	This study
pDS11HS75A	pET28a carrying site-directed mutant (S75A) version of *smc01003*, expressed with N-terminal His tag	This study
pDS12	BamHI-restricted pDS10 in pRK404	This study
pDS13	BglII-restricted pDS11 in pRK404	This study
pDS31	BamHI-restricted pDS30 in pRK404	This study
pDS11HBAD	pBAD24 carrying *smc01003*, expressed with N-terminal His tag	This study
pDS11HS75ABAD	pBAD24 carrying site-directed mutant (S75A) version of *smc01003*, expressed with N-terminal His tag	This study
pNG28	BamHI-restricted pET17b in pRK404	González-Silva *et al*., 2011

a. Tc^R, Tp^R, Km^R, Sp^R, Gm^R, Sm^R, Cb^R, Cm^R: tetracycline, trimethoprim, kanamycin, spectinomycin, gentamicin, streptomycin, carbenicillin, chloramphenicol resistance respectively.

(Table S6) genes encoding potential (lyso)phospholipases were amplified from *S. meliloti* 1021 genomic DNA. Suitable restriction sites for cloning of the genes were introduced by PCR with oligonucleotides. After restriction with NdeI and BamHI (or NdeI and XhoI in the case of *smc01003*), the PCR-amplified DNA fragments were cloned into a pET17b or a pET28a vector that had been digested with NdeI and BamHI (or NdeI and XhoI in the case of *smc01003*). The gene *smc01003* was recloned from pET28a as a NcoI-XhoI fragment into pBAD24 that had been digested with NcoI and SalI in order to obtain pDS11HBAD. Expression plasmids for use in *E. coli* are listed in Table 1. Plasmids carrying the respective genes were digested with BamHI (or BglII in the case of *smc01003*) and cloned into the broad-host-range vector pRK404 that had been digested with BamHI. In that way, we obtained *smc00930*-containing pDS12, *smc01003*-containing pDS13, and *smc04041*-containing pDS31 plasmids used for expression of these genes in *S. meliloti*.

Construction of site-directed mutant SMc01003-S75A

After having shown the functionality of the N-terminally His-tagged SMc01003, the pDS11H plasmid was used as a template for site-directed mutagenesis reactions in order to replace the active-site serine residue (S75) of SMc01003 by an alanine. The mutation was constructed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and the mutagenic primers t223g and t223g_antisense (Table S6) which were designed using the QuikChange Primer Design Program available online (www.genomics.agilent.com/primerDesignProgram.jsp). The mutation in the resulting plasmid pDS11H-S75A was confirmed by Sanger sequencing (Eurofins Medigenomics, Ebersberg, Germany). The site-directed version of *smc01003* was obtained as NcoI-XhoI fragment from pDS11H-S75A and cloned into the NcoI-SalI restricted pBAD24 vector obtaining pDS11H-S75ABAD.

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

Lipid compositions of bacterial strains were determined following labelling with [1-^{14}C]acetate (45–60 mCi mmol^{-1}; Perkin Elmer). Cultures of *E. coli* (1 ml) were grown in LB medium and inoculated from precultures grown in the same medium. In the case of *E. coli* BL21(DE3) x pLysS strains harbouring pET17b derivatives, cultures were induced at an $OD_{620} = 0.3$ with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) and labelled with 1 μCi of [1-^{14}C]acetate for 4 h whereas *E. coli* RZ6 strains harbouring pBAD24-derived plasmids were induced (or not) at an $OD_{620} = 0.1$ with 0.2% of arabinose, labelled with 1 μCi of [1-^{14}C]acetate until cultures reached $OD_{620} = 0.3$. In the case of *S. meliloti* strains, cultures (1 ml) were grown in TY medium and inoculated from precultures grown in the same medium. Starting at an $OD_{620} = 0.3$ labelling with 1 μCi of [1-^{14}C]acetate was performed for a period of either 4 or 28 h. At the end of the respective incubation periods, cells were harvested by centrifugation, and resuspended in 100 μl of water. Lipids were extracted according to the method of Bligh and Dyer (1959) and the chloroform phase was separated by one-dimensional thin-layer chromatography (1D-TLC) on high-performance TLC aluminum sheets (silica gel 60; Merck). For separation of distinct phospholipids, chloroform : methanol : acetic acid (130:50:20, v/v) was employed as mobile phase. For the separation of FA from polar lipids, ethyl acetate : hexane : acetic acid (60:40:5; v/v) was used as the mobile phase (Pech-Canul *et al*., 2011), whereas neutral lipids were separated with hexane : diethylether : acetic acid (70:30:4, v/v) (Flieger *et al*., 2000). Radioactive lipids were visualized using a Phosphor-Imager (Storm 820; Molecular Dynamics) and quantification was performed using ImageQuant TL (Amersham Biosciences).

Preparation of [^{32}P]-labelled phospholipids and of [^{14}C]-labelled DAG

For the preparation of ^{32}P-labelled phospholipids, *S. meliloti* 1021 was grown in minimal medium that contained reduced concentration (0.2 mM) of inorganic phosphate (Pi). To a culture of *S. meliloti* 1021 (1 ml), 100 μCi [^{32}P] orthophosphate (specific radioactivity 285.6 Ci mg^{-1}) was added at a cell density of 2×10^8 cells ml^{-1} and labelling was performed for 24 h. Extracted lipids were dissolved in $CHCl_3$:MeOH (1:1, v/v) and quantified by liquid scintillation counting.

The preparation of ^{14}C-labelled DAG was performed in several steps. First, *S. meliloti* cultures (1 ml) grown in complex TY medium were labelled with 1 μCi [1-^{14}C]acetate for 28 h as described above. Lipid extracts were separated by 1D-TLC in chloroform : methanol : acetic acid (130:50:20, v/v) and radiolabelled PC was extracted from the silica gel matrix. The sinorhizobial PC (313 000 cpm) was then incubated with 0.1 U of phospholipase C from *Clostridium perfringens* in 50 mM Tris/HCl (pH 7.2), 0.5% Triton X-100 and 10 mM $CaCl_2$, and after 2 h of incubation, the treated lipids were extracted according to Bligh and Dyer (1959) and separated by TLC in hexane : diethylether : acetic acid (70:30:4, v/v). Diacylglycerol (175 000 cpm) was scraped from the silica plate, extracted and quantified by scintillation counting.

Preparation of cell-free extracts for analysis of the potential SMc01003, SMc00930 or SMc04041 lipases

To examine the lipolytic activity of *smc04041*, *smc01003* and *smc00930*, the cloned genes that code for the predicted lipases were expressed in distinct *E. coli* strains. Liquid cultures (1 l) of exponentially growing *E. coli* BL21(DE3) x pLysS harbouring pET17b or pET28a derivatives were induced with 0.1 mM IPTG or *E. coli* cultures harbouring pBAD24 derivatives were induced with 0.2% arabinose at a density of 4×10^8 cells ml^{-1} and incubated for 4 h at 30°C. After harvesting cells at 4°C, each cell pellet was resuspended in 10 ml of 50 mM Tris-HCl buffer, pH 9.0. Cell suspensions were passed three times through a cold French pressure cell at 20 000 lb in^2. Unbroken cells and cell debris were removed by centrifugation at $4000 \times g$ for 10 min at 4°C to obtain cell-free extracts as supernatants. Protein concentrations were determined by the method of Dulley and Grieve (1975).

Enzymatic assays

In order to determine the enzyme activities of SMc04041, SMc01003 or SMc00930, cell-free extracts in which *smc04041*, *smc01003* or *smc00930* had been overexpressed were incubated with *p*-nitrophenyl (*p*-NP) esters of different fatty acyl chain lengths (C10–C18), with radiolabelled lipids, or with unlabelled mono- or DAGs.

For spectrophotometric measurements, we used an enzymatic assay similar to the one described for the PLP Yvdo from *B. subtilis* (Kato *et al*., 2010). Our standard assay mixture (1.0 ml) contained 625 μM *p*-NP ester, 50 mM Tris–HCl buffer (pH 8.5), 150 mM NaCl, 0.2% Triton X-100, 2.5% dimethylsulfoxide and the enzyme (1–100 μg protein of cell-free extracts). The reaction mixture without the enzyme was preincubated for 3 min at 30°C. The reaction was initiated by the addition of the enzyme and the absorbance was followed at 400 nm.

When cell-free extracts were incubated with radiolabelled lipids, the standard enzymatic assay (100 μl) contained 150 000 cpm ^{32}P-labelled phospholipids or 5000 cpm ^{14}C-labelled DAG in 50 mM Tris/HCl (pH 8.5), 150 mM NaCl, 0.2% Triton X-100 and the enzyme (50 μg protein). The reaction was initiated by the addition of the enzyme, and after 2 h of incubation at 30°C, the reaction was stopped by the addition of 250 μl of methanol and 125 μl of chloroform, and the lipid fraction was extracted as described.

A similar assay was used when different unlabelled mono- or DAGs (200 nmol per assay) were tested as substrates. DL-α-palmitin (SIGMA M1640), DL-α-stearin (M2015), dilauroyl-*sn*-glycerol, 1,2-dipalmitoyl-*sn*-glycerol, 1-palmitoyl-2-oleoyl-*sn*-glycerol or 1,2 dioleoyl-*sn*-glycerol (AVANTI POLAR LIPIDS) were incubated with cell-free extracts for 0, 2 or 4 h, and the reactions were stopped by the addition of 250 μl of methanol and 125 μl of chloroform. The lipidic fraction was split into two equivalent parts one used for the determination of free FA and the other for one-dimensional TLC analysis. The unlabelled, separated lipids

were detected by oxidative charring using ceric sulphate in sulphuric acid (Villaescusa and Pettit, 1972).

Determination of free FA

For the quantification of free FA, 20 μg of tridecanoic acid (C13:0) was added as an internal standard to each half of a lipid extract (previously dried under a stream of nitrogen). Methanol (800 μl) was added together with 50 μl of 1-ethyl-3-(3-dimethylamino-propyl-carbodiimide) (100 mg ml^{-1} in methanol). After 2 h of incubation at 22°C, the reaction was stopped by adding 400 μl of saturated NaCl solution. The methyl esters were extracted twice with 1 ml of hexane each time followed by centrifugation. The upper hexane phases were pooled into a new glass vial and dried under a nitrogen stream. For quantitative analysis, fatty acid methyl esters were dissolved in 125 μl of hexane and 1 μl was used for analysis by GC/MS using a Clarus 600T MS instrument coupled to a Clarus 600 gas chromatography system. Fatty acid species were identified using retention times and mass spectral information by comparison with the bacterial acid methyl esters mix standard (BAME 47080-U; Sigma-Aldrich). The relative amounts of fatty acid methyl esters were determined by comparing the areas under the peaks on the chromatogram to the area under the peak of the internal fatty acid standard (C13:0).

Fluorescence microscopy

Escherichia coli colonies were grown on LB agar at 37°C for ~ 24 h. Cells were then scraped from the edge of the colony and the centre of the colony. For cells overexpressing SMc01003, samples from the centre of the colony were scraped from the clearing in the centre of the colony. The cells were then resuspended in 1X T-base containing 1 μg ml^{-1} FM 4–64, 2 μg ml^{-1} DAPI and 0.5 μM SYTOX Green and transferred to an agarose pad (20% LB, 1% agarose) for visualization. Cells were visualized on an Applied Precision DV Elite optical sectioning microscope equipped with a Photometrics CoolSNAP-HQ^2 camera and deconvolved using softWoRx v5.5.1 (Applied Precision). The median focal plane is shown. The phase contrast and FM images were adjusted for best visualization, and the DAPI and SYTOX Green intensities were normalized based on intensity and exposure length to reflect intensities relative to the treatment with the highest fluorescence intensity (Lamsa *et al*., 2012), so they reflect relative intensities of DAPI and SYTOX Green between the images.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. The overall lipid profile of *S. meliloti* mutants deficient in SMc00930, SMc01003, SMc04041 is similar to the wild type profile. Lipid profile analysis of *S. meliloti* wild type strain and mutants deficient in potential lipases. Strains were labelled with ^{14}C-acetate for 28 h and extracted lipids were separated by one-dimensional thin-layer chromatography using chloroform : methanol : acetic acid (130:50:20, v/v) as the mobile phase. Lipids of *S. meliloti* 1021 wild type, DXSC1 (*smc04041*-deficient), DSXC2 (*smc01003*-deficient), DXSC3 (*smc00930*-deficient). FA, fatty acids; CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; DMPE, dimethyl-PE; PC, phosphatidylcholine.

Fig. S2. Membrane phospholipids from *S. meliloti* are not consumed by SMc01003 or SMc00930 under the conditions tested. Total ^{32}P-radiolabelled phospholipids from *S. meliloti* were treated with cell-free extracts of *E. coli* BL21(DE3) x pLysS harbouring the *smc01003*-expressing vector, the empty pET17b vector, the *smc00930*-expressing vector or with phospholipase A_2 from *Crotalus adamanteus* (PLA_2). After 5 h incubation, lipids were extracted and separated by one-dimensional thin-layer chromatography using chloroform : methanol : acetic acid (130:50:20, v/v) as the mobile phase. CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; MMPE, monomethyl-PE; PC, phosphatidylcholine.

Fig. S3. Ser75 is required for the DAG lipase activity of SMc01003. Cell-free extracts from *E. coli* BL21(DE3) × pLysS harbouring the empty vector pET28a (–) or had expressed His-tagged SMc01003, or the His-tagged site-directed mutant version SMc01003-S75A were incubated with ^{14}C-labelled diacylglycerol and after 24 h of incubation at 30°C, the lipidic fractions were separated by one-dimensional thin-layer chromatography using hexane : diethylether : acetic acid (70:30:4, v/v) (left panel). Cell-free extracts of the three strains were also analysed for production of the His-tagged proteins using Western blot (right panel). Protein extracts were separated by polyacrylamide (12%) gel electrophoresis in the presence of sodium dodecyl sulfate and after blotting the electrophoretogram to a membrane, Western blot detection was performed using anti-His as primary antibody similarly as described (Solís-Oviedo *et al.*, 2012). Bovine serum albumin (79.7 kD), ovalbumin, (43 kD), carbonic anhydrase 34.1 kD), soybean trypsin inhibitor (27 kD) and lysozyme (17.6 kD) were used as molecular weight markers. FA, fatty acids; DAG, diacylglycerol; MAG, monoacylglycerol.

Fig. S4. DAG lipase SMc01003 is associated with *S. meliloti* cells. Cultures of *S. meliloti* wild type or the *smc01003*-deficient mutant were cultivated on TY medium until bacterial suspensions reached an $OD_{620} = 1.2$. Cells were harvested by centrifugation, resuspended in buffer and cell-free extracts were prepared as described in *Experimental procedures*. Spent cell-free supernatants were concentrated about 100-fold in Amicon Ultra centrifugal filters (30 K) in order to be able to use protein preparations obtained from the same initial volume of bacterial suspension as the cell-free bacterial extracts. Cell-free extracts (100 μg protein per assay and derived from about 1 ml of initial cell suspension) and concentrated spent supernatants (derived from about 1 ml spent supernatant) of *S. meliloti* wild type or the *smc01003*-deficient mutant were incubated with ^{14}C-labelled diacylglycerol for 24 h at 30°C. Lipidic fractions were extracted and separated by one-dimensional thin-layer chromatography using hexane : diethylether : acetic acid (70:30:4, v/v). FA, fatty acids; DAG, diacylglycerol; MAG, monoacylglycerol.

Fig. S5. Lysis phenotype in bacterial colonies of *E. coli* is due to SMc01003 enzymatic activity. Growth of bacterial colonies on LB solid media of *E. coli* RZ6 carrying the empty vector (pBAD24) or the SMc01003-expressing plasmid (pDS11HBAD) or the plasmid (pDS11H-S75ABAD) expressing the site-directed mutant version SMc01003-S75A in the absence (0%) or presence of arabinose (0.05% or 0.2%) at 37°C for 36 h.

Fig. S6. Phylogenetic tree of characterized patatin-like bacterial phospholipases (PLP) and homologues to SMc00930 and SMc01003. For the construction of the tree

(see Appendix S1) the following patatin-like proteins were selected: ORFs from *Agrobacterium tumefaciens* str. C58 (Atu1751: NP_354736; Atu0691: NP_353715), YvdO from *Bacillus subtilis* subsp. *subtilis* str. 168 (NP_391333), ORFs from *Bradyrhizobium japonicum* USDA 110 (blr5550: NP_772190; blr2776: NP_769416), ORFs from *Brucella abortus* 2308 (BAB1_1099: YP_414495; BAB2_0203: YP_418429), ORFs from *Burkholderia cenocepacia* J2315 (BCAL2127: YP_002231254; BCAM2172: YP_002234772), RssA from *Escherichia coli* K-12 (P0AFR0), PLPs from *Legionella pneumophila* subsp. *pneumophila* str. Philadelphia 1(VipD: YP_096826; VpdB: YP_095258; VpdA: YP_096418; VpdC: YP_095455), ORFs from *Mesorhizobium loti* MAFF303099 (mlr0925: NP_102623; mlr8257: NP_1083920), a PLP from *Myxococcus xanthus* DK 1622 (MXAN_3852: YP_632034), ExoU from *Pseudomonas aeruginosa* PA103 (AAC16023), PlpD from *Pseudomonas aeruginosa* PAO1 (NP_252029), ORFs from *Sinorhizobium fredii* NGR234 (NGR_c04200: YP_002824969; NGR_c12550: YP_002825789), ORFs from *Rhizobium tropici* CIAT 899 (RTCIAT899_CH09385: YP_007333809; RTCIAT899_CH04025: YP_007332764), an ORF from *Rhodopseudomonas palustris* BisB18 (RPC_3127: YP_532988), a PLP from *Rickettsia typhi* str. Wilmington (RT_0522: YP_067473), SMc01003 and SMc00930 from *Sinorhizobium meliloti* 1021 (SMc01003: NP_385524; SMc00930: NP_384909). Likely orthologue to SMc01003 or SMc00930 are highlighted in light or dark grey respectively. Bootstrap support values for these clades are higher than 90% and aLRT statistic is above 0.99. Distances between sequences are expressed as 0.8 changes per amino acid residue.

Table S1. Membrane lipid composition of *Escherichia coli* BL21(DE3) pLysS expressing *smc00930* (pDS10), *smc01003* (pDS11), or *smc04041* (pDS30) and *E. coli* BL21(DE3) x pLysS harbouring the empty vector (pET17b) after 4 h of induction and ^{14}C-acetate labelling. The values shown are mean values ± SD derived from three independent experiments. FA, fatty acids; PG, phosphatidylglycerol; CL, cardiolipin; PE, phosphatidylethanolamine.

Table S2. Membrane lipid composition of *Sinorhizobium meliloti* 1021 wild type expressing *smc00930* (pDS12), *smc01003* (pDS13) or *smc04041* (pDS31) and *S. meliloti* 1021 harbouring the empty vector (pNG28) after 4 h of ^{14}C-acetate labelling. The values shown are mean values ± SD derived from three independent experiments. FA, fatty acids; PG, phosphatidylglycerol; CL, cardiolipin; PE, phosphatidylethanolamine; MMPE, monomethyl-PE; DMPE, dimethyl-PE; PC, phosphatidylcholine.

Table S3. SMc01003 and SMc00930 contribute to the formation of free fatty acids in *S. meliloti*. Membrane lipid composition of *S. meliloti* 1021 wild type, mutants deficient in SMc01003 (DXSC2), FadD (FadD2), SMc01003 and FadD (FDXSC2), double mutant FDXSC2 harbouring the SMc01003-expressing plasmid pDS13, or the SMc00930-expressing plasmid pDS12, or the empty plasmid (pNG28). Labelling with ^{14}C-acetate was performed for 28 h, lipids were extracted, separated by 1D-TLC and quantified. The values shown are mean values ± SD derived from three independent experiments.

Table S4. The predicted PLPs SMc01003 and SMc00930 from *S. meliloti* hydrolyse *p*-nitrophenylacyl substrates. Using a spectrophotometric assay, cell-free extracts of *E. coli* BL21(DE3) x pLysS, containing the empty pET17b vector, or the vector from which *smc00930*, or *smc01003* genes had been expressed, were assayed for their ability to hydrolyse *p*-nitrophenyl (*p*-NP) ester substrates of varying fatty acyl chain length (10, 12, 14, 16 or 18). Specific activities (μmol nitrophenol min^{-1} mg $protein^{-1}$) for different *p*-NP ester substrates are given. These values were obtained after subtracting minor activities present in the extract from *E. coli* BL21(DE3) x pLysS harbouring the empty pET17b vector. The values shown are mean values ± standard deviation derived from three independent experiments.

Table S5. Construction of knock-out mutants in potential lipase genes.

Table S6. Oligonucleotides used for construction of different phospholipase/lysophospholipase expression plasmids or a site-directed mutant. Sites for recognition by restriction enzymes are underlined.

Appendix S1. Phylogenetic analyses of protein sequences for tree construction.

SUPPLEMENTAL INFORMATION

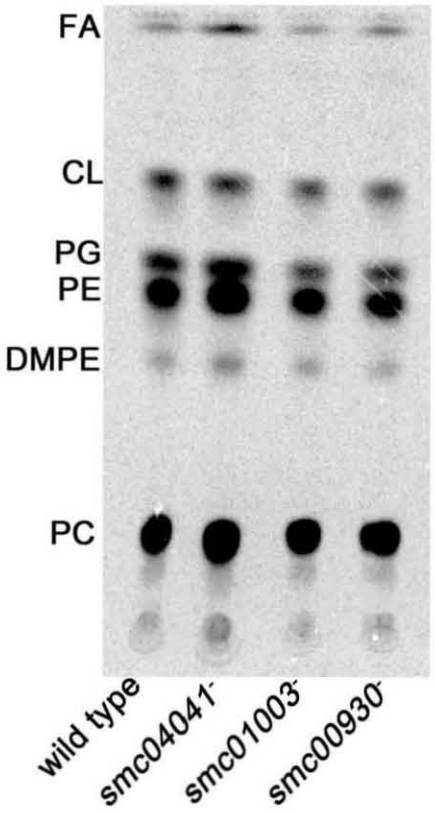

Fig. S1. The overall lipid profile of *S. meliloti* mutants deficient in SMc00930, SMc01003, SMc04041 is similar to the wild type profile. Lipid profile analysis of *S. meliloti* wild type strain and mutants deficient in potential lipases. Strains were labeled with ^{14}C-acetate for 28 h and extracted lipids were separated by one-dimensional thin-layer chromatography using chloroform:methanol:acetic acid (130:50:20, v/v) as the mobile phase. Lipids of *S. meliloti* 1021 wild type, DXSC1 (*smc04041*-deficient), DSXC2 (*smc01003*-deficient), DXSC3 (*smc00930*-deficient). FA, fatty acids; CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; DMPE, dimethyl-PE; PC, phosphatidylcholine.

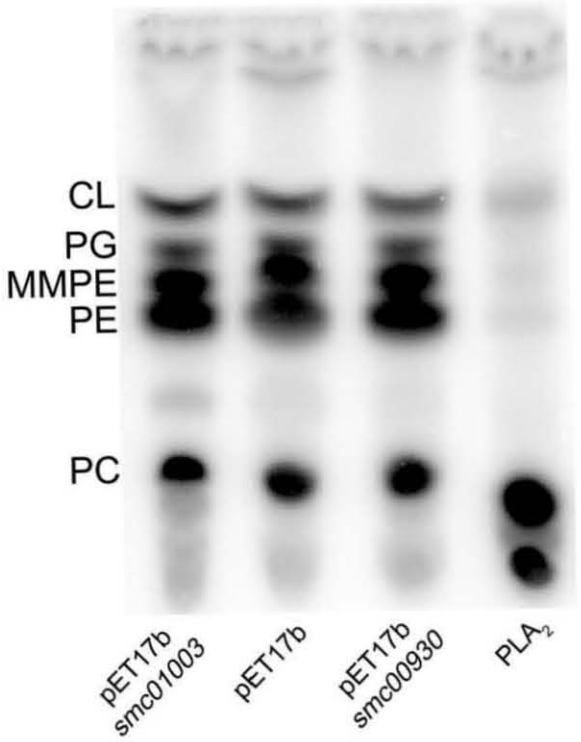

Fig. S2. Membrane phospholipids from *S. meliloti* are not consumed by SMc01003 or SMc00930 under the conditions tested. Total ^{32}P-radiolabled phospholipids from *S. meliloti* were treated with cell-free extracts of *E. coli* BL21(DE3) x pLysS harboring the *smc01003*-expressing vector, the empty pET17b vector, the *smc00930*-expressing vector, or with phospholipase A_2 from *Crotalus adamanteus* (PLA_2). After 5 h incubation, lipids were extracted and separated by one-dimensional thin-layer chromatography using chloroform:methanol:acetic acid (130:50:20, v/v) as the mobile phase. CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; MMPE, monomethyl-PE; PC, phosphatidylcholine.

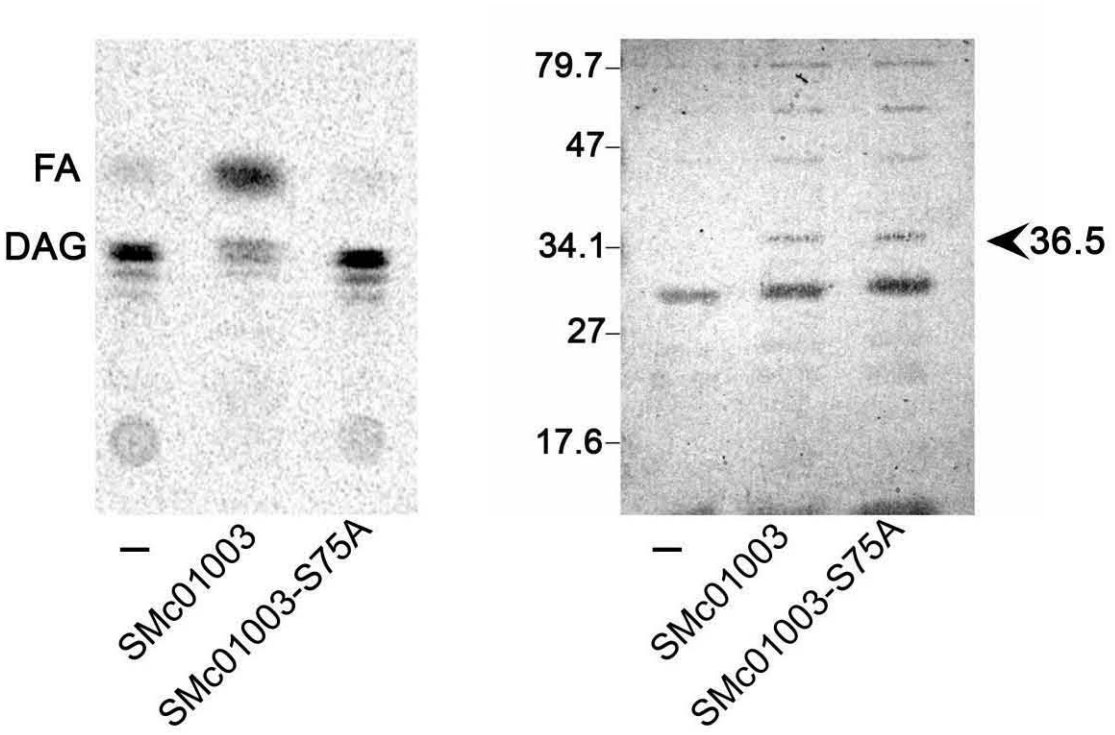

Fig. S3. Ser75 is required for the DAG lipase activity of SMc01003. Cell-free extracts from *E. coli* BL21(DE3) × pLysS harboring the empty vector pET28a (-) or had expressed His-tagged SMc01003, or the His-tagged site-directed mutant version SMc01003-S75A were incubated with ^{14}C-labeled diacylglycerol and after 24 h of incubation at 30 ^{o}C, the lipidic fractions were separated by one-dimensional thin-layer chromatography using hexane:diethylether:acetic acid (70:30:4, v/v)(left panel). Cell-free extracts of the three strains were also analyzed for production of the His-tagged proteins using Western blot (right panel). Protein extracts were separated by polyacrylamide (12%) gel electrophoresis in the presence of sodium dodecylsulfate and after blotting the electrophoretogram to a membrane, Western blot detection was performed using anti-His as primary antibody similarly as described (Solís-Oviedo *et al.*, 2012). Bovine serum albumin (79.7 kD), ovalbumin, (43 kD), carbonic anhydrase 34.1 kD), soybean trypsin inhibitor (27 kD), and lysozyme (17.6 kD) were used as molecular weight markers. FA, fatty acids; DAG, diacylglycerol; MAG, monoacylglycerol.

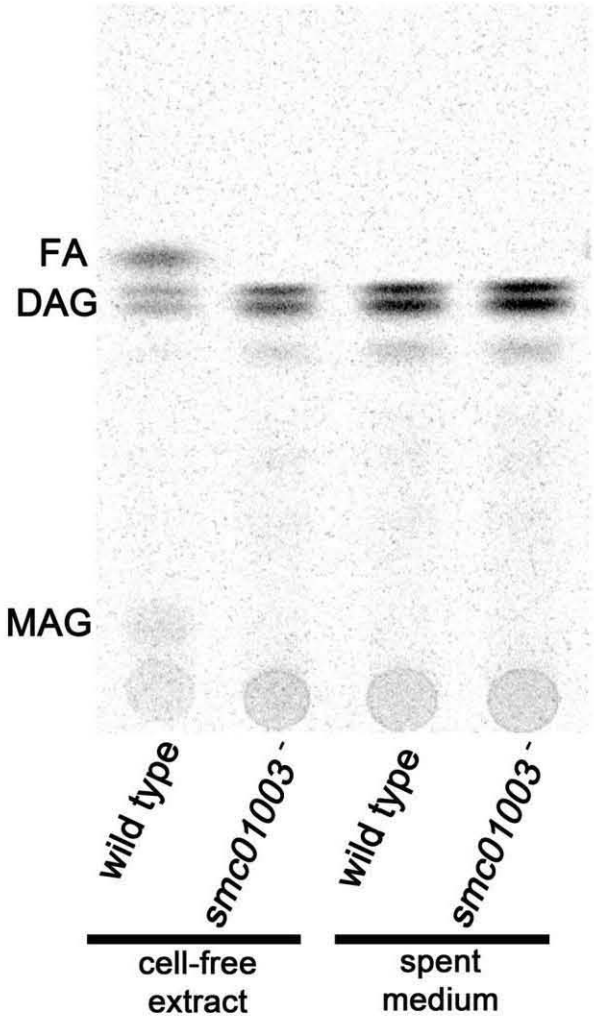

Fig. S4. DAG lipase SMc01003 is associated with *S. meliloti* cells. Cultures of *S. meliloti* wild type or the *smc01003*-deficient mutant were cultivated on TY medium until bacterial suspensions reached an OD_{620} = 1.2. Cells were harvested by centrifugation, resuspended in buffer, and cell-free extracts were prepared as described in Experimental Procedures. Spent cell-free supernatants were concentrated about 100-fold in Amicon Ultra centrifugal filters (30K) in order to be able to use protein preparations obtained from the same initial volume of bacterial suspension as the cell-free bacterial extracts. Cell-free extracts (100 µg protein per assay and derived from about 1 ml of initial cell suspension) and concentrated spent supernatants (derived from about 1 ml spent supernatant) of *S. meliloti* wild type or the *smc01003*-deficient mutant were incubated with ^{14}C-labeled diacylglycerol for 24 h at 30 ^{o}C. Lipidic fractions were extracted and separated by one-dimensional thin-layer chromatography using hexane:diethylether:acetic acid (70:30:4, v/v). FA, fatty acids; DAG, diacylglycerol; MAG, monoacylglycerol.

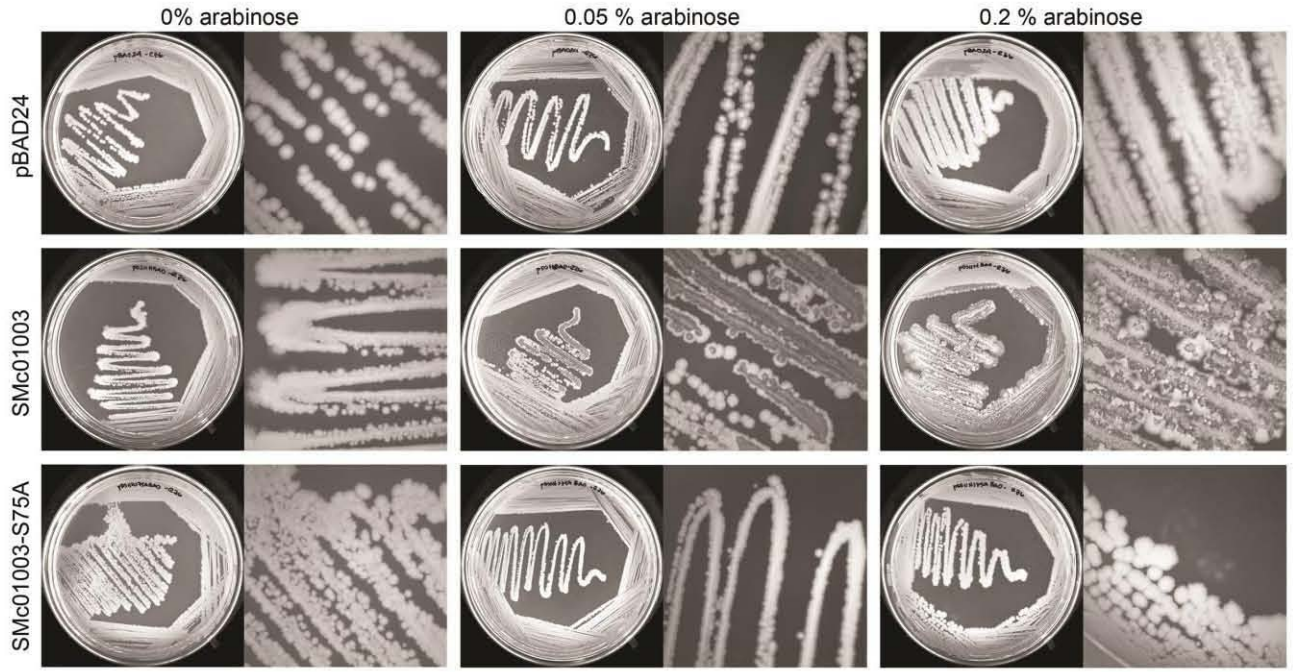

Fig. S5. Lysis phenotype in bacterial colonies of *E. coli* is due to SMc01003 enzymatic activity.

Growth of bacterial colonies on LB solid media of *E. coli* RZ6 carrying the empty vector (pBAD24) or the SMc01003-expressing plasmid (pDS11HBAD), or the plasmid (pDS11H-S75ABAD) expressing the site-directed mutant version SMc01003-S75A in the absence (0%) or presence of arabinose (0.05% or 0.2%) at 37^{o}C for 36 h.

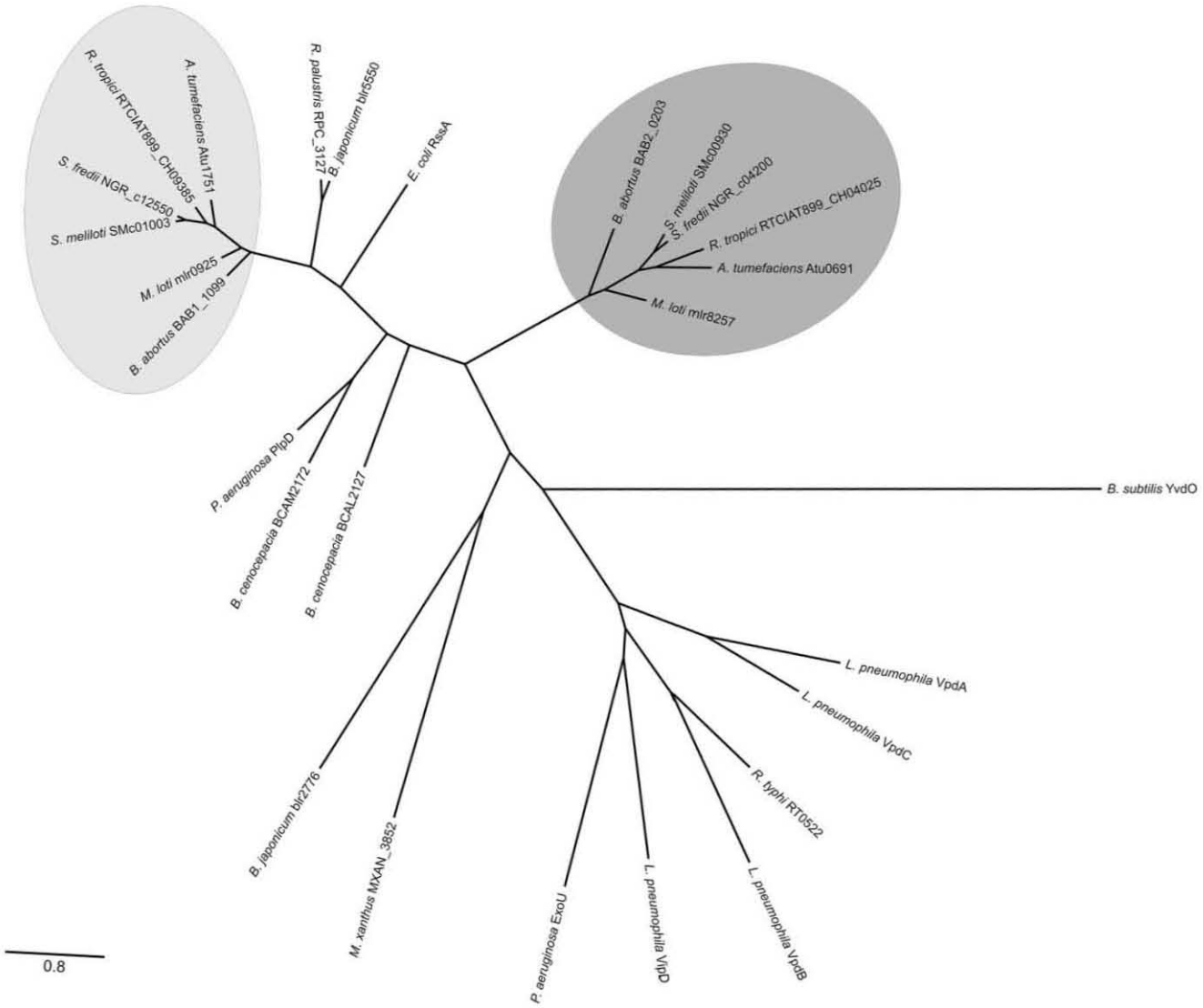

Fig. S6. Phylogenetic tree of characterized patatin-like bacterial phospholipases (PLP) and homologs to SMc00930 and SMc01003. For the construction of the tree (see Experimental Procedures) the following patatin-like proteins were selected: ORFs from *Agrobacterium tumefaciens* str. C58 (Atu1751: NP_354736; Atu0691: NP_353715), YvdO from *Bacillus subtilis* subsp. *subtilis* str. 168 (NP_391333), ORFs from *Bradyrhizobium japonicum* USDA 110 (blr5550: NP_772190; blr2776: NP_769416), ORFs from *Brucella abortus* 2308 (BAB1_1099: YP_414495; BAB2_0203: YP_418429), ORFs from *Burkholderia cenocepacia* J2315 (BCAL2127: YP_002231254; BCAM2172: YP_002234772), RssA from *Escherichia coli* K-12 (P0AFR0), PLPs from *Legionella pneumophila* subsp. *pneumophila* str. Philadelphia 1(VipD: YP_096826; VpdB: YP_095258; VpdA: YP_096418; VpdC: YP_095455), ORFs from *Mesorhizobium loti* MAFF303099 (mlr0925: NP_102623; mlr8257: NP_1083920), a PLP from *Myxococcus xanthus* DK 1622 (MXAN_3852: YP_632034), ExoU from *Pseudomonas aeruginosa* PA103 (AAC16023), PlpD from *Pseudomonas aeruginosa* PAO1 (NP_252029), ORFs from *Sinorhizobium fredii* NGR234 (NGR_c04200: YP_002824969; NGR_c12550: YP_002825789), ORFs from *Rhizobium tropici* CIAT 899 (RTCIAT899_CH09385: YP_007333809; RTCIAT899_CH04025: YP_007332764), an ORF from *Rhodopseudomonas palustris* BisB18 (RPC_3127: YP_532988), a PLP from *Rickettsia typhi* str. Wilmington (RT_0522: YP_067473), SMc01003 and SMc00930 from *Sinorhizobium meliloti* 1021 (SMc01003: NP_385524; SMc00930: NP_384909). Likely orthologs to SMc01003 or SMc00930 are highlighted in light or dark grey, respectively. Bootstrap support values for these clades are higher than 90% and aLRT

statistic is above 0.99. Distances between sequences are expressed as 0.8 changes per amino acid residue.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Phylogenetic analyses of protein sequences for tree construction

Sequences of putative patatin-like phospholipases were retrieved with PSI-BLAST (Schäffer *et al.*, 2001) using SMc00930 and SMc01003 as query. Other bacterial lipases with patatin-like domains whose activities had been experimentally studied (see Fig. 1) were also included in the multiple sequence alignment. The alignment was performed with MAFFT (Katok and Toh, 2008) in the http://www.ebi.ac.uk/Tools server (Goujon *et al.*, 2010), using the BLOSUM62 substitution matrix, a gap open penalty of 2.2 and a gap extension penalty of 0.05. The multiple sequence alignment was then edited with Jalview (Waterhouse *et al.*, 2009), where the presence of the four conserved blocks (Fig.1) in the protein sequences was confirmed. The phylogenetic tree was constructed with PhyML 3.0 (Guindon *et al.*, 2010), using the LG amino acid substitution model, four substitution rate categories, a BIONJ starting tree, both NNI and SPR options for tree improvement, a proportion of invariable sites of 0.02, optimizing the topology and branch lengths, and estimating the Gamma shape parameter. To compute branch support, 100 bootstrap replicas were performed and, in addition, the approximate Likelihood-Ratio Test (aLRT) statistic with the SH-like interpretation was calculated.

Table S1. Membrane lipid composition of *Escherichia coli* BL21(DE3) pLysS expressing *smc00930* (pDS10), *smc01003* (pDS11), or *smc04041* (pDS30) and *E. coli* BL21(DE3) x pLysS harboring the empty vector (pET17b) after 4 h of induction and ^{14}C-acetate labeling. The values shown are mean values ± SD derived from three independent experiments. FA, fatty acids; PG, phosphatidylglycerol; CL, cardiolipin; PE, phosphatidylethanolamine.

Lipid	Composition (% of total ^{14}C)			
	E. coli x pET17b	*E. coli* x pDS10	*E. coli* x pDS11	*E. coli* x pDS30
FA	5.1±0.9	17.6±1.9	17.3±1.5	8.2±0.3
PG	9.0±1.0	4.8±1.8	8.6±0.1	10.7±1.6
CL	1.3±0.4	2.7±1.0	4.6±2.6	5.1±1.8
PE	84.7±1.7	75.0±1.8	69.5±2.0	76.0±0.4

Table S2. Membrane lipid composition of *Sinorhizobium meliloti* 1021 wild type expressing *smc00930* (pDS12), *smc01003* (pDS13), or *smc04041* (pDS31) and *S. meliloti* 1021 harboring the empty vector (pNG28) after 4 h of ^{14}C-acetate labeling. The values shown are mean values ± SD derived from three independent experiments. FA, fatty acids; PG, phosphatidylglycerol; CL, cardiolipin; PE, phosphatidylethanolamine; MMPE, monomethyl-PE; DMPE, dimethyl-PE; PC, phosphatidylcholine.

Lipid	Composition (% of total ^{14}C)			
	S. meliloti x pNG28	*S. meliloti* x pDS12	*S. meliloti* x pDS13	*S. meliloti* x pDS31
FA	0.9±0.6	4.4±1.2	2.5±1.0	1.0±1.1
PG	25.7±1.1	30.1±1.6	30.5±1.2	24.3±2.1
CL	3.1±1.1	1.6±0.5	1.8±0.8	2.7±0.9
PE + MMPE	42.6±6.9	38.0±5.8	39.5±5.8	42.9±4.8
DMPE	6.6±2.3	3.3±1.1	4.7±1.1	5.7±0.9
PC	21.1±6.2	22.6±6.3	21.1±5.3	23.4±3.7

Table S3. SMc01003 and SMc00930 contribute to the formation of free fatty acids in *S. meliloti*. Membrane lipid composition of *S. meliloti* 1021 wild type, mutants deficient in SMc01003 (DXSC2), FadD (FadD2), SMc01003 and FadD (FDXSC2), double mutant FDXSC2 harboring the SMc01003-expressing plasmid pDS13, or the SMc00930-expressing plasmid pDS12, or the empty plasmid (pNG28). Labeling with ^{14}C-acetate was performed for 28 h, lipids were extracted, separated by 1D-TLC and quantified. The values shown are mean values ± SD derived from three independent experiments.

Lipid	Composition (% of total ^{14}C)	
	Free fatty acids	Polar membrane lipids
Wild type	0.4±0.2	99.6±0.2
DXSC2	0.2±0.1	99.8±0.2
FadD2	14.1±1.3	85.9±1.3
FDXSC2	3.0±0.4	97.0±0.4
FDXSC2x pDS12	14.7±1.9	85.3±1.9
FDXSC2x pDS13	15.6±1.2	84.4±1.2
FDXSC2x pNG28	7.6±0.2	92.4±0.2

Table S4. The predicted PLPs SMc01003 and SMc00930 from *S. meliloti* hydrolyze *p*-nitrophenylacyl substrates. Using a spectrophotometric assay, cell-free extracts of *E. coli* BL21(DE3) x pLysS, containing the empty pET17b vector, or the vector from which *smc00930*, or *smc01003* genes had been expressed, were assayed for their ability to hydrolyze *p*-nitrophenyl (*p*-NP) ester substrates of varying fatty acyl chain length (10, 12, 14, 16, or 18). Specific activities (µmol nitrophenol/mg protein x min) for different *p*-NP ester substrates are given. These values were obtained after subtracting minor activities present in the extract from *E. coli* BL21(DE3) x pLysS harboring the empty pET17b vector. The values shown are mean values ± standard deviation derived from three independent experiments.

Fatty acyl length in *p*-NP ester	10	12	14	16	18
SMc01003	27.1±2.1	18.4±2.0	6.9±0.7	14.3±1.5	12.2±0.9
SMc00930	1822±283	1828±283	2860±117	5505±394	3392±65

Table S5. Construction of knock-out mutants in potential lipase genes.

S. meliloti deficient-mutant	Mutant characteristics and oligonucleotide primers used for construction	Restriction site	PCR product (bp)
DXSC1	***smc04041* replaced by spectinomycin cassette**		
	primer pair for upstream region:		
	ATGTAGTCGACTAGCTAGCCGGTCACAGGC	SalI	
	AAAGGATCCGGCATCGAGTGAAGGAGC	BamHI	965
	primer pair for downstream region:		
	AAAGGATCCAGGCGATGGCCGCGATCCTG	BamHI	
	AAGAATTCGTCTATTCAGATCGAACC	EcoRI	901
DXSC2	***smc01003* deleted by joining downstream and upstream region**		
	primer pair for upstream region:		
	ACTGGAATTCGCGCATATGAACGCCGAAG	EcoRI	
	ACTGCCCGGGCGAAAGCCTCCAATTC	SmaI	947
	primer pair for downstream region:		
	ACTGCCCGGGCTATCAGGAAGCCAAGATC	SmaI	
	ACTGTCTAGAGCGGCACGGACTGAAACTC	XbaI	1028
DXSC3	***smc00930* replaced by kanamycin cassette**		
	primer pair for upstream region:		
	ACTGAAGCTTTGCCGGAGACCGGCCACGCGCGAG	HindIII	
	ACTGCCCGGGCAGGCCGAGATCACGCGGAATCTGAG	SmaI	986
	primer pair for downstream region:		
	AAACCCGGGACCAAGGACGAGGTG	SmaI	
	AACATCTAGATATCAATGCGCCATTCTGG	XbaI	844

For inactivation of a potential lipase-encoding gene, the upstream and downstream regions were PCR-amplified (XL-PCR kit; Applied Biosystems) with a specific pair of oligonucleotide primers from genomic DNA of *S. meliloti*, introducing restriction sites (underlined). After subsequent cloning of upstream and downstream region into pUC18 (Yanisch-Perron *et al.*, 1985){or pBBR1MCS-5 (Kovach *et al.*, 1995) in the case of *smc00930*}, an appropriate resistance-conferring cassette {or nothing in the case of *smc01003*} was cloned between upstream and downstream region. Either a spectinomycin resistance-conferring cassette was obtained as a BamHI fragment from pHY109 (Østerås *et al.*, 1998), or a kanamycin resistance-conferring cassette was obtained as a SmaI fragment from pTB3131 (Kretzschmar *et al.*, 2001). The regions usually flanking the sinorhizobial gene and the appropriate resistance-conferring cassette located in between those regions was cloned into the suicide vector pK18*mobsacB* (Schäfer *et al.*, 1994), the construct was introduced into wild type *S. meliloti* 1021, and double recombinants in which the appropriate resistance-conferring cassette had replaced the sinorhizobial gene were obtained following a procedure described previously (Sohlenkamp *et al*., 2004). Mutations in which the original sinorhizobial gene was replaced by a resistance-conferring cassette were confirmed by Southern hybridization.

Table S6. Oligonucleotides used for construction of different phospholipase/lysophospholipase expression plasmids or a site-directed mutant. Sites for recognition by restriction enzymes are underlined.

Primers Sequence	**(5´-3´)**
Primers for expression plasmids	
oLOP149	AGGAATACATATGCTGAACTGGACATTCACG
oLOP150	ACGGCTCGAGTCAACGGGACAGCGGTTC
oLOP151	AGGAATACATATGACGGAGGTGGCGATGG
oLOP152	AAAGGATCCTTATATCCCTTTCCTCCACCG
oLOP190	AGGAATACATATGACCACGCTCCTTCACTC
oLOP191	AAAGGATCCTTAAGCCTCTTCCAACTGTCG
Primers for site-directed mutagenesis	
t223g	ATGATTGCGGGCACAGCGATCGGTGCGC
t223g_antisense	GCGCACCGATCGCTGTGCCCGCAATCAT

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Lysophospholipase from *Sinorhizobium meliloti* affects nodule morphology during symbiosis with alfalfa

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Running title: lysophospholipase from *Sinorhizobium meliloti*

Six keywords: lysophospholipid, membrane turnover, lysophospholipase, fatty acid, multilobed nodule

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Summary

Phospholipids form membranes which separate the interior of a cell from the outside world. After their biosynthesis, membrane phospholipids can be subject to turnover, giving rise to the remodeling of membranes but also for lipid signaling. In stationary phase of growth, bacteria produce a considerable amount of free fatty acids which are usually activated to their coenzyme A thioester derivatives and subsequently degraded by β-oxidation. In *Sinorhizobium meliloti*, two predicted patatin-like phospholipases A (SMc00930 and SMc01003) contribute to the formation of free fatty acids. Surprisingly, neither SMc00930 nor SMc01003 can use phospholipids as substrates. Here we report that SMc00930 can degrade 1- or 2-lysophospholipids to a fatty acid and the glycerophosphoalcohol backbone. Specifically we show that SMc00930 can degrade 1- or 2-lysophosphatidylglycerol. A SMc00930-deficient mutant of *S. meliloti* provokes an unexpected phenotype upon symbiosis with its legume host plant alfalfa as an increase of multilobed nodules can be observed. One might speculate that in a SMc00930-deficient sinorhizobial mutant, a lysophospholipid might accumulate, which could act as signal for the generation of new meristems and therefore the establishment of the multilobed nodule structures.

Introduction

Bacteria have the ability to remodel their membrane phospholipids by removing parts of the molecule with the help of phospholipases or transferases. Such fragmented lipids can be either totally degraded or they can be recycled and function again as a membrane lipid. Although lipid cycles are better known from in eukaryotic membranes, some studies give evidence for lipid cycles in the model bacterium *Escherichia coli*. *E. coli* possesses the classic diacylglycerol (DAG) cycle and the lysophosphatidylethanolamine (LPE) cycle and the enzymes involved are well known (Rock, 2008). Degradation of membrane phospholipids often involves an outer membrane phospholipase A (OMPLA) yielding lysophospholipids (LPL), such as LPE, as products. (Nishijima *et al.,* 1977). LPLs can be further degraded in *E. coli* by the lysophospholipase L2 (PldB) to a fatty acid and the respective glycerophosphoalcohol. In other bacteria even LPLs are thought to have important functions. In *Yersinia pseudotuberculosis* LPE is increased when growing at elevated temperatures or in conditions were glucose is added to the growth medium (Barkholdina *et al.,* 2004). LPE is accumulated in the membranes of *Helicobacter pylori* by phase variation in the OMPLA-encoding *pldA* gene (Tannaes *et al.,* 2001) and its accumulation permits bacterial adaptation to the acidic environment. In addition, OMPLA mutants in *Y. pseudotuberculosis* and *H. pylori* are defective in the colonization of mice (Bos *et al.,* 2005; Karlyshev *et al.,* 2001) suggesting that the activity of OMPLA is related to pathogen infection due to adaptation to different environments. Phospholipases like OMPLA generate a wide variety of lipid products which are involved in control of cellular signaling, adaptation of the cell or as precursors of new molecules. Although the importance of these enzymes in bacterial pathogenicity is not questioned, much remains to be studied regarding the regulation of these enzymes and their physiological role in lipid turnover.

Sinorhizobium meliloti is a soil bacterium that can form a nitrogen-fixing root nodule symbiosis with legume host plants, such as alfalfa. The formation and remodeling of membrane lipids in *S. meliloti*

has been subject of intense studies and is understood to some extent (Geiger *et al*., 2013; Sohlenkamp and Geiger, 2015). *S. meliloti* must be able to adapt to different life styles and environments, such extreme as inside a legume nodule or in soil where nutrients usually are scarce, including limited availability of phosphorus sources.

When the symbiotic bacterium *S. meliloti,* is under phosphate-limiting conditions it degrades its own zwitterionic phospholipids phosphatidylethanolamine (PE) and phosphatidylcholine (PC) to phosphoalcohol and diacylglycerol (DAG) by the activity of the phospholipase C PlcP (Zavaleta-Pastor *et al.,* 2010). DAG is the lipid anchor for biosynthesis of the phosphorus-free membrane lipid diacylglyceryl-N,N,N-trimethylhomoserine (DGTS). In this case, membrane phospholipids provide the building block for a non-phosphorus-containing lipid, formed under phosphate limitation. When growing *S. meliloti* under low osmolarity conditions, anionic cyclic glucans are formed (Breedveld and Miller, 1995; Wang *et al.,* 1999) and as the second product DAG is formed. Recently we discovered a DAG lipase (DglA) activity that seems to be responsible for the removal of excessive DAG in *S. meliloti* (Sahonero-Canavesi *et al.,* 2015).

In this work, we show that the predicted patatin like-phospholipase SMc00930 uses lysophospholipids as substrates and contributes to the formation of free long-chain fatty acids. A SMc00930-deficient mutant of *S. meliloti* provokes an increase in multilobed nodule structures on its host plant alfalfa.

Results

SMc00930 contributes to the liberation of free long-chain fatty acids

A *fadD*-deficient mutant of *S. meliloti* accumulates free fatty acids in the stationary phase of growth which are probably derived from membrane lipids (Pech-Canul *et al.,* 2011). Of two predicted

patatin-like phospholipases (DglA and SMc00930), both contribute to the formation of free fatty acids in *E. coli* but also in its native host *S. meliloti* (Sahonero-Canavesi *et al.,* 2015) but the precise nature of the liberated fatty acids was not determined. In order to know the fatty acid species that are liberated by SMc00930, we overexpressed the *smc00930* gene in a *fadD*$^{-}$*, smc01003*$^{-}$ (FDXSC2) double mutant. The identification and quantification of the free fatty acid content by GC-MS showed an increase for most long-chain fatty acids by at least a factor 1.1 when SMc00930 had been expressed (Table 1). In the case of the most abundant *cis*-vaccenic acid (C18:1) there was 40% more of the free fatty acid when SMc00930 had been expressed (Table 1).

Lysophospholipids derived from S. meliloti membrane are consumed by smc00930 activity

As SMc00930 could not use phospholipids as substrates (Sahonero-Canavesi *et al.,* 2015), we studied whether lysophospholipids served as substrates for SMc00930. For that purpose, we prepared ^{32}P-labeled 1-lysophospholipids (1-LPL)(obtained by treatment with phospholipase A_1) or 2-lysophospholipids (2-LPL)(obtained by treatment with phospholipase A_2) which were then incubated with cell-free extracts of *E. coli* BL21(DE3) in which SMc00930 had been expressed from pDS10 or with cell-free extracts of *E. coli* BL21(DE3) harboring the empty vector pET17b. When total 1- and 2-LPL were incubated with a cell-free extract of *E. coli* in which SMc00930 had been expressed, nearly all the LPL compounds disappeared (Figure 1, D and E) which was not the case when LPLs were incubated with an *E. coli* extract devoid of SMc00930. Analysis of the aqueous phases after extraction, indicated the much of the radioactivity lost from the lysophospholipid-containing butanol phase is encountered as increases of radioactivity in the aqueous phases.

Quantification of the aqueous phases showed that when 1-LPL or 2-LPL were incubated with cell-free extracts of *E. coli* in which SMc00930 had been expressed, ^{32}P-labeled water-soluble

compounds were increased about three times when compared to an incubation with cell-free extracts of *E. coli* harboring the empty vector (Table 2). The resulting water-soluble products are probably distinct glycerophosphoalcohol (GPA) molecules which were developed by paper chromatography (Figure 1 C and 1F) and show a clear increase in ^{32}P-containing molecules after SMc00930 treatment. The precise structure of the ^{32}P-containing water-soluble products is not known to date.

SMc00930 is not specific for 1- or 2-lysophospholipids isomers

As the mixture of 1- and 2-LPL were degraded by SMc00930, we studied whether any of the individual LPL isomers were preferably consumed by the SMc00930 lysophospholipase. Individual isomers of lysophosphatidylglycerol (LPG) were incubated with cell-free extract of *E. coli* in which SMc00930 has been overexpressed or which harbored the empty pET17b vector. Both, 1- and 2-LPG isomers were similarly degraded by SMc00930 (Figure 2). This result suggests that there is no specificity for any of the LPG isomers.

Alfalfa plant nodules formed after smc00930-mutant inoculation dramatically change in their morphology

As reported previously, mutants of *S. meliloti* deficient in SMc00930, SMc01003, SMc04041, or wild type were able to form nitrogen-fixing root nodules with similar numbers of nodule meristems (Sahonero-Canavesi *et al.*, 2015). However, a closer inspection of nodule morphologies indicated that when plants were inoculated with the SMc00930-deficient mutant DXSC3 (75% single-lobed, 25% multilobed), multilobed nodules increased in relative abundance (Table 3) and the latter effect was even more pronounced when inoculated with the SMc00930- and SMc01003-deficient double mutant DXSC5 (57% single-lobed, 43% multilobed) was

employed (Table 3) when compared with the wild type (86% single-lobed, 13% multilobed). Histological inspections of longitudinal nodule sections showed that each lobe possesses its own apical meristem (Figure 3), suggesting that multilobed nodules are the result of generation of additional meristems after the initial onset of a nodule meristem formation. The multilobed phenotype, provoked by the SMc00930-deficient mutant DXSC3, seems to be repressed when SMc00930 is expressed *in trans* from plasmid pDS12 (83% single-lobed, 17% multilobed) which is not the case when the empty broad host range plasmid is present in DXSC3 (61% single-lobed, 39% multilobed)(Table 3). Also, in the SMc00930- and SMc01003-deficient double mutant DXSC5, the multilobed phenotype seems to be partially repressed when SMc00930 (57% single-lobed, 43% multilobed) or SMc01003 (67% single-lobed, 33% multilobed) are expressed *in trans* in comparison to DXSC5 haboring the empty pRK404 vector (48% single-lobed, 52% multilobed)(Table 3).

Discussion

SMc00930 is a lysophospholipase

Lysophospholipase L2 (PldB) from *E. coli* is located to the inner membrane, hydrolyzes 2-acyl-glycerophosphoethanolamine, and although it is among the best studied bacterial lysophopholipases (Rock, 2008), its precise physiological role remains unknown. In many pathogens encountered in aquatic environments, such as *Vibrio cholerae*, an outer membrane-associated lysophospholipase VolA can cleave exogenously provided lysophosphatidylcholine and use liberated fatty acids as carbon sources (Pride *et al.,* 2013). In our previous study we could show that SMc00930 uses *p*-nitrophenyl esters (C10-C18) efficiently as substrates (Sahonero-Canavesi et al. 2015), demonstrating the highest activities with palmitoyl-*p*-

nitrophenol. In the present work we now show that SMc00930 can act on mixtures of 1-lysophospholipids (generated by phospholipase A_1 treatment) or on mixtures of 2-lysophospholipids (generated by phospholipase A_2 treatment) as well as on purified 1-lysophosphatidylglycerol and 2-lysophosphatidylglycerol. Unfortunately, a precise determination of the substrate spectrum for SMc00930 has not been possible to date because cell-free extracts of *E. coli* harbor intrinsic lysophospholipase activities that interfere with kinetic determinations for SMc00930. Clearly, a purification of the SMc00930 enzyme should be achieved before substrate specificities are to be determined. Presently, the intracellular localization of SMc00930 is not clear either.

Role of lysophospholipids in bacteria

In bacteria, PE hydrolysis to LPE is frequently catalyzed by the outer membrane phospholipase A (OMPLA) PldA, which exists in the outer membrane of many Gram-negative bacteria, but not in *S. meliloti* and to date we do not know any phospholipase A from *S. meliloti*. PldA in *Helicobacter pylori* increases LPE levels and as a consequence virulence and survival at acidic pH are increased (Tannaes *et al.,* 2001).

LPC, which is a degradation product of PC by the activity of a phospholipase A_2, has been demonstrated to act as a signal in the arbuscular mycorrhizal symbiosis, by activating the phosphate transporter genes StPT3 and StPT4 (Drissner *et al.,* 2007). Furthermore, MtN5 a family of plant small proteins which are induced during the early phases of the symbiosis between *Medicago truncatula* and *S. meliloti* (Gamas *et al.,* 1996), binds LPC and is required for the successful establishment of the symbiosis (Pii *et al.,* 2009). In this context it is worth noting that SMc00930 is a lysophospholipase and in its absence as, for example, in a SMc00930-

deficient mutant, certain lysophospholipids might accumulate due to a lack of consumption by SMc00930 and act as signal in the Rhizobium-legume symbiosis.

Presence of multilobed structures in legume plants

Some legume plants can form nodule-like structures even in the absence of rhizobia (Truchet *et al.,* 1989; Blauenfeldt *et al.,* 1994). In alfalfa plants, these axenically formed structures are often multilobed empty nodules but none of them is able to fix atmospheric nitrogen (Truchet *et al.,* 1989). Similar phenotypes can be observed with other legumes, such as *M. truncatula* (Fox *et al.,* 2011), *Trifolium repens* (Blauenfeldt *et al.,* 1994), *Crotalaria* sp (Sy *et al.,* 2001), and *Sesbania rostrata* (Mathis *et al.,* 2005). Therefore, this multilobed morphology occurs naturally on legume plants, and seems to be to some extent time-dependent. Also, other factors might affect how many lobes are encountered per base where the nodule originates from the root. For example, during a co-inoculation experiment of *Pseudomonas fluorescens* WSM3457 and *Ensifer* (*Sinorhizobium) medicae* WSM419, *M. truncatula* plants formed nodules with multiple meristems and developed into cluster-like multilobed nodules, structures not seen to this extent when plants were inoculated with WSM419 only. This co-inoculation enhanced symbiotic effectiveness of *M. truncatula* especially at low inoculums of *E. medicae* WSM419 (Fox *et al.,* 2011).

Bacterial surface composition affects nodule colonization and morphology

S. melitoti mutants, that are unable to synthesize exopolysaccharide EPS I, can form pseudonodule structures, however, such mutants can usually not colonize the nodules (Niehaus *et al.,* 1993). However, after prolonged incubation with EPS I-deficient *S. meliloti* mutants, delayed

infections by the mutants might occur with colonized lobes of the pseudonodules, but with an abnormal morphology. These nodule structures reported by Niehaus *et al.* (Niehaus *et al.,* 1993) resemble the nodule morphology observed by us here for the lysophospholipase (SMc00930)- or lysophospholipase (SMc00930)- and DglA lipase deficient mutants (Figure 3, B and C). Also, a mutant of *Azorhizobium caulinodans* (ORS571-oac2), affected in its surface polysaccharides, shows a defective interaction with its host *S. rostrata* by inducing structures with retarded development and continued generation of new infection centers, leading to multilobed ineffective nodules (Mathis *et al.,* 2005). Based on these findings, we propose that lysophospholipids might act as signals or mediators at the plant-bacterium interface, contributing to a successful symbiosis.

Experimental Procedures

Bacterial strains, plasmids, and growth conditions

Sinorhizobium meliloti strains were grown either in complex tryptone/yeast extract (TY) medium that contained 4.5 mM $CaCl_2$ (Beringer, 1974) or in minimal medium (Sherwood, 1970) with succinate (8.3 mM) replacing mannitol as the carbon source at 30°C on a gyratory shaker. The bacterial strains and plasmids used and their relevant characteristics are shown in Table 4. *E. coli* strains were cultured on Luria-Bertani (LB) medium (Miller, 1972) at 37°C or at 30°C when the SMc00930 protein was expressed. Antibiotics were added to media in the following concentrations (µg/ml) when required: spectinomycin 400, gentamicin 70, nalidixic acid 40, tetracycline 10, neomycin 200, chloramphenicol 80, in the case of *S. meliloti*, and spectinomycin 200, carbenicillin 100, tetracycline 20, gentamicin 10, kanamycin 50, chloramphenicol 20 in the case of *E. coli*. Plasmids were mobilized into *S. meliloti* strains by diparental mating using the *E. coli* S17-1 donor

strain as described previously (Simon *et al.*, 1983). Double mutant DXSC5 was constructed by general transduction (Finan *et al.,* 1984).

Preparation of [^{32}P]-labeled lysophospholipids

^{32}P-labeled phospholipids from *S. meliloti* 1021 were prepared as previously described (Sahonero-Canavesi *et al.*, 2015). For phospholipase treatments, either total lipid extracts (3 x 10^6 cpm, respectively) or individual phospholipid classes (PC, PE, PG), that had been obtained by thin-layer chromatographic separation, were used. The respective lipids were dissolved in $CHCl_3$:MeOH (1:1, v/v) and dried down under a stream of nitrogen in the presence of Triton X-100. Treatment with phospholipase A_1 from *Mucor javanicus* (100 units) was performed in 50 mM boric acid-borax buffer, pH6.0 in the presence of 0.8% Triton X-100 in 100 µl for 2 h at 37°C. Treatment with phospholipase A_2 from *Crotalus adamanteus* (1 unit) was performed in 100 mM Tris-HCl buffer, pH7.0 in the presence of 2 mM $CaCl_2$ and 0.2% Triton X-100 in 100 µl for 15 min at 37°C. At the end of the incubation, the enzyme was inactivated for 10 min at 100°C. In each case, lysophospholipids were extracted by adding 200 µl of water and 150 µl of n-butanol. After thorough mixing, samples were centrifuged for 5 min at 13000 rpm in an Eppendorf centrifuge and the upper organic butanol phases were transferred to new tubes and dried under vacuum similarly as described previously (Bjerve *et al.,* 1974). After the phospholipase A treatments, mixtures of 1-lysophospholipids (1.16 x 10^6 cpm) or of 2-lysophospholipids (1.23 x 10^6 cpm) were obtained.

Preparation of cell-free extracts for analysis of the SMc00930 lysophospholipase

To examine the lipolytic activity of *smc00930*, the cloned gene was expressed in *E. coli.* Liquid cultures (1 liter) of exponentially growing *E. coli* BL21(DE3) x pLysS, harboring pET17b

derivatives were induced with 0.1 mM isopropyl-β-D-thiogalactoside at a cell density of 4 x 10^8 cells ml^{-1} and incubated for 4 h at 30°C. After harvesting cells at 4°C, each cell pellet was resuspended in 10 ml of 50 mM Tris-HCl buffer, pH 8.5. Cell suspensions were passed three times through a cold French pressure cell at 20,000 lb in^{-2}. Unbroken cells and cell debris were removed by centrifugation at 4,000 × *g* for 10 min at 4°C to obtain cell-free extracts as supernatants. Protein concentrations were determined as described previously (Dulley and Grieve, 1975).

When cell-free extracts were incubated with radiolabeled lipids, the standard enzymatic assay (100 µl) contained 250,000 cpm ^{32}P-labeled of a lysophospholipid mixture or 5000 cpm of purified lyso-PGs in 50 mM Tris-HCl (pH 8.5), 150 mM NaCl, 0.2% Triton X-100, and the enzyme (50 µg protein). The reaction was initiated by the addition of the enzyme and after 4 h of incubation at 30^{o}C, 150 µl of butanol and 200 µl of water were added, and after extraction, the lipid-containing butanol fraction was separated in chloroform:methanol:ammonia (130:50:10; v/v) in the first dimension and chloroform: methanol:acetone:acetic acid (30:10:40:10:5; v/v) in second dimension as mobile system.

Determination of free fatty acids

For the quantification of free fatty acids, to a total lipid extract (previously dried under a stream of nitrogen), methanol (800 µl) was added together with 50 µl of 1-ethyl-3-(3-dimethylamino-propyl-carbodiimide) (100mg/ml in methanol). After 2 h of incubation at 22^{o}C, the reaction was stopped by adding 400 µl of saturated NaCl solution. The methyl esters were extracted twice with 1 ml of hexane each time followed by centrifugation. The upper hexane phases were pooled into a new glass vial and dried under a nitrogen stream. For quantitative analysis, fatty acid methyl esters were dissolved in 125 µl of hexane and 1 µl was used for analysis by gas chromatography. Fatty acid

species were identified using retention times and mass spectral information by comparison with the bacterial acid methyl esters mix standard (BAME 47080-U; Sigma-Aldrich).

Plant assays

Alfalfa (*Medicago sativa L.*) plants were grown hydroponically in a nitrogen-free medium, which contained about 0.7 mM phosphate. To test the infectivities of the rhizobial strains, bacterial cells were pregrown to stationary phase in complex TY medium containing 4.5 mM $CaCl_2$ at 30°C. Cells were diluted in sterile saline, and for each strain, 30 individual plants were inoculated with 10^5 cells. After inoculation, the number of nodulated plants and the number of nodules per plant were recorded every 3 days until no more changes in the total nodule numbers were observed. Plants were incubated in a plant growth chamber at 22°C using a 12-h/12-h day/night cycle.

Microscopic analysis of nodules

For the structural analysis of nodules, samples were collected from 6 old week roots. After fixation, they were dehydrated (5–100% ethanol series), pre-embedded in ethanol/histoclear mixtures (3:1, 1:1, and 1:3) and embedded in 100% parafin. The sample was then sliced in ultrathin sections (X nm) with a microtome (Leica, Wetzlar, Germany) and mounted on glass grids. They were then stained with 0.01% methylene blue and visualized by light microscopy.

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Tables

Table 1. SMc00930 liberates mainly long-chain fatty acids from *S. meliloti*.

Composition of free fatty acids of the FadD-, DglA-deficient double mutant of *S. meliloti* 1021 (FDXSC2) harboring the SMc00930-expressing plasmid (pDS12) or the empty plasmid (pNG28). The values shown are mean values derived from two independent experiments and values are given in relative abundance (area).

Fatty acid	**FDXSC2 x pNG28**	**FDXSC2 x pDS12**	**Fold**
C14:1	6,297,245	6,916,338	1.1 x
C14:0	2,393,447	2,584,617	1.1 x
C15:0	1,511,159	5,283,495	3.5 x
C16:1	10,732,685	14,202,629	1.3 x
C16:0	86,111,228	94,011,400	1.1 x
C17:0C	5,408,026	9,692,446	1.8 x
C17:0	3,445,722	3,947,540	1.1 x
C18:1	790,449,971	1,094,091,263	1.4 x
C18:0	56,783,026	65,879,582	1.2 x
C19:0C	67,412,158	82,590,260	1.2 x

Table 2. SMc00930 acts as a lysophospholipase. ^{32}P-labeled 1-lysophospholipids (obtained by treatment with phospholipase A_1) or 2-lysophospholipids (obtained by treatment with phospholipase A_2) (250000 cpm in each case) were treated with cell-free extracts from *E. coli* BL21(DE3) x pLysS, containing the empty pET17b vector or pDS10 from which *smc00930* had been expressed. Values are given in total cpm found in the aqueous phase at the end of the enzymatic assays.

Phospholipase Treatment	**pET17b**	**pET17b x pDS10**
A_1	58,240	194,720
A_2	59,280	171,280

Table 3. SMc00930 affects nodule morphology of alfalfa nodules. Alfalfa plants (30 for each strain) were inoculated with *S. meliloti* wild type, mutant DXSC3 (*smc00930*$^-$), double mutant DXSC5 (*smc00930*$^-$, *smc01003*$^-$), or mutants carrying the empty vector (pNG28) or plasmids that express *smc00930* (pDS12) or *smc01003* (pDS13). Meristems of single lobe (MSL) nodules (one meristem/base) were distinguished from meristems of multilobulated (MML) nodules (several meristems/base). Values are the means of three independent experiments ± standard deviations. The quotient MSL/MML is indicated.

Strain	**MSL**	**MML**	**MSL/MML**	**Total**
Wild type	147±26	23±3	6.4	170±30
smc00930$^-$	112±15	38±2	2.9	150±17
smc00930$^-$ *empty vector*	49±8	31±8	1.6	80±7
smc00930$^-$*, smc00930*	100±5	20±8	5.0	120±13
smc00930 ,smc01003$^-$	61±14	46±2	1.3	107±16
smc00930 ,smc01003$^-$ *empty vector*	56±8	60±46	0.93	116±41
smc00930 ,smc01003$^-$*smc00930*	67±15	49±46	1.4	116±38
smc00930 ,smc01003smc01003	76±15	37±28	2.1	113±42

Table 4. Bacterial strains and plasmids.

Strain or plasmid	**Relevant characteristics**[a]	**Reference**
Sinorhizobium meliloti		
S. meliloti 1021 our	wild type used throughout this study	López-Lara *et al.*, 2005
Sm 1021 derivatives		
DXSC3	*smc00930*::*km*	Sahonero-Canavesi *et al.*, 2015
DXSC5	*smc00930*::*km, smc01003*::*deletion*	This study
FadD2	*smc02162*::*km*	Sahonero-Canavesi *et al.*, 2015
FDXSC2	*smc02162*::*km*, *smc01003*::*deletion*	Sahonero-Canavesi *et al.*, 2015
E. coli		
DH5α	*rec*A1, ϕ80 *lac*ZΔM15, host for cloning	Hanahan, 1983
S17-1	*thi*, *pro*, *rec*A, hsdR, *hsd*M+, RP4Tc::Mu, Km::Tn7;Tp^R, Sm^R, Sp^R	Simon *et al.*, 1983
BL21(DE3)	expression strain	Studier *et al.*, 1990
Plasmids		
pET17b	expression vector, Cb^R	Novagen
pLysS	production of lysozyme for repression of T7 polymerase, Cm^R	Studier *et al.*, 1990
pRK404	broad host-range vector, Tc^R	Ditta *et al.*, 1985
pK18*mobsacB*	suicide vector, Km^R	Schäfer *et al.*, 1994
pTB3131	plasmid carrying Km^R cassette	Kretzschmar et al., 2001
pDS10	pET17b carrying *smc00930*	Sahonero-Canavesi *et al.*, 2015
pDS11	pET17b carrying *smc01003*	Sahonero-Canavesi *et al.*, 2015
pDS12	BamHI-restricted pDS10 in pRK404	Sahonero-Canavesi *et al.*, 2015
pDS13	BglII-restricted pDS11 in pRK404	Sahonero-Canavesi *et al.*, 2015
pNG28	BamHI-restricted pET17b in pRK404	Gonzalez-Silva *et al.*, 2011

[a]Tc^R, Tp^R, Km^R, Sp^R, Gm^R, Sm^R, Cb^R, Cm^R :tetracycline, trimethoprim, kanamycin, spectinomycin, gentamicin, streptomycin, carbenicillin, choramphenicol resistance, respectively.

Figures and Figure Legends

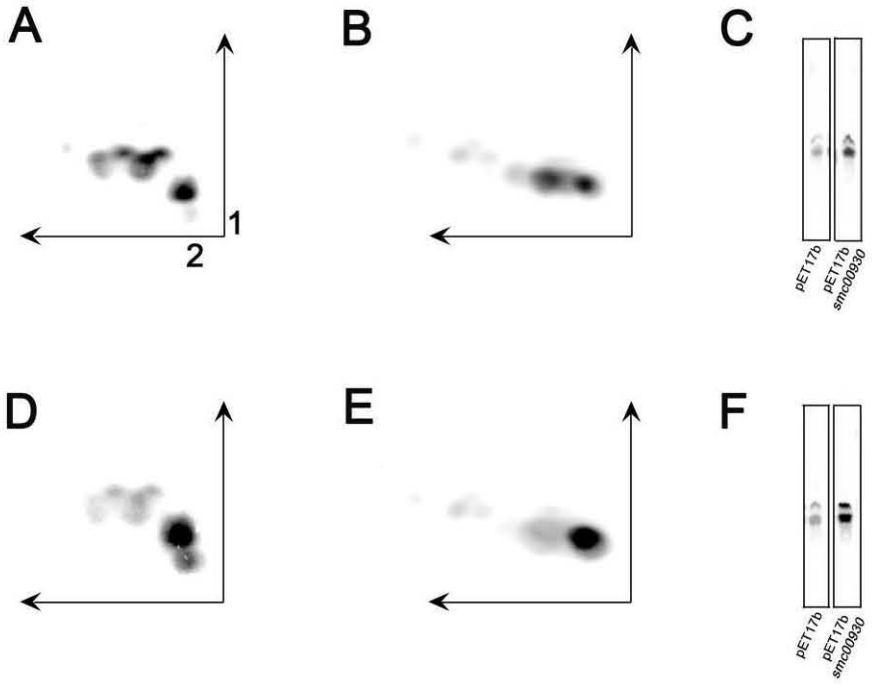

Figure 1. Mixtures of lysophospholipids from *S. meliloti* are consumed by SMc00930 activity. Total ^{32}P-radiolabled lysophospholipids, which had been obtained by phospholipase A_1 (A, B) or phospholipase A_2 (D, E) treatment, were incubated with cell-free extracts of *E. coli* BL21(DE3) x pLysS harboring the *smc00930*-expressing vector pDS10 (B, E) or the empty pET17b vector (A, D). After 4 h incubation, lipids were extracted and separated by two-dimensional thin-layer chromatography using chloroform:methanol:ammonia (in first dimension) and chloroform: methanol:acetone:acetic acid (in second dimension) as the mobile phase. The aqueous phases resulting of the same assays, were developed by paper chromatography using phenol:water (70:30; v/v) as the mobile phase (C, F). Water-soluble products after treatment of 1-lysophospholipids with cell-free extracts of *E. coli* BL21(DE3) x pLysS harboring the *smc00930*-expressing vector pDS10 or the empty pET17b vector (C), or after treatment of 2-lysophospholipids with cell-free extracts of *E. coli* BL21(DE3) x pLysS harboring the *smc00930*-expressing vector pDS10 or the empty pET17b vector (F).

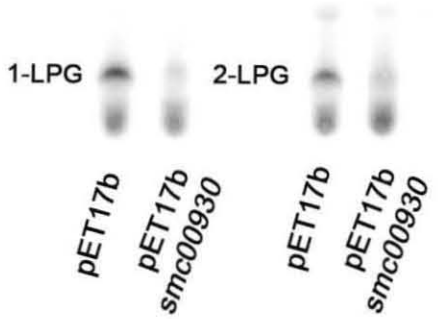

Figure 2. SMc00930 degrades 1-lysophosphatidylglycerol and 2-lysophosphatidylglycerol. ^{32}P-labeled 1-lysophosphatidylglycerol (1-LPG) or 2-lysophosphatidylglycerol (2-LPG), that had been obtained by phospholipase A_1 and A_2 treatment, respectively, were incubated with cell-free extracts of *E. coli* BL21(DE3) × pLysS expressing SMc00930 or containing the empty pET17b vector for 4 h, lipids were extracted, and separated by one-dimensional thin-layer chromatography using chloroform:methanol:acetone:acetic acid:water (30:10:40:10:5; v/v) as the mobile phase.

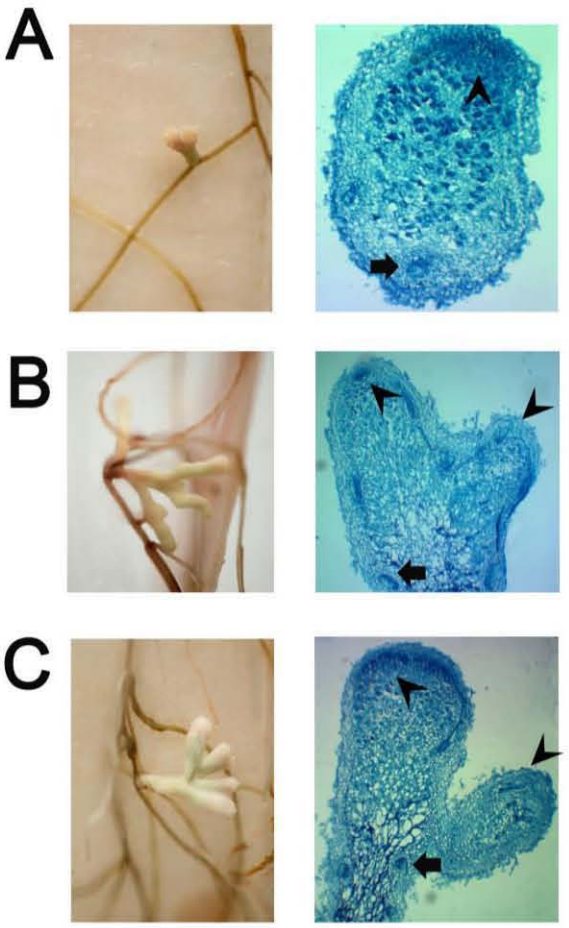

Figure 3. Symbiotic phenotypes of *Sinorhizobium meliloti* wild type and mutant strains on nodules of alfalfa plants. Single-lobulated nodule frequently found when inoculated with *S. meliloti* wild type strain 1021 (A, left panel) in contrast to multilobulated nodules found more frequently when inoculated with *S. meliloti* mutant DXSC3 (*smc00930*$^{-}$)(B, left panel) and with *S. meliloti* double mutantDXSC5 (*smc00930*$^{-}$, *smc01003*$^{-}$)(C, left panel). Longitudinal sections showing the meristematic zone (arrowhead), the vascular bundles (small arrows show histologies of 6 week old stained nodules (right panels) generated by wild type (A), mutant DXSC3 (*smc00930*$^{-}$)(B), or double mutant DXSC5 (*smc00930*$^{-}$, *smc01003*$^{-}$)(C).

7 Resultados adicionales

Con el fin de conocer la topología y una posible localización de SMc00930 y SMc01003 dentro de la célula bacteriana, se analizaron las secuencias de aminoácidos que componen ambas proteínas utilizando diversas herramientas bioinformáticas.

Adicionalmente, durante la caracterización bioquímica de SMc01003 mediante ensayos enzimáticos (Ver Sahonero-Canavesi *et al.,* 2015), se encontró actividad enzimática en los extractos libres de células correspondientes a la cepa que lleva el vector vacío. Este resultado indica que existen actividades similares en el fondo genético donde es sobre-expresada esta proteína. Con el fin de obtener resultados más claros respecto a su especificidad, se realizaron intentos de purificación de la proteína SMc01003 mediante cromatografía de afinidad a níquel y cromatografía de intercambio iónico.

7.1 Predicción de la topología de SMc00930 y SMc01003

En el análisis de la secuencia de aminoácidos de SMc00930, al utilizar el programa OCTOPUS (Viklund & Elofsson, 2008) se detectaron dos segmentos transmembranales (TM), mientras que con el programa DAS (Cserzö *et al.*, 1997) se detectan 3 segmentos TM, TMHMM2.0 (Krogh *et al.,* 2001) por contraparte, no predice ningún segmento TM para SMc00930. Al utilizar el programa TOPCONS (Tsirigos *et al.,* 2015), que realiza un consenso entre distintas herramientas de predicción de hélices transmembranales y péptido señal, predice un péptido señal en los primeros 27 aminoácidos de la proteína (Figura 8).

En el caso de SMc01003, el programa OCTOPUS, DAS y TMHMM2.0 sugieren dos segmentos TM, (Figura 9). Mientras que el programa TOPCONS durante el consenso predice que no existen hélices transmembranales. Este análisis sugiere que SMc01003 puede estar parcialmente asociado a la membrana.

El resultado de los análisis bioinformáticos sugirió distintas topologías para SMc00930, mientras que las predicciones para SMc01003 resultan más concluyentes respecto a su topología.

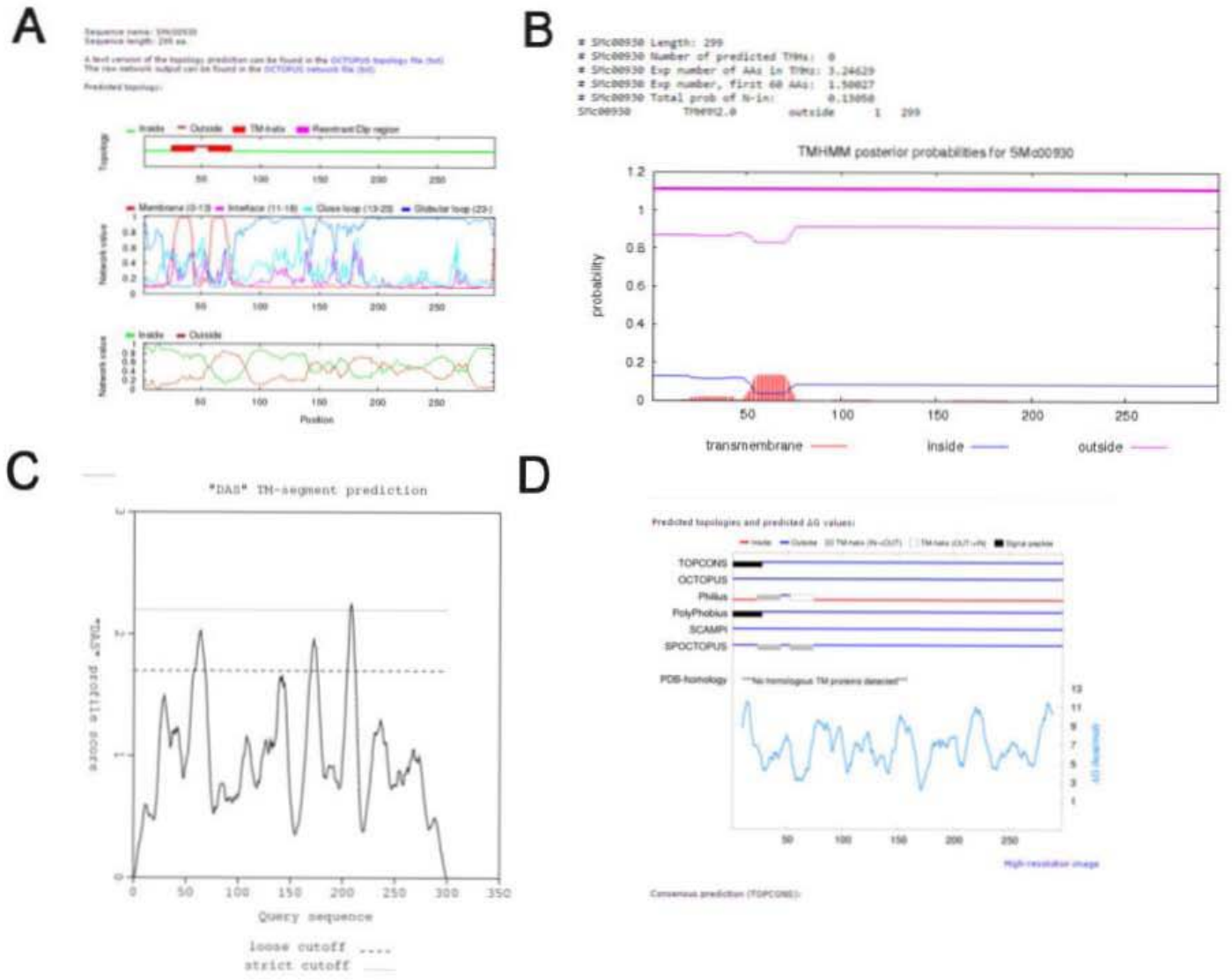

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Figura 8. Resultado de la predicción estructural para SMc00930 utilizando distintos programas bioinformáticos. Resultados obtenidos al utilizar **A)** OCTOPUS **B)** TMHMM 2.0 **C**) DAS **D)** TOPCONS.

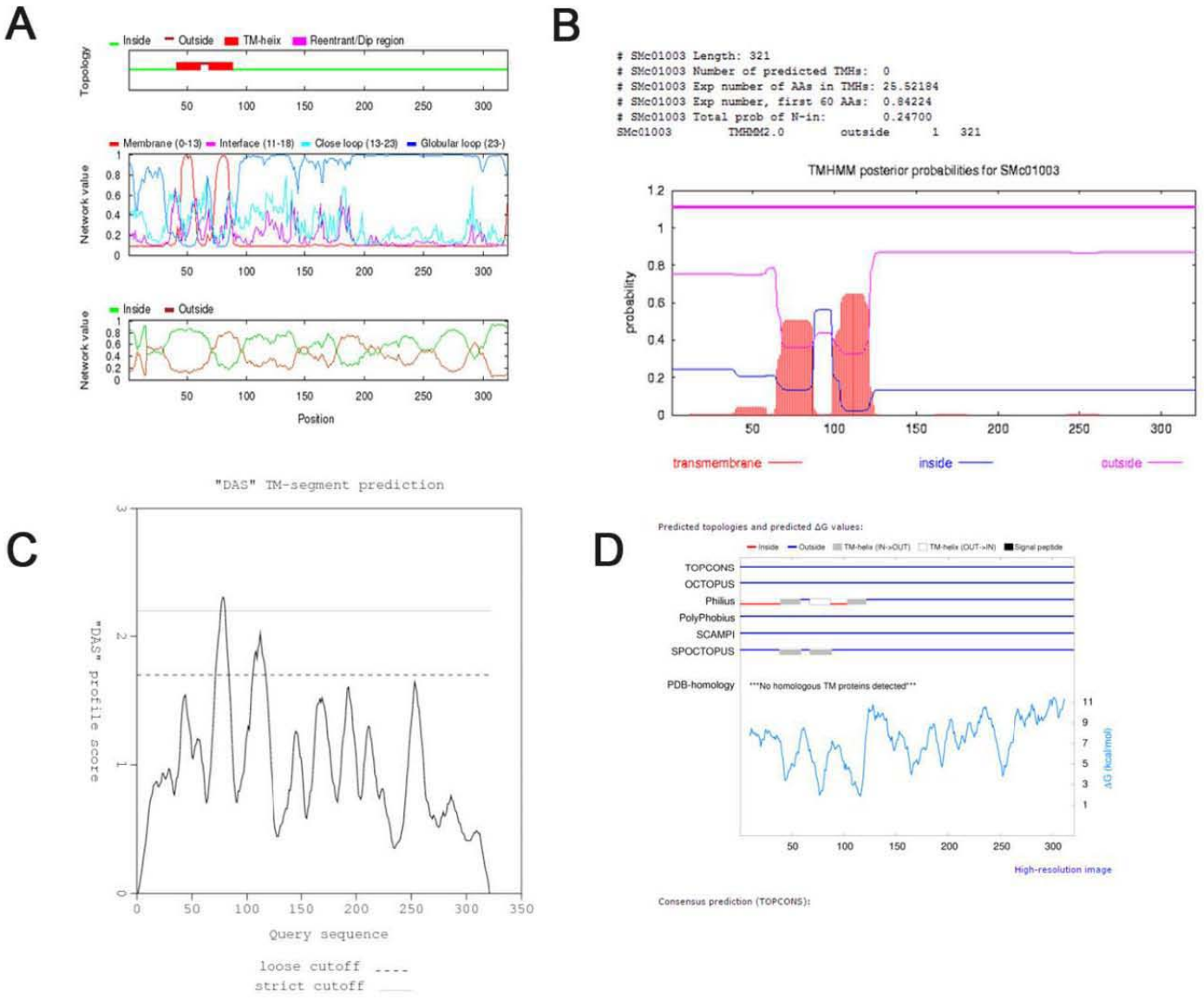

Figura 9. Resultado de la predicción estructural para SMC01003 utilizando distintos programas bioinformáticos. Resultados obtenidos al utilizar **A)** OCTOPUS **B)** TMHMM 2.0 **C)** DAS **D)** TOPCONS.

7.2 Intento de purificación de SMc01003 mediante una columna de afinidad al níquel resulta en la pérdida de la actividad enzimática.

Para realizar la purificación de SMc01003 mediante una columna de afinidad a níquel, se reclonó el gen *smc01003* a partir de pDS11 en el vector pET28Aque proporciona colas de histidina dando como producto el vector pDS11H (Tabla 1 en: Sahonero Canavesi *et al.,* 2015). A continuación, se realizó el crecimiento y la inducción de las cepas de *E. coli* BL21

(DE3) pLysS que llevan el vector vacío pET28A, como las que sobre-expresan a SMc01003. Cultivos líquidos (20 mL) de *E. coli* se indujeron con 0.1 mM IPTG durante la fase exponencial de crecimiento y se incubaron por 4 h a 30^{o}C. Después de centrifugar las células, las pastillas celulares fueron resuspendidas en la solución de lisis. Las suspensiones celulares se congelaron y descongelaron 3 veces para romper las células. Posteriormente, se centrifugaron a 12000 rpm por 10 min a 4^{o}C para obtener los extractos libres de células.

Una vez obtenidos los extractos libres de células, se realizó el protocolo de purificación en condiciones nativas (Ni-NTA 31314-QIAGEN):

Solución de lisis: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol ajustado a pH 8 con NaOH

Solución de lavado I: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazol ajustado a pH 8 con NaOH

Solución de lavado II: 50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazol ajustado a pH 8 con NaOH

Solución de elución: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazol ajustado a pH 8 con NaOH

El extracto libre de células (600 µl) se cargó en las columnas Ni-NTA previamente equilibradas con la solución de lisis (600 µl). A continuación se realizaron dos lavados independientes (600 µl c/u)(solución de lavado I y II) y finalmente dos pasos de elución (200 µl x 2) (solución de elución). Todos los pasos se realizaron mediante centrifugación (2 min a 2000 rpm) y se realizaron a 4^{o}C, se recuperaron las fracciones eluidas en todos los pasos mencionados y se guardaron para su posterior análisis.

La fracción eluida con 250 mM de imidazol que poseía la proteína purificada, se evaluó mediante un gel SDS-PAGE (Datos no mostrados) y se sometió a un ensayo donde no se detectó actividad enzimática en la fracción enriquecida al utilizar *p*-nitrofenil palmitato como sustrato. Los resultados sugieren que SMc01003 en presencia de 250 mM de imidazol ya no es funcional y como consecuencia se perdió la actividad.

Para comprobar si existe la posibilidad de recuperar la actividad de la proteína eliminando el imidazol de la fracción purificada de la proteína, se concentró y dializó el eluato que

poseía a SMc01003 respecto a una solución amortiguadora que no contenía imidazol y donde la proteína era normalmente activa (Tris-HCl 50 mM pH 8.5).

Una vez completado este paso, se realizó un ensayo enzimático donde nuevamente no se detectó ninguna actividad enzimática (Datos no mostrados). Este último resultado indica que la presencia del imidazol en una fracción enriquecida de SMc01003 afecta la funcionalidad de la proteína de manera irreversible.

Como una segunda estrategia para purificar la proteína sin presencia de imidazol se realizó intentos de purificación mediante cromatografía de intercambio iónico.

7.3 Purificación de SMc01003 mediante cromatografía de intercambio iónico en presencia de detergente.

El punto isoeléctrico calculado para SMc01003 es de 7.2. Además, distintas herramientas bioinformáticas predicen que posee al menos dos hélices transmembranales sugiriendo que podría encontrarse asociada a la membrana (Figura 9). Basado en esta información se realizó una cromatografía de intercambio aniónico utilizando 50 mM Tris-HCl pH 9 1 mM 3-[(3-colamidopropil) dimetilamonio]-1-propanosulfonato (CHAPS) como el buffer de equilibrio. Se utilizó el cambiador de aniones dietilaminoetil celulosa DE52 (Whatman) como la fase sólida en la columna. El extracto libre de células se obtuvo a partir de 1 L de cultivo de cepas de *E.* coli BL21 (DE3) pLysS que sobreexpresan el plásmido pDS11, así como las que llevan el plásmido vacío (Tabla 1 en Sahonero-Canavesi *et al.,* 2015). Durante la cromatografía se utilizó una velocidad de flujo de 12 ml/hr controlado mediante una bomba peristáltica P1 (Pharmacia Biotech) y una columna con 1 cm de diámetro de DE52 previamente acondicionada en la solución de equilibrio. Durante el procedimiento, se equilibró la columna con 40 ml de la solución de equilibrio, y después se pasaron 40 ml del extracto libre de células. A continuación, se realizaron dos lavados con la solución de equilibrio (40 ml x 2). Consecutivamente se eluyó la proteína utilizando 5 volúmenes (200 ml) de un gradiente lineal de 0 a 1.5 M de NaCl en la solución de equilibrio. Este último paso se realizó utilizando un mezclador de gradientes (GM200-Scientific Co). Todas las fracciones provenientes de la columna (extracto libre de células, lavados y elusión) se colectaron con la ayuda de un colector de fracciones (4 ml por fracción)(RediFrac-

Pharmacia Biotech) y todo el procedimiento se realizó a 4°C. Las fracciones obtenidas se analizaron mediante un ensayo enzimático en placa. El ensayo enzimático consiste en el cambio de coloración del substrato una vez que este ha sido hidrolizado. Las fracciones en las que se detectó un cambio en la coloración fueron consideradas para un posterior análisis individual. El análisis de la determinación de la actividad específica para las fracciones evaluadas (31-40 para el extracto del vector vacío y 39-43 para SMc01003)(Figura 10) demuestra que se recupera un 72% del total de la actividad de la proteína sometida al proceso de purificación, obteniendo un factor 5.6 de enriquecimiento. Además, el análisis de conductividad de estas mismas fracciones indica que el pico más alto de actividad para SMc01003 eluye aproximadamente entre 70 y 110 mM de NaCl mientras que el pico más alto de actividad detectado para el extracto libre de células del vector vacio eluye aproximadamente entre 35 y 70 mM de NaCl. Esto último indica que la actividad enzimática de SMc01003 purificada no coincide con la actividad de las lipasas o fosfolipasas intrínsecas de *E. coli*, sin embargo la fracción de actividad para el fondo genético coincide para las fracciones más altas en extractos libres de células de SMc01003. En consecuencia, aunque pudimos enriquecer DglA 5.6 veces, no pudimos separar la actividad de DglA por completo de las actividades intrínsecas de *E. coli.*

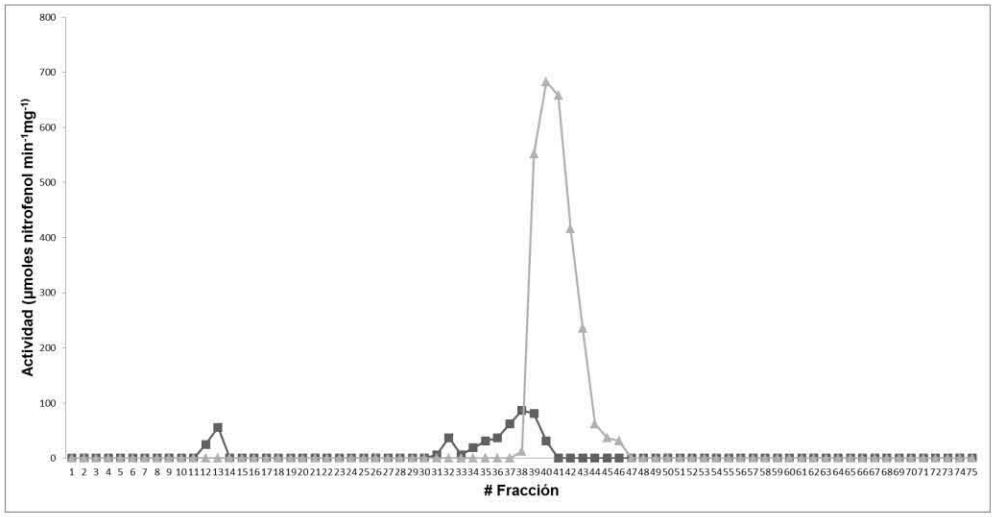

Figura 10. Resultado del análisis enzimático de las distintas fracciones resultantes del intercambio iónico Las fracciones provenientes de la cromatografía de intercambio iónico se colectaron y se analizaron mediante un ensayo enzimático donde se utilizó *p*-nitrofenil palmitato como sustrato. Las fracciones analizadas para los extractos del vector vacío (pET17b) se muestran en cuadrados negros y las fracciones provenientes para los extractos de SMc01003 en triángulos grises.

8 Discusión

Durante la fase estacionaria de crecimiento, la proteína FadD activa los ácidos grasos (AG) endógenos a sus derivados de CoA para así poder incorporarse al metabolismo de la bacteria. (Pech-Canul *et al.,* 2011). Los ácidos grasos corresponden a los usualmente asociados a los lípidos de membrana. Sorpresivamente, las actividades enzimáticas responsables de la liberación de estos ácidos grasos resultaron ser una diacilglicerol lipasa y una lisofosfolipasa. El estudio de las enzimas que degradan a los lípidos juega un papel importante en el entendimiento de los ciclos de lípidos y su contribución en la adaptación de las bacterias a distintas condiciones fisiológicas.

SMc01003 y SMc00930 están involucrados en la liberación de ácidos grasos a partir de la membrana bacteriana

El análisis de los ácidos grasos provenientes de una cepa deficiente en FadD demostró que la identidad de estos es similar a la usualmente encontrada asociada a los lípidos de membrana (Pech-Canul *et al.,* 2011). En una mutante doble en FadD y SMc01003, la cantidad de ácidos grasos disminuye a niveles usualmente encontrados en la cepa silvestre. Este resultado indica que la liberación de ácidos grasos es responsabilidad de la actividad de SMc01003. Adicionalmente, la acumulación de los ácidos grasos es restaurada al colocar una copia de SMc01003 o SMc00930 en *trans*. Aunque hasta el momento no se ha logrado identificar la función fisiológica de ambas lipasas, sugerimos que podrían estar involucradas en la adaptación de la bacteria durante la fase estacionaria de crecimiento.

Se sabe que la liberación de ácidos endógenos es un fenómeno conservado en organismos filogenéticamente distantes (Kaczmarzyk & Fulda, 2010). En *Pseudomonas aeruginosa,* mutantes deficientes en FadD se encuentran afectadas en la utilización de los ácidos grasos del hospedero como fuente de carbono y como consecuencia en su adaptación al entorno (Kang *et al.,* 2010). En contraste con *P. aeruginosa, S. meliloti* consume ácidos grasos derivados de la propia membrana bacteriana, sin embargo la utilización de ácidos grasos como fuente de carbono es un mecanismo de adaptación conservado. En general, cuando las células se encuentran en un entorno en el que las condiciones son variables y poco favorables respecto a los nutrientes, un fenómeno como este podría resultar importante (Kaczmarzyk & Fulda, 2010).

Diacilglicerol (DAG), una fuente de ácidos grasos en *S. meliloti*

La caracterización molecular de SMc01003 y SMc00930 demostró actividad de diacilglicerol lipasa y lisofosfolipasa respectivamente. Este resultado indica que tanto el DAG y los lisofosfolípidos (LPL) son formados transitoriamente en las células y estos posteriormente son consumidos por las actividades de SMc01003 y SMc00930 descritas durante este estudio.

En *E. coli* la principal fuente de DAG es fosfatidilglicerol (PG). PG puede donar glicerol-1-fosfato y transferirlo a los oligosacáridos derivados de la membrana (MDO) mediante MdoB. Posteriormente, una cinasa de DAG convierte este último en ácido fosfatídico (PA) y PA puede ingresar nuevamente a la biosíntesis *de novo* de los fosfolípidos de membrana (Rock, 2008). De manera similar en *S. meliloti* durante la formación de los glucanos cíclicos aniónicos, la transferencia del grupo fosfoglicerol a los glucanos cíclicos neutros da como resultado la formación de DAG (Wang *et al.*, 1999). En condiciones de limitación de fosfato, la bacteria *S. meliloti* degrada a PC mediante la actividad de PlcP y lo convierte en fosfocolina y DAG (Zavaleta-Pastor *et al.*, 2010). En este caso el DAG resultante puede servir como base para la formación de lípidos de membrana sin fósforo. El análisis de los lípidos neutros en una cepa deficiente de SMc01003 realizado en medio TY (baja osmolaridad), confirmó la acumulación transitoria de DAG en la mutante, indicando que en esta condición DAG se forma en la cepa silvestre pero es consumido por la actividad de SMc01003. Adicionalmente, aunque las condiciones fisiológicas estudiadas durante este trabajo no fueron limitantes respecto a la concentración de fosfato, posiblemente una de las funciones fisiológicas de SMc01003 podría consistir en degradar niveles excesivos de DAG durante la formación del mismo en cualquiera de las condiciones ya conocidas (Figura 11). Finalmente, no debemos descartar que existan actividades aún desconocidas que durante la fase estacionaria sean capaces de producir DAG.

El monoacilglicerol (MAG) genera lisis en células bacterianas

Durante la expresión de SMc01003 en distintas cepas de *E. coli,* se observaron fenotipos de lisis en las colonias durante la fase estacionaria. El análisis de las células que se

encontraban en el centro de las colonias demostró que las células se encontraban lisadas. El MAG y los ácidos grasos libres son los productos de la actividad de SMc01003. Algunas moléculas de MAG, han demostrado poseer una fuerte actividad antimicrobiana contra distintas especies del genero *Bacillus* (Nakayama *et al.*, 2015) y *Staphylococcus aureus* (Dolezalova *et al.*, 2010). Se sabe que la actividad de SMc01003 también puede degradar a MAG en glicerol y ácidos grasos libres. Además no existe evidencia directa de la actividad antimicrobiana del MAG sobre *E. coli*. Sin embargo, proponemos que el MAG formado por la actividad de SMc01003 podría generar inestabilidad en las membranas de las bacterias más antiguas de la colonia (las del centro) y así provocar su lisis. Este supuesto, será necesario probarlo directamente con MAG derivado de la degradación de DAG por SMc01003, así como con moléculas químicamente conocidas.

Los lisofosfolípidos (LPL) también son el origen de la liberación de ácidos grasos y pueden estar involucrados en la señalización planta-bacteria

La formación de LPL puede darse mediante la transferencia de una de las cadenas de ácidos grasos del fosfolípido, mediada por una aciltransferasa o por la hidrólisis de una de las cadenas de ácidos grasos por la actividad de una fosfolipasa A. En general, niveles elevados de LPL son conocidos por ser citolíticos y poseer propiedades que perturban la membrana (Weltzien, 1979). PldA, que se encuentra en la membrana externa de *Helicobacter pylori* incrementa los niveles de LPE y como consecuencia incrementa la virulencia y la sobrevivencia en pH ácido (Tannaes *et al.*, 2001). En *Yersinia pseudotuberculosis* los niveles de LPE aumentan por la actividad de OMPLA cuando crece en temperaturas elevadas o en condiciones donde se añade glucosa al medio (Barkholdina *et al.,* 2004). Adicionalmente, mutantes en OMPLA de *Y. pseudotuberculosis* son incapaces de colonizar ratones (Karlyshev *et al.,* 2001) sugiriendo que la actividad de OMPLA está relacionada con la infección de patógenos así como la adaptación a distintos ambientes. Nuestros resultados sugieren que SMc00930 es una lisofosfolipasa que libera ácidos grasos para ser consumidos por la propia bacteria. La lisofosfolipasa de *Vibrio cholerae* VolA y el transportador de ácidos grasos de cadena larga FadL se encuentran localizados en la membrana externa de la célula, y en conjunto están involucrados en la utilización de los ácidos grasos exógenos como fuente de carbono (Pride *et al.,* 2014). Las predicciones

obtenidas mediante distintos programas bioinformáticos para la topología de SMc00930 arrojan resultados poco consistentes entre ellos, por lo tanto no se pudo realizar un consenso respecto a su posible localización. Consideramos importante conocer la localización de SMc00930 mediante su análisis experimental, de esta manera poder entender la función de SMc00930.

Finalmente, durante la caracterización del perfil de lípidos membranales de la mutante deficiente en SMc00930, no encontramos diferencias en los niveles de LPL. Por lo tanto, no fue posible evaluar de qué manera afecta la presencia de LPL a la fisiología de *S. meliloti.* Probablemente los LPL no se estén acumulando en la mutante deficiente de SMc00930 ya que pueden existir otras posibles lisofosfolipasas aún desconocidas que se encarguen de consumir este compuesto. Además, la función molecular de SMc00930 aún se encuentra parcialmente esclarecida y será necesario realizar una caracterización más profunda donde se conozca estructuralmente las moléculas de LPL consumidas y formadas por esta actividad.

Como se mencionó anteriormente, la formación de LPL en las células bacterianas se da principalmente por la actividad de fosfolipasas A. En el genoma de *S. meliloti* no existe ningún ortólogo a OMPLA, una de las fosfolipasas más estudiadas en bacterias Gram negativas. En un estudio reciente se caracterizó una fosfolipasa del tipo A1 en *Brucella melitensis* que está involucrada con la resistencia a la polimixina (Kerrinnes *et al.,* 2015). En una búsqueda de posibles ortólogos a esta fosfolipasa (http://operons.ibt.unam.mx/gct3V1401/) se encontraron dos posible candidatos (anotados como SMc00613 y AcvB). Además, existe una base de datos que integra la información respecto a la secuencia, estructura y función de lipasas, esterasas y proteínas afín, donde se proponen al menos16 proteínas que puedan poseer alguna de estas funciones en *S. meliloti* (Fischer & Pleiss, 2003). Será de gran importancia determinar si alguna de estas candidatas tiene actividad de fosfolipasa A y generan LPL que son consumidos posteriormente por la actividad de SMc00930.

Durante la caracterización de una mutante deficiente en SMc00930 en simbiosis con la planta de alfalfa *Medicago sativa,* encontramos cambios en la morfología de los nódulos. Las plantas de alfalfa inoculadas con la mutante deficiente en SMc00930 presentaron un mayor número de nódulos multilobulados que la cepa silvestre. Anteriormente se había

demostrado que la lisofosfatidilcolina (LPC) actúa como una señal en la simbiosis de la micorriza con *Medicago truncatula*, activando genes transportadores de fosfato StPt3 y StPt4 (Drissner *et al.,* 2007). Aunado a esto, MtN5 una familia de proteínas que proviene de las plantas, es inducida durante fases tempranas de la simbiosis entre *M. truncatula* y *S. meliloti* (Gamas *et al.,* 1996). Además MtN5 posee la capacidad de unir LPC y es requerida para el establecimiento de una simbiosis funcional (Pii *et al.*, 2009). Tomando en cuenta todo lo anterior, será de gran importancia esclarecer si los LPL derivados de *S. meliloti* pueden afectar la nodulación, más específicamente la diferenciación de la morfología de los nódulos en la planta.

Lipasas bacterianas

Durante la realización de esta tesis, se logró estudiar un aspecto novedoso de las lipasas bacterianas. Aunque la presencia de lipasas es común en bacterias, en general el conocimiento de estas se extiende respecto a su aplicación biotecnológica (Jaeger *et al*., 1997)(Hasan *et al.,* 2006). Las lipasas pertenecen a diferentes familias de proteínas que no poseen similitud entre su secuencia, sin embargo poseen la misma arquitectura de plegamiento α/β hidrolasa (Nardinidi & Dijkstra, 1999). Además, dentro de su secuencia de aminoácidos, poseen la firma clásica de las lipasas G-X-S-X-G y una triada catalítica compuesta por Serina-Histidina-Aspartato (Jaeger *et al.,* 1994).

Dentro de los principales géneros de bacterias que producen lipasas se encuentran *Bacillus, Pseudomonas y Burkholderia.* (Gupta *et al.,* 2004). Sin embargo la distribución de las lipasas se encuentra ampliamente entre todos los organismos vivos. Aunque la mayoría de las lipasas mencionadas anteriormente han sido bien caracterizadas por su especificidad a distintos sustratos, no existe ninguna indicación de que estas puedan consumir lípidos intrínsecos bacterianos y de esta manera estar involucradas con procesos de reciclaje de lípidos.

Durante el estudio de SMc01003 no logramos obtener una fracción enriquecida de esta proteína donde la actividad residual de las lipasas propias de *E. coli* no coincida con la actividad estudiada durante este proyecto (Figura 10). Con el fin de estudiar la especificidad por la naturaleza de las moléculas lipídicas, será necesario realizar la

purificación tanto de SMc01003 como de SMc00930. El estudio de las enzimas que modifican a los lípidos permite ampliar el conocimiento respecto a los procesos de edición de los bloques formadores de membrana y así contribuir con el estudio de los ciclos de lípidos en bacterias.

9 Conclusiones

Durante el desarrollo de este trabajo, se identificaron 3 genes en el genoma de *S. meliloti* que codifican para posibles lipasas. Estos 3 genes fueron caracterizados durante la realización de esta tesis. El resultado de este trabajo permite concluir que:

1. La sobre-expresión de los 3 genes que codifican para posibles lipasas (SMc01003, SMc00930 y SMc04041) genera una acumulación de ácidos grasos libres en *E. coli* indicando que están implicados en la liberación de ácidos grasos.

2. La disminución en la cantidad de ácidos grasos libres, en una mutante deficiente de *fadD* y *smc01003*, confirma que *smc01003* está involucrada en la liberación de ácidos grasos en *S. meliloti.* Adicionalmente, este fenotipo es restaurado al colocar copias intactas de *smc01003* o de *smc00930*.

3. SMc01003 consume DAG *in vivo* y degrada especies conocidas de DAG *in vitro*. Adicionalmente, la actividad enzimática de SMc01003 se encuentra asociada a las células y degrada DAG derivado de la membrana de *S. meliloti.* SMc01003 codifica para una diacilglicerol lipasa intrínseca.

4. SMc00930 codifica para una lisofosfolipasa y posee actividad preferentemente sobre el sustrato artificial *p*-nitrofenil palmitato.

5. SMc00930 degrada lisofosfolípidos formados a partir de componentes membranales de *S. meliloti*. Se demostró que específicamente lisofosfatidilglicerol (LPG) puede ser consumido *in vitro*.

6. Mutantes deficientes de SMc00930 o dobles mutantes en SMc00930 y SMc01003, demostraron un cambio en la morfología de los nódulos formados durante la simbiosis con la planta de alfalfa.

Aunado a esto, contribuimos con el avance del conocimiento al respecto de los ciclos de lípidos en la bacteria *Sinorhizobium meliloti*, una propuesta de esto se encuentra detallada en la figura 11.

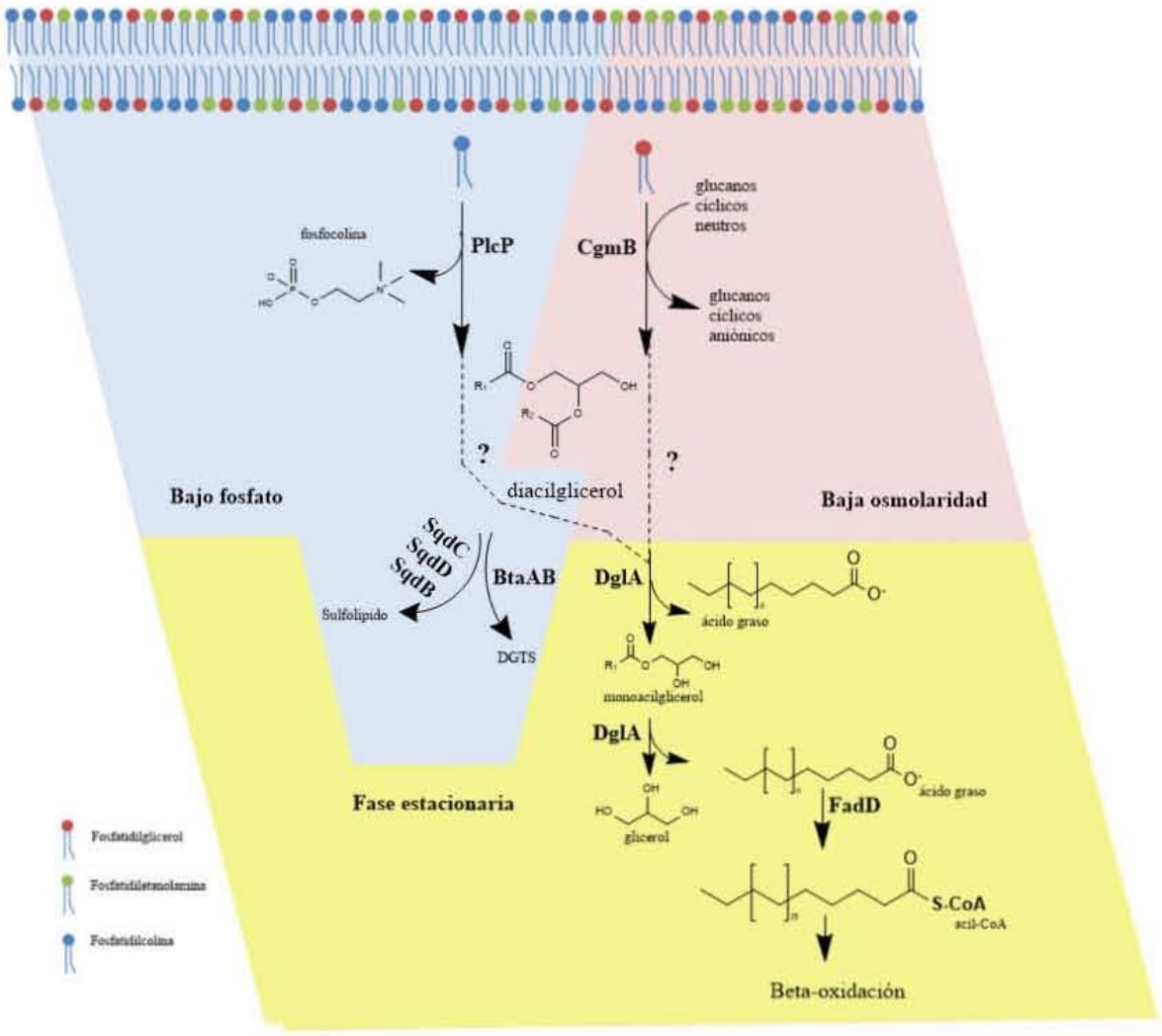

Figura 11. Modelo propuesto para la formación y degradación del DAG en *Sinorhizobium meliloti.* En condiciones de limitación de fosfato, la fosfolipasa C (PlcP) degrada fosfatidilcolina (PC) en fosfocolina y DAG. En Baja osmolaridad, CgmB transfiere el grupo fosfoglicerol de PG hacia los glucanos cíclicos. El DAG formado puede ser degradado por DglA y los ácidos grasos libres resultantes son consumidos por β-oxidación.

10 Perspectivas

1. Purificar SMc01003 y SMc00930 para caracterizar en detalle su especificidad por los sustratos.

2. Caracterizar la actividad enzimática de SMc01003 en más detalle utilizando sustratos con cadenas cortas y medianas de ácidos grasos (*p*-nitrofenil ésteres, DAG´s y MAG´s).

3. Caracterizar la actividad enzimática de SMc00930 utilizando sustratos conocidos de lisofosfolípidos.

4. Evaluar los lisofosfolípidos como posible moléculas señalizadoras en la simbiosis entre *S. meliloti* y la alfalfa.

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