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Diacylglycerol lipase from Sinorhizobium meliloti

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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.

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-

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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 $3 \times e^{-38}$ 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 (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 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) × 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) × 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). 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* × pET17b or with an extract of *E. coli* × 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 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) × 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) × 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) × 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) × 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 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) × 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) × pLysS × 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) × 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 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 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) × 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) × 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 × *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 1× 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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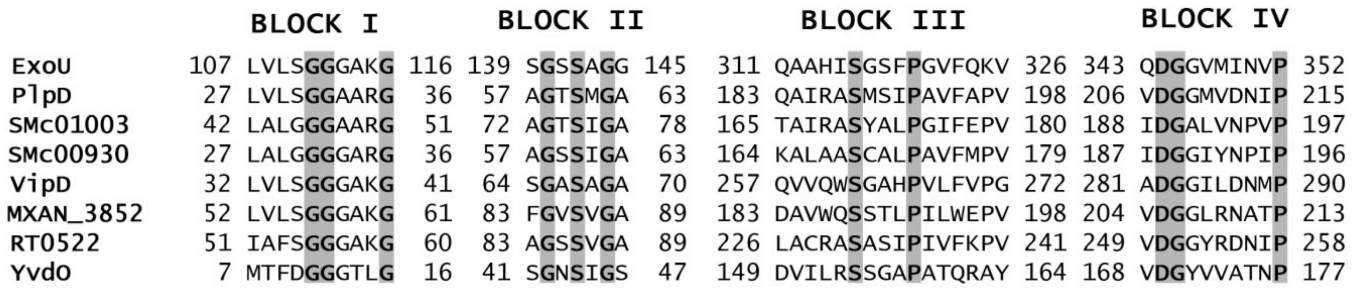

Fig. 1.

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*).

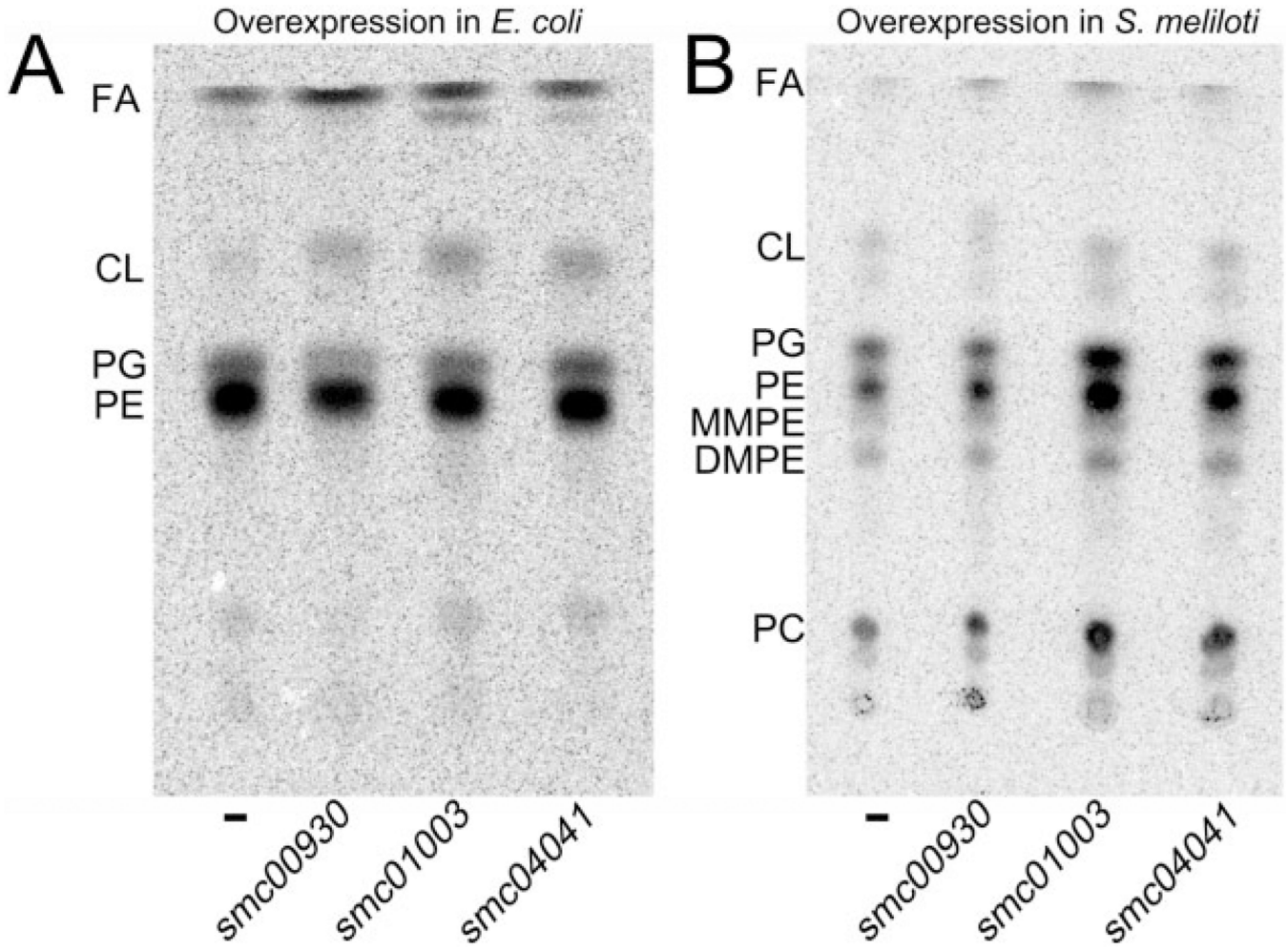

Fig. 2.

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) × 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.

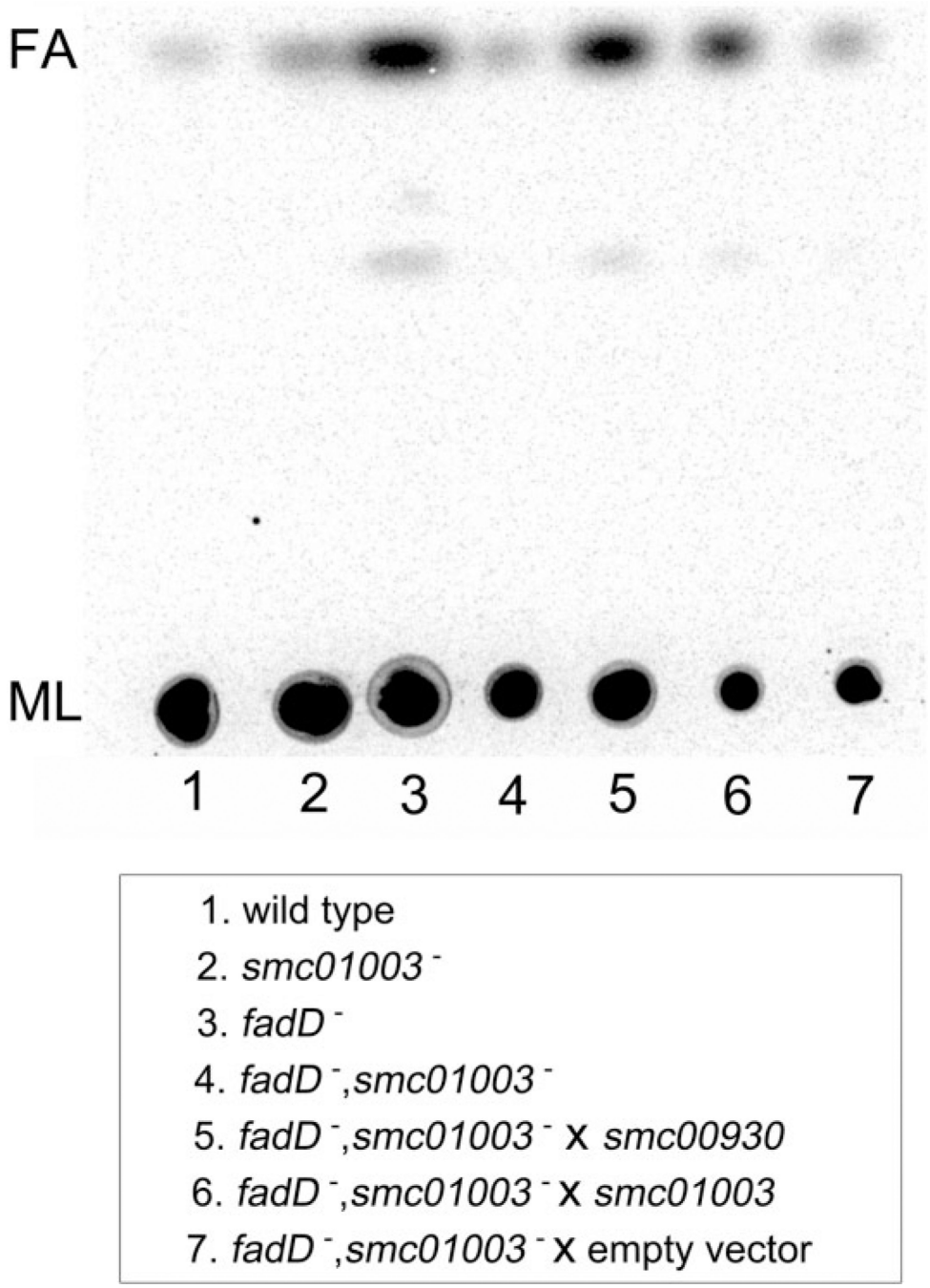

Fig. 3.

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 14C-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.

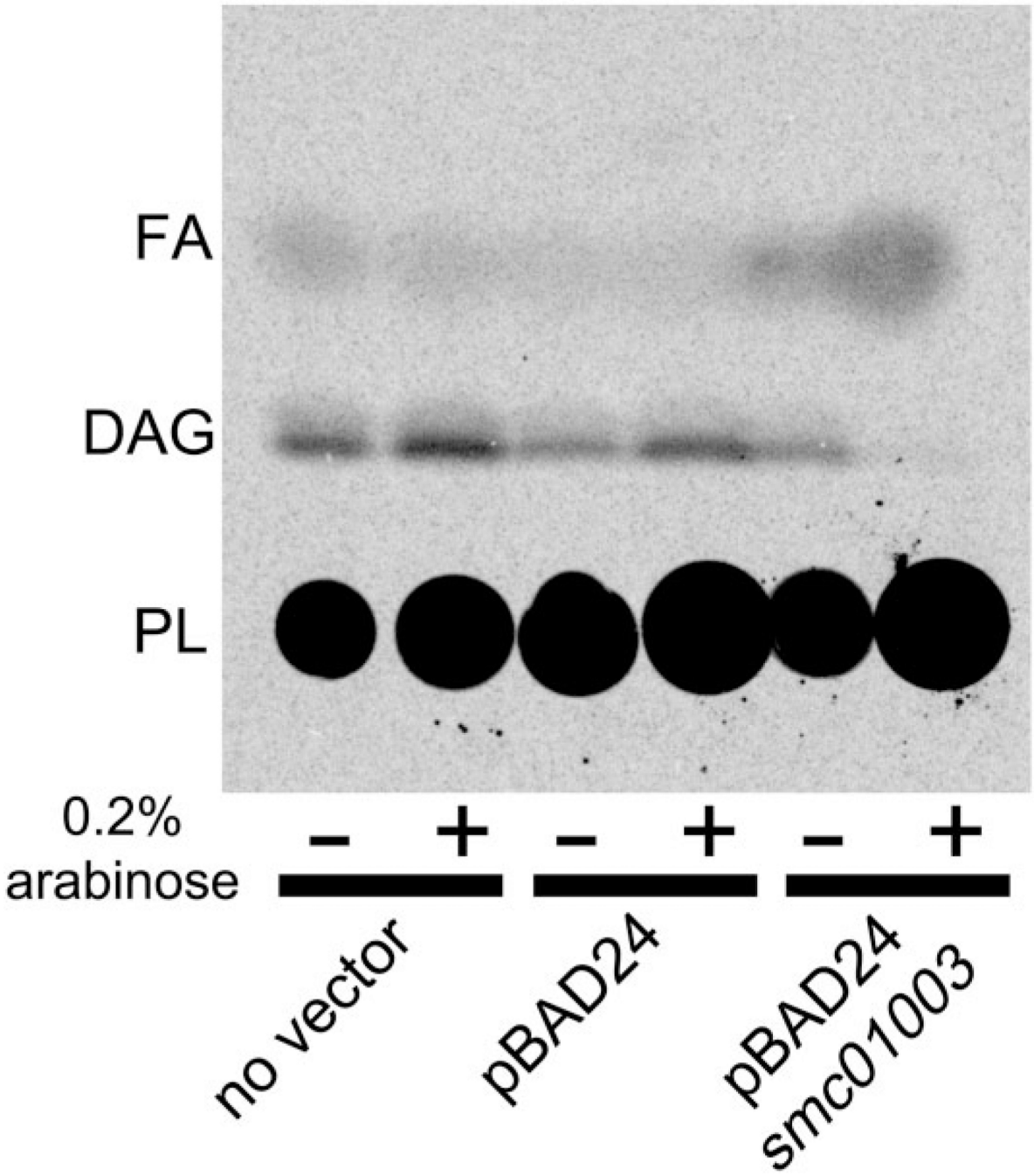

Fig. 4.

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.

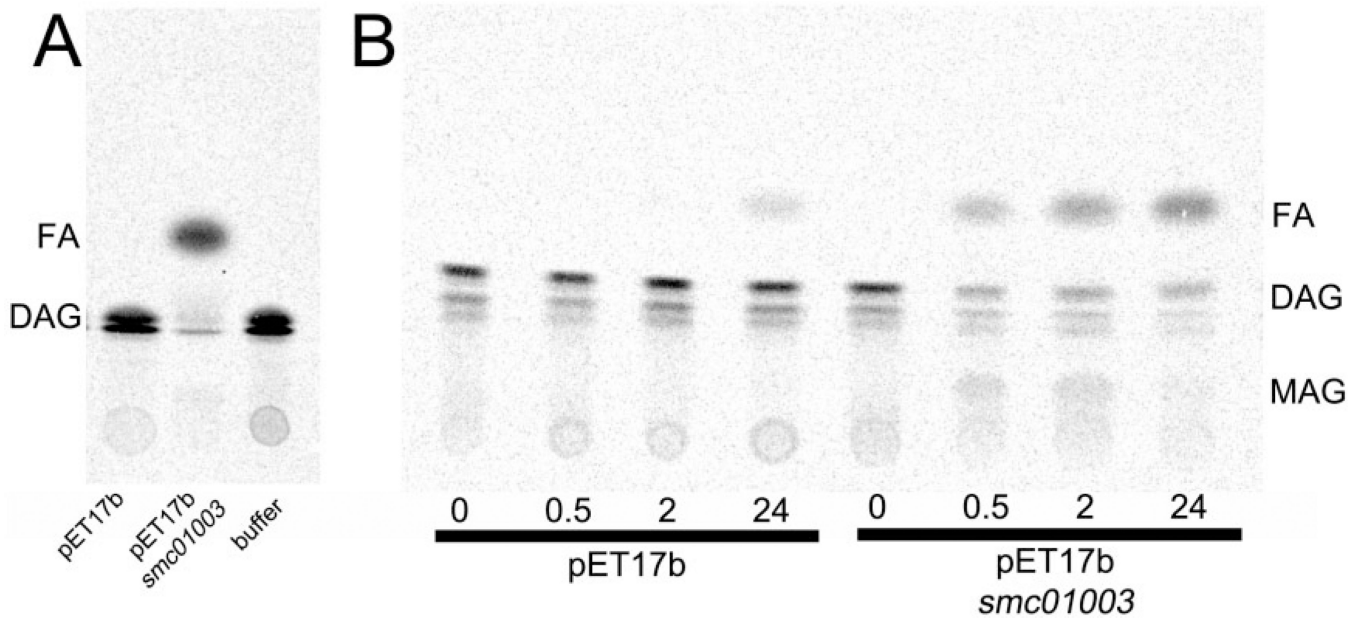

Fig. 5.

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.

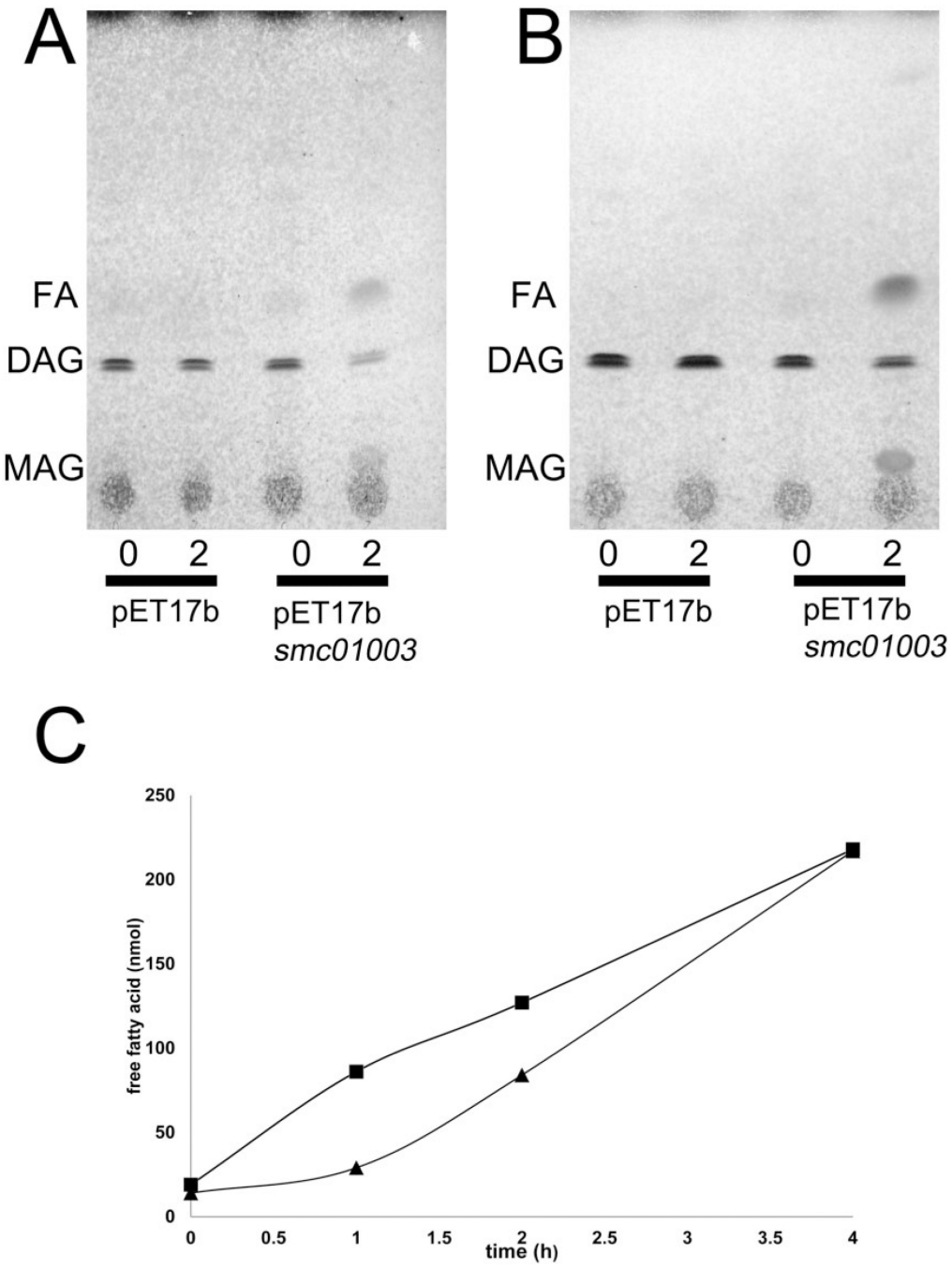

Fig. 6.

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.

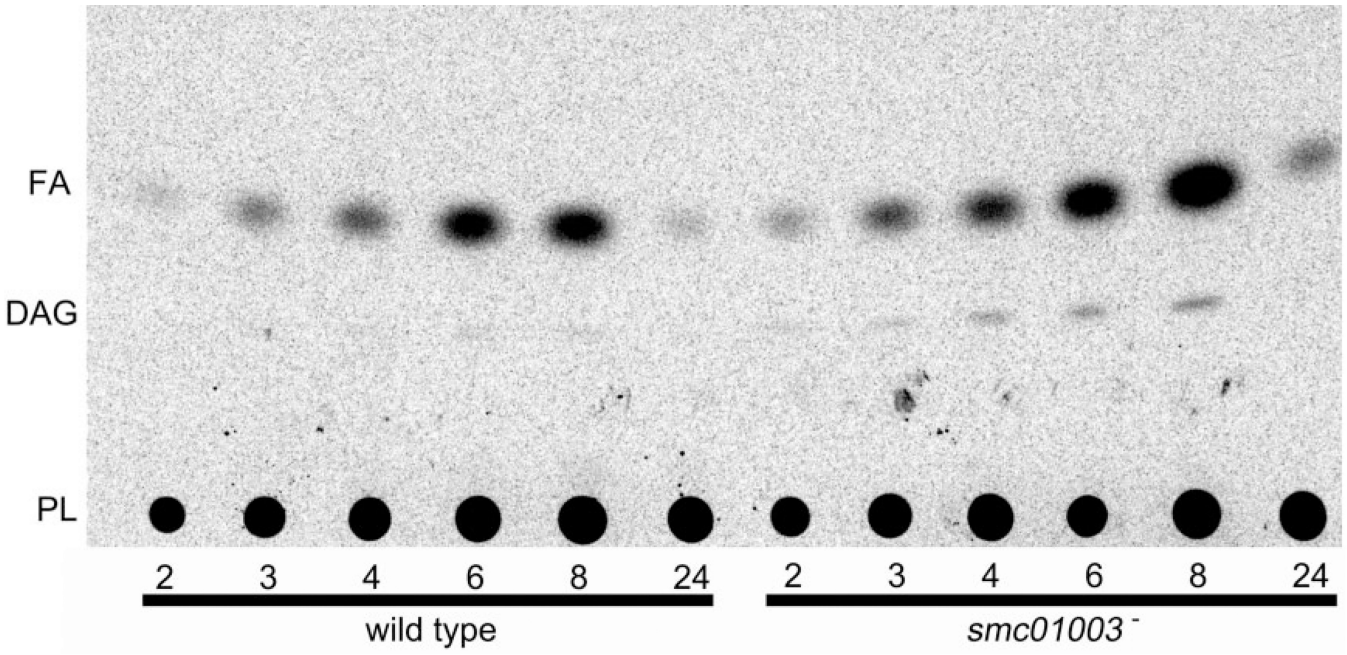

Fig. 7.

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.

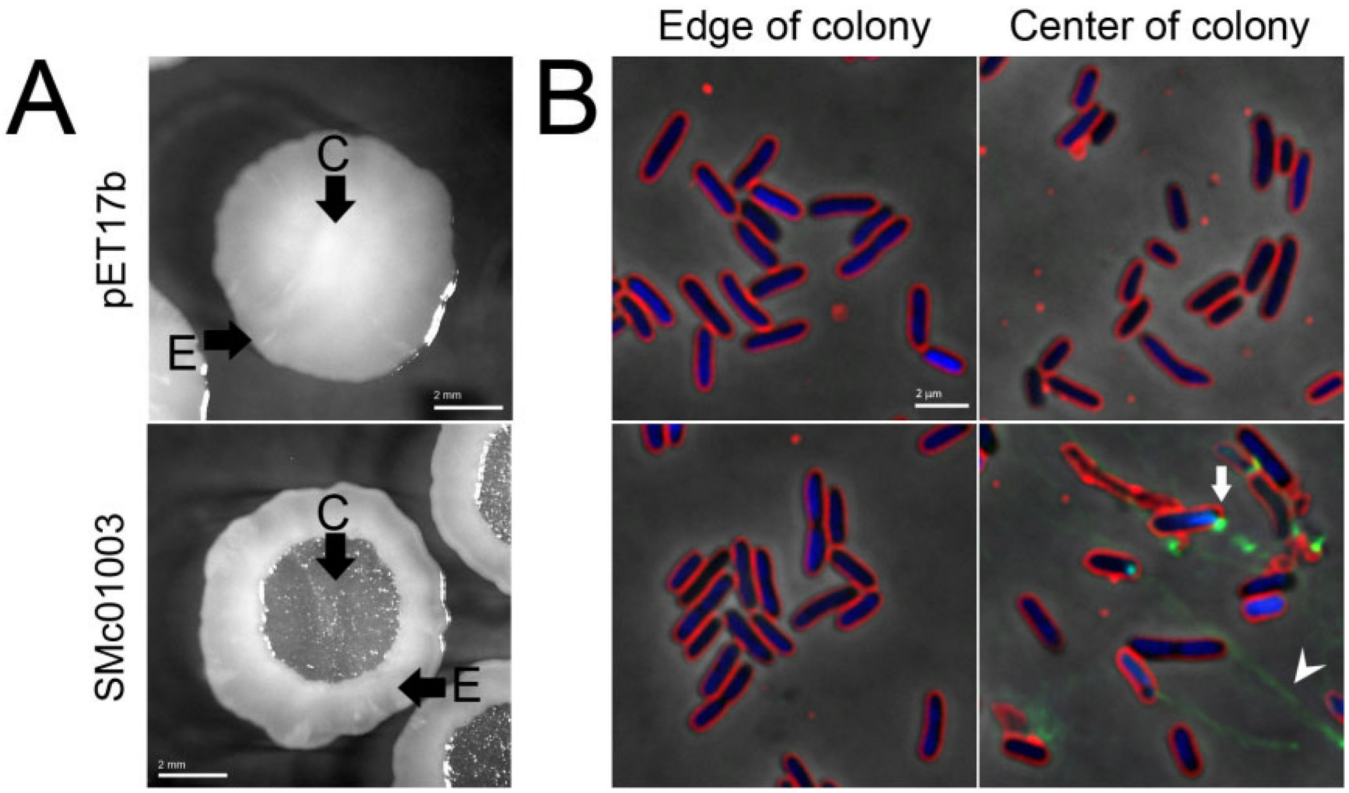

Fig. 8.

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) × 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.

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.