Cyclopropane fatty acyl synthase in *Sinorhizobium meliloti*

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Cyclopropane fatty acyl synthases (CFA synthases) are enzymes that catalyse the addition of a methylene group across *cis* double bonds of monounsaturated fatty acyl chains in lipids. We have investigated the function of two putative genes, *cfa1* and *cfa2*, proposed to code for CFA synthases in *Sinorhizobium meliloti*. Total fatty acid composition and fatty acid distributions within lipid classes for wild-type and *cfa1* and *cfa2* mutant strains grown under P_i starvation and in acidic culture conditions were obtained by GC/MS and by infusion ESI/MS/MS, respectively. For wild-type cells and the *cfa1* mutant, total cyclopropane fatty acids (CFAs) increased by 10 % and 15 % under P_i starvation and acidic conditions, respectively; whereas in the *cfa2* mutant, CFAs were less than 0.1 % of wild-type under both growth conditions. Reporter gene fusion experiments revealed that *cfa1* and *cfa2* were expressed at similar levels in free-living cells. Thus under the conditions we examined, *cfa2* was required for the cyclopropanation of lipids in *S. meliloti* whereas the role of *cfa1* remains to be determined. Analysis of intact lipids revealed that cyclopropanation occurred on *cis*-11-octadecenoic acid located in either the *sn*-1 or the *sn*-2 position in phospholipids and that cyclopropanation in the *sn*-2 position occurred to a greater extent in phosphatidylcholines and sulfoquinovosyldiacylglycerols under acidic conditions than under P_i starvation. The *cfa2* gene was also required for cyclopropanation of non-phosphorus-containing lipids. Principal components analysis revealed no differences in the cyclopropanation of four lipid classes. We concluded that cyclopropanation occurred independently of the polar head group. Neither *cfa1* nor *cfa2* was required for symbiotic nitrogen fixation.

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INTRODUCTION

The soil bacterium *Sinorhizobium meliloti* infects the roots of *Medicago sativa* (alfalfa), forming nodules and establishing a beneficial symbiotic relationship in which the bacteria convert atmospheric nitrogen into a usable source of nitrogen for the plants (Garg & Geetanjali, 2007). In the last two decades, extensive research has been conducted to understand the biochemistry and genetics of nitrogen-fixing bacteria and their symbiosis with leguminous plants,

given the importance of this process for sustainable agriculture (Cowie *et al.*, 2006; Galibert *et al.*, 2001; MacLean *et al.*, 2007; Weidner *et al.*, 2003). Soil bacteria encounter numerous adverse conditions in the environment, including increased soil salinity, acidic pH conditions and limited inorganic phosphate (P_i) (Cheng *et al.*, 2005; Garau *et al.*, 2005; Ibragimova *et al.*, 2006).

Phospholipids constitute up to 95 % of *S. meliloti* lipids; they include phosphatidylcholines (PCs), monomethylphosphatidylethanolamines (MMPEs), phosphatidylethanolamines (PEs), dimethylphosphatidylethanolamines (DMPEs), phosphatidylglycerols (PGs) and cardiolipins (Geiger *et al.*, 1999). Minor lipid classes (5 %) include ornithine lipids (OLs) and sulfoquinovosyldiacylglycerols (SLs). Under P_i-limiting conditions, *S. meliloti* partially substitutes membrane phospholipids with non-phosphorus-containing lipids, which can represent up to 70 % of total lipids; these lipids include 1,2-diacylglycerol-3-O-4'-(*N,N,N*-trimethyl)-homoserine lipids (TMHSs), SLs and OLs (Lopez-Lara *et al.*, 2005). Recently, we showed for the first time that the percentage composition of

Abbreviations: CFA, cyclopropane fatty acid/acyl; DMPE, dimethylphosphatidylethanolamine; FAME, fatty acid methyl ester; MMPE, monomethylphosphatidylethanolamine; PC, phosphatidylcholine; PCA, principal components analysis; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SL, sulfoquinovosyldiacylglycerol; TMHS, 1,2-diacylglycerol-3-O-4'-(*N,N,N*-trimethyl)-homoserine. Relative positions of fatty acyl chains in lipids are represented by the fatty acid in the *sn*-1 position first followed by the fatty acid in the *sn*-2 position, separated by / as recommended by LIPID MAPS (<http://www.lipidmaps.org/>).

A supplementary table and figure are available with the online version of this paper.

cyclopropane fatty acids (CFAs) increased twofold under P_i starvation conditions (Saborido Basconcillo & McCarry, 2008). Two CFAs, *cis*-9,10-methylene hexadecanoic acid [17:0cyclo(9,10)] and *cis*-11,12-methylene octadecanoic acid [19:0cyclo(11,12)] and their biochemical precursors, *cis*-9-hexadecenoic acid [16:1(9)] and *cis*-11-octadecenoic acid [18:1(11)], have been identified in *S. meliloti* (Jarvis & Tighe, 1994; Saborido Basconcillo & McCarry, 2008). Cyclopropane fatty acyl synthases (CFA synthases) are enzymes that catalyse the addition of a methylene group from *S*-adenosylmethionine across *cis* double bonds of monounsaturated fatty acyl chains in phospholipids (Grogan & Cronan, 1997). Cyclopropanation is a post-synthetic modification of phospholipids which reaches a maximum during the stationary growth phase (Grogan & Cronan, 1997). While CFA synthase in *Escherichia coli* has been well studied (Courtois *et al.*, 2004; Courtois & Ploux, 2005; Wang *et al.*, 1992), there appear to have been no studies as yet in *S. meliloti*. Two putative CFA synthases (SMc00358 and SMc02645) have been annotated as *cfa1* and *cfa2* genes in *S. meliloti* but no genetic or metabolite analyses have been conducted to support these functional assignments. Here we report what we believe to be the first efforts in this area.

While the biological roles of CFAs in bacteria are not completely understood (Cronan, 2002), it has been demonstrated that cyclopropane-containing lipids protect bacteria from adverse conditions such as acidity (Ballen *et al.*, 1998; Brown *et al.*, 1997; Chang & Cronan, 1999; Kim *et al.*, 2005), freeze-drying (Munoz-Rojas *et al.*, 2006), desiccation (Boumahdi *et al.*, 2001) and exposure to pollutants (Fang *et al.*, 2007; Mrozik *et al.*, 2005, 2006). There is some debate as to whether an increase in cyclopropane content of bacterial membranes leads to a decrease in membrane fluidity (Loffhagen *et al.*, 2007; Munoz-Rojas *et al.*, 2006). Furthermore, CFAs are involved in the pathogenesis of *Mycobacterium tuberculosis* (Glickman *et al.*, 2000; Rao *et al.*, 2005) and have antifungal activity in the antagonistic fluorescent pseudomonads (Ayyadurai *et al.*, 2007), making CFAs targets for the development of new drugs for the treatment of antibiotic-resistant strains of *M. tuberculosis* (Bhatt *et al.*, 2007; Guianvarc'h *et al.*, 2006; Sekanka *et al.*, 2007). Since CFAs are important in bacteria–host interactions for some pathogens, we have examined their possible role in *S. meliloti* during the alfalfa root-nodulation symbiosis.

Typically, CFA synthase studies have involved analysis of total fatty acids by gas chromatography coupled to mass spectrometry (GC/MS) or fatty acid analysis of lipid classes isolated using thin-layer chromatography (TLC) (Correa *et al.*, 1999; Grogan & Cronan, 1997). However, intact lipids can be analysed directly by the infusion of crude extracts without the need for chromatography, using a combination of tandem mass spectrometric techniques (ESI/MS/MS); this approach is known as 'shotgun lipidomics' (Han & Gross, 2003, 2005a, b). In this work, we used a shotgun lipidomics strategy to determine fatty

acid distributions within five lipid classes (phospholipids and non-phosphorus-containing lipids). Principal components analysis was used to identify trends in the fatty acid distributions of lipid classes. MS/MS experiments were used to determine fatty acid compositions in lipids and their relative positions in lipids (*sn*-1 vs *sn*-2), which provided insights into the selectivity of CFA synthases. Total fatty acids were analysed as fatty acid methyl esters (FAMES) by GC/MS using a micro-scale method which was developed to assure good recoveries of CFAs (Saborido Basconcillo & McCarry, 2008).

The objectives of this work were: (1) to study and compare the effects of P_i starvation and acidic conditions on lipid cyclopropanation in *S. meliloti*; (2) to determine whether the putative *cfa1* and *cfa2* genes encoded CFA synthases; (3) to determine substrate selectivity of CFA synthases in *S. meliloti* such as preference towards certain lipid classes; and (4) to determine whether CFA synthases exhibited a preference for monounsaturated fatty acyl chains located at either the *sn*-1 or *sn*-2 position in lipids.

METHODS

Bacterial strains and culture conditions. *S. meliloti* RmP110 is a *pstC*⁺ derivative of strain 1021 and thus has a wild-type Pho regulon phenotype (Yuan *et al.*, 2006a). For P_i -limitation experiments, *S. meliloti* RmP110 cultures were grown in MOPS-buffered minimal medium (Bardin *et al.*, 1996) with added Co^{2+} (10 ng $CoCl_2$ ml⁻¹), containing glucose (15 mM) as the carbon source, in the presence of 2 mM P_i or in the absence of added P_i . *S. meliloti* cells grown overnight in LBmc broth [Luria–Bertani (LB) broth containing 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$] were washed with MOPS-buffered minimal medium (2 and 0 mM P_i), subcultured in this medium and grown for 36–40 h at 30 °C with shaking. Cultures used in the pH study were initially grown overnight in LBmc, washed with 0.85% NaCl and subcultured in M9 minimal medium containing glucose (15 mM), at pH 7.0 and pH 5.5 followed by an incubation of 36–40 h at 30 °C with shaking. In all cases, 500 ml late-exponential-phase cultures (OD₆₀₀ 0.6–0.9) were centrifuged at 5000 g for 20 min, and the cell pellets were resuspended in 2.5 ml medium and divided into 250 µl aliquots in Eppendorf tubes. Each tube was centrifuged, the supernatant discarded and the wet pellets flash-frozen in liquid nitrogen and stored at –80 °C until further use. The pellets (equivalent to 50 ml of original culture) corresponded to 61.8 ± 4.5 mg wet weight with 2 mM P_i ($n=9$) and 27.6 ± 1.2 mg with 0 mM P_i ($n=9$) from MOPS media or 40.5 ± 0.9 mg with pH 7.0 ($n=9$) and 37.0 ± 2.0 mg with pH 5.5 ($n=9$) from M9 media. Lipid extracts prepared from cell pellets were used for fatty acid and intact lipid analyses.

Construction of *S. meliloti* *cfa1* and *cfa2* mutants. The *S. meliloti* SMc00358 and SMc02645 genes are annotated as *cfa1* and *cfa2*, respectively (<http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi>). The *smc02644* gene, annotated as encoding a putative transcriptional accessory protein, is located on the same strand 204 nt downstream of *cfa2*, while *smc00359*, encoding a putative transmembrane protein putatively involved in electron transport, lies 275 nt downstream of *cfa1*. Since neither of the downstream genes appeared to be involved in cyclopropanation, we chose to investigate the role of the *cfa* genes via the generation of *cfa* insertion mutants. Single-crossover mutations in the *cfa* genes were constructed in the RmP110 background, as RmP110 has a

wild-type Pho regulon phenotype and we wished to examine the effects of phosphate limitation on fatty acid cyclopropanation (Yuan *et al.*, 2006b). Accordingly, 263 bp and 271 bp fragments were PCR amplified from within the 5' regions of *cfa1* and *cfa2*, respectively, using primers 5'-GCTCTAGAGCTATCGCATATG-ATGAAGTCGTTTC-3' (*Xba*I site in bold) and 5'-TGCATGCAT-GTCACAACAGCTTCTGGATCGGATAGG-3' (*Nsi*I site in bold) for *cfa1* and primers 5'-GCTCTAGAGCGGGATTGTTGAATCTAC-TTCGC-3' (*Xba*I site in bold) and 5'-CCATCGATGGTCACGC-GACGATCGAATTGGTGAAGG-3' (*Cl*aI site in bold) for *cfa2*. Each of these fragments was cloned into plasmid pTH1360 (Yuan *et al.*, 2006b) using appropriate restriction sites and the resultant plasmids from *E. coli* DH5 α were recombined into the *S. meliloti* RmP110 genome via triparental mating using MT616 as a helper strain (Finan *et al.*, 1986). Single-crossover recombinants were selected on LB plates containing 200 μ g streptomycin ml⁻¹ and 200 μ g neomycin ml⁻¹. Representative colonies for each cointegrate mutant were streak-purified three times before further use. Plasmid pTH1360 contains a promoterless *gusA* gene downstream of the multiple cloning site for the generation of integrated transcriptional fusions. The structure of the recombinants was confirmed via the direct sequencing of purified and sheared genomic DNA preparations from transconjugant strains using a reverse primer from the 3' region of *gusA* (5'-TTGGGGTTT-CTACAGGACGTAAC-3'). This primer could generate DNA sequence reading through the recombined region and into the *S. meliloti* genome, to confirm that the recombination occurred in the appropriate target sequences.

β -Glucuronidase activity assays. The expression of the *gusA* gene, encoding β -glucuronidase, was measured using *S. meliloti* cultures grown in three different media: LBmc, MOPS-buffered minimal medium with 2 and 0 mM P_i, and M9 at pH 7.0 and pH 5.5. To 100 μ l of cell culture, 400 μ l of GusA assay buffer [50 mM Na₃PO₄ pH 7.4, 1 mM EDTA, 10 mM DTT, 0.01 % SDS and 0.44 mg ml⁻¹ of substrate *p*-nitrophenyl- α -D-glucopyranoside (pNPG) (Cowie *et al.*, 2006)] was added, mixed well and incubated at room temperature until the development of yellow colour. The reaction was stopped by the addition of 500 μ l of a solution of Na₂CO₃ (1 M); mixtures were spun down for 2 min at 10 000 g to remove cell debris and the supernatant was used to measure A₄₀₅. The enzyme activity values were expressed as Miller units [activity = (A₄₀₅ × 1000)/(time (min) × culture volume (ml) × OD₆₀₀)].

Fatty acid methyl ester (FAME) analysis. Fatty acids were analysed as their FAMEs by GC/MS. FAMEs were prepared from dried lipid extracts using a micro-scale one-vial method that employed sodium methoxide in methanol as described previously (Saborido Basconcillo & McCarry, 2008).

Intact lipid analysis. Dried lipid extracts were prepared from wet cell pellets (50 ml culture) as described previously and dissolved in methanol/chloroform (1:1, v/v, 200 μ l) (Saborido Basconcillo & McCarry, 2008). Aliquots were diluted fivefold with methanol containing the internal standards 1,2-diheneicosanoylphosphatidylcholine [PC(21:0/21:0), 32 μ mol] and 1,2-dimyristoylphosphatidylethanolamine [PE(14:0/14:0), 28 μ mol]; lithium chloride (2.5 mM) was added only for analyses conducted in the positive ionization mode. For analysis in the negative ionization mode, aliquots were diluted fivefold with methanol containing 1,2-dilauroylphosphatidylglycerol [PG(12:0/12:0), 3 μ mol] as the internal standard. A Waters Quattro Ultima triple-quadrupole mass spectrometer equipped with a microelectrospray ion source and operating under the MassLynx software was used for the analysis of bacterial lipid extracts. The collision gas (N₂) pressure was 2 × 10⁻³ bar and collision energies ranged between 25 and 50 eV. Bacterial lipid extracts were continuously infused at 1 μ l min⁻¹ using a Harvard syringe pump

in the positive ion mode with or without the addition of lithium chloride and in the negative ion mode (no LiCl added). PCs, PEs and TMHs were analysed as [M + Li]⁺ ions using neutral loss scans of 189 (Hsu *et al.*, 1998), 147 (Hsu & Turk, 2000) and 74 mass units (authors' unpublished data) respectively. PGs and SLs were analysed as their [M - H]⁻ ions in the negative ionization mode using parent ion scans of *m/z* 153 (Griffiths, 2003; Han & Gross, 2005a) and 225 ions (authors' unpublished data). In all experiments 100 spectra were averaged to afford a single mass spectrum. Peak areas obtained in each MS/MS spectrum were normalized to the area of the internal standard and used to obtain relative percentage compositions of molecular species in each lipid class.

Statistical analysis. Each bacterial culture was sampled then analysed in triplicate and all data are expressed as means. Wild-type cultures were grown in triplicate over a period of 1 year and analysed for fatty acid composition (See Supplementary Table S1, available with the online version of this paper). The analytical variabilities for fatty acid analyses were ≤20% for fatty acids with percentage composition exceeding 1%. The biological variabilities were ≤20% over a 1 year period.

The SPSS statistical package, version 15.0, was used for statistical analysis. Student's *t*-test was used to evaluate the significance of the differences when only two groups were compared, whereas analysis of variance (ANOVA) was used for multiple group comparisons (*P* ≤ 0.05). Principal components analyses (PCAs) were performed using the Multivariate Analysis add-in for Excel, version 1.3 (Bristol Chemometrics).

RESULTS

Total CFAs increase with P_i starvation and acidity

Table 1 presents the fatty acid composition data for *S. meliloti* wild-type and the *cfa1* and *cfa2* mutants under two stressed conditions: P_i starvation compared to 2 mM P_i and pH 5.5 compared to pH 7.0. The levels of the major CFA [19:0cyclo(11,12)] increased significantly under P_i starvation conditions while the minor CFA [17:0cyclo(9,10)] showed no change. Acidic conditions (pH 5.5) caused significant increases in both CFAs in the wild-type (pH 7.0). The total increases in CFAs were 10% and 15% under P_i starvation and acidic conditions, respectively.

Total fatty acid analyses were performed using a one-vial transmethylation method that employed sodium methoxide in methanol, the reagent of choice for the conversion of cyclopropane-containing lipids into their FAMEs (Grogan & Cronan, 1997). While most studies of CFA synthases have employed basic hydrolysis followed by an acid-catalysed methylation with HCl to generate FAMEs from bacterial lipids (Boumahdi *et al.*, 2001; Brown *et al.*, 1997; Loffhagen *et al.*, 2007; Mrozik *et al.*, 2006; Munoz-Rojas *et al.*, 2006), this methodology produced lower yields of CFAs from lipid extracts of *S. meliloti* compared to the sodium methoxide in methanol procedure (Saborido Basconcillo & McCarry, 2008). CFAs [17:0cyclo(9,10) and 19:0cyclo(11,12)] were chromatographically resolved from their monounsaturated counterparts (17:1 and 19:1) (Saborido Basconcillo & McCarry, 2008).

Table 1. Relative percentage compositions of fatty acids in *S. meliloti* wild-type and *cfa1* and *cfa2* mutants in the presence of inorganic phosphate (2 mM P_i) relative to P_i starvation, and at pH 7.0 relative to pH 5.5

ND, Not detected. Mean values in bold presented errors of less than 10 %.

Fatty acid	Relative percentage composition of <i>S. meliloti</i> , mean values (n=3)											
	Wild-type				<i>cfa1</i> mutant				<i>cfa2</i> mutant			
	P _i		pH		P _i		pH		P _i		pH	
	2 mM	0 mM	7.0	5.5	2 mM	0 mM	7.0	5.5	2 mM	0 mM	7.0	5.5
14:0	0.2	0.3	0.4	0.4	0.3	0.3	0.5	0.4	0.3	0.3	0.4	0.3
16:0	13.0	11.9	12.5	12.1	14.4	10.9*	14.1	11.1	15.7†	15.3‡	17.9†	12.4§
17:0	0.2	0.2	0.1	0.4 	0.2	0.2	0.2	0.3	0.3	0.2	0.2	0.4
18:0	3.6	1.8 	4.0	2.7	3.1	1.3*	4.8	2.6	4.9	2.4	8.1	2.9§
16:1(9)	1.2	1.4	0.6	0.7	1.5	1.3	1.1	0.9	3.3†	3.6‡	2.3†	2.2
18:1(11)	54.4	46.9 	59.7	46.4 	53.0	44.8*	57.2	48.8*	75.4†	78.2‡	71.0	81.8§‡
17:0cyclo(9,10)	1.6	2.1	1.6	2.7 	1.9	2.4	1.6	2.3	ND†	ND‡	ND†	ND‡
19:0cyclo(11,12)	25.8	35.5 	20.9	34.6 	25.6	38.7*	20.6	33.6*	ND†	ND‡	ND†	ND‡

**cfa1* mutant under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) from *cfa1* mutant under normal growth conditions (2 mM P_i or pH 7.0).

†Under normal growth conditions (2 mM P_i or pH 7.0), *cfa2* mutant is significantly different from wild-type ($P \leq 0.05$).

‡Under stressed conditions (0 mM P_i or pH 5.5), *cfa2* mutant is significantly different from wild-type ($P \leq 0.05$).

§*cfa2* mutant under stressed conditions (pH 5.5) is significantly different ($P \leq 0.05$) from *cfa2* mutant under normal growth conditions (pH 7.0).

||Wild-type under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) from wild-type under normal growth conditions (2 mM P_i or pH 7.0).

Cyclopropanation occurs in the *sn*-1 and *sn*-2 positions in lipids

A shotgun lipidomics methodology was used to determine fatty acid distributions for seven lipid classes (PCs, PEs, MMPEs, PEs, PGs, SLs, TMHSs). In this paper, we focus on four lipid classes: PCs and PGs, which are the most abundant phospholipids in *S. meliloti* under normal growth conditions, and TMHSs and SLs, the major lipids under P_i starvation conditions (Geiger *et al.*, 1999). Lipid profiles of PCs and PGs are shown in Fig. 1 for the wild-type under normal growth conditions. The ions observed at *m/z* 764.6, 766.7, 778.8, 780.8, 792.8, 806.8 and 820.8 corresponded to the [M+Li]⁺ adducts of PCs with fatty acid compositions of 34:2, 34:1, 35:2, 35:1, 36:2, 37:2 and 38:2, respectively (Fig. 1a). Similarly, the profiles of PGs revealed the presence of lipids with fatty acid compositions corresponding to 34:2, 34:1, 35:2, 35:1, 36:2, 37:2 and 38:2 (Fig. 1b). MS/MS experiments were conducted to identify which lipid species contained CFAs and the relative positions of these fatty acids (*sn*-1 vs *sn*-2) in lipids.

Collision-induced dissociation (CID) of [M+Li]⁺ adducts of PCs produces fragment ions due to the neutral losses of the lithiated polar head group ([M+Li-189]⁺) and the fatty acyl chains (Hsu *et al.*, 1998; Hsu & Turk, 2003). Characteristic fragment ions are observed at *m/z* [M+Li-59-RCOOH]⁺, [M+Li-RCOOLi]⁺ and [M+Li-RCOOH]⁺. Moreover, the [M+Li-59-RCOOH]⁺

ions produced by the loss of fatty acids from the *sn*-1 position are more intense than those produced by the loss of *sn*-2 fatty acids (Hsu & Turk, 2003), which allowed the assignment of CFAs to the *sn*-1 or *sn*-2 positions. The PC-35:2, PC-35:1, PC-37:2 and PC-38:2 species all contained CFAs as demonstrated by the presence of fragment ions at *m/z* [M+Li-59-268.2]⁺ or [M+Li-9-296.3]⁺, corresponding to neutral losses of [N(CH₃)₃+17:0cyclo] or [N(CH₃)₃+19:0cyclo], respectively. The lipid PC-37:2, the most abundant cyclopropane-containing PC, was shown to have the 19:0cyclo and 18:1 fatty acids attached to the *sn*-1 and *sn*-2 positions, respectively (Fig. 2a). The fragment ion at *m/z* 465.3 derived from the neutral loss of 18:1 was more intense than those corresponding to the loss of 19:0cyclo at *m/z* 451.4 (Fig. 2a, inset); based on the work of Hsu & Turk (2003) these data were interpreted as PC-37:2 corresponding primarily to PC(18:1/19:0cyclo). However, since these ions did not provide the 2:1 to 3:1 ratio of ion intensities reported by Hsu & Turk (2003), PC-37:2 may contain some of the PC(19:0cyclo/18:1) isomer.

Daughter ion spectra in the negative ionization mode of [M-H]⁻ ions of PG-33:1, PG-35:2, PG-35:1 and PG-37:2 showed the presence of CFAs indicated by carboxylate ions at *m/z* 267.2 and 295.3. The relative positions (*sn*-1 vs *sn*-2) of CFAs in PGs were determined because the [RCOO]⁻ ions from fatty acids in the *sn*-2 position are more abundant than ions from the *sn*-1 position (Hsu &

Fig. 1. PC (a) and PG (b) profiles of *S. meliloti* wild-type and the *cfa1* and *cfa2* mutants in the presence of 2 mM P_i . NL, neutral loss scan.

Turk, 2001). The daughter ion spectrum of PG-37:2 clearly showed that this lipid corresponded to PG(19:0cyclo/18:1) (Fig. 2b).

The profiles for SLs and TMHSs, derived from wild-type and both knockout mutants with P_i starvation, are shown in Fig. 3. The daughter ion spectra of $[M-H]^-$ ions of SLs with fatty acid compositions of SL-35:2, SL-35:1, SL-37:2 and SL-38:2 showed that these lipids contained CFAs, as confirmed by the presence of $[RCOO]^-$ fragment ions in the MS/MS spectra at m/z 267.2 and 295.3 for 17:0cyclo and 19:0cyclo, respectively. These spectra were interpreted as corresponding to lipids with fatty acid combinations of

SL(18:1/17:0cyclo), SL(19:0cyclo/16:0), SL(18:1/19:0cyclo) and SL(19:0cyclo/19:0cyclo), respectively. Lipid profiles of the TMHSs using neutral loss scans of 74 provided profiles similar to those observed for PCs (Fig. 1b) formed by TMHS-35:2, TMHS-35:1 and TMHS-37:2 lipids. The relative positions of fatty acids (*sn*-1 vs *sn*-2) in SLs and TMHSs could not be determined since the fragmentation mechanisms of these lipids have not been characterized due to the unavailability of analytical standards. However, cyclopropanation occurred in both the *sn*-1 and the *sn*-2 positions, demonstrated by the presence of 38:2 lipids.

Fig. 2. Daughter ion spectra of 37:2 lipids of (a) PCs and (b) PGs in *S. meliloti*.

Cyclopropanation of PCs and SLs occurs more extensively with acidity than with P_i starvation

Tables 2 and 3 show relative percentage compositions in wild-type and *cfa* mutant cells for PCs and PGs, respectively. Increased acidity and P_i starvation caused an increase in cyclopropane-containing species (35:2, 35:1, 37:2, 38:2) in all lipid classes, while species that contained the biochemical precursors of CFAs (34:1 and 36:2) decreased significantly. PGs showed a similar trend; however, only PG-37:2 increased significantly while PG-35:2 and PG-35:1 did not increase as observed for PCs (Table 3). PG-38:2 was a minor component and did not increase with P_i starvation, while PC-38:2 increased up to 4%. PEs and SLs that contained CFAs also increased with P_i starvation, reaching a maximum of 7% and 14%, respectively, for 37:2 species (data not shown).

Under P_i starvation, cyclopropanation increased in all lipid classes in general, but occurred to a larger extent in PCs and SLs than in PGs and PEs. Cyclopropanation was more pronounced under acidic conditions than under P_i starvation; this phenomenon was manifested by further transformation of 37:2 lipids to 38:2 lipids in PCs (Table 2) instead of a build-up of 37:2 lipids as observed in PGs (Table 3). The same pattern as observed in PCs was present in SLs under acidic conditions, as SL-37:2 was further cyclopropanated to SL-38:2.

cfa2 is required for the cyclopropanation of phospholipids and non-phosphorus-containing lipids in *S. meliloti*

PGs and PCs containing CFAs (35:2, 35:1, 37:2, 38:2) decreased significantly (87–100%) in *cfa2* mutant cells grown under P_i starvation or in acidic conditions (Tables 2 and 3). In addition, cyclopropane-containing SLs and TMHSs were not detected in *cfa2* cultures (Fig. 3). Thus, *cfa2* is required for cyclopropanation of phospholipids and non-phosphorus-containing lipids. On the other hand, the lipid profiles of PCs, PGs, SLs and TMHSs in the *cfa1* mutant cells showed no significant differences compared to wild-type cells (Tables 2 and 3). Fatty acid composition data showed that CFAs were not detected in the *cfa2* mutant, while the biochemical precursor of the major CFA (*cis*-11-octadecenoic acid) exhibited a significant increase (Table 1). Fatty acid composition data of *cfa1* mutants were statistically identical to the wild-type under all conditions examined. It is noteworthy that CFAs in *cfa2* mutants were not detected by GC/MS analyses, while ESI/MS/MS techniques detected low levels (<1%) of cyclopropane-containing lipids.

Cyclopropanation in *S. meliloti* is independent of lipid class

PCA was applied to the fatty acid distributions of lipid classes (PCs, PEs, PGs, SLs) in the wild-type and the *cfa1*

Fig. 3. Infusion electrospray mass spectra of non-polar extracts of *S. meliloti* wild-type and *cfa1* and *cfa2* mutants, showing the profiles of (a) SLs and (b) TMHSs with P_i starvation. NL, neutral loss scan.

and *cfa2* mutants under all growth conditions in order to identify trends in the dataset (Fig. 4). In the scores plots (Fig. 4a, c), lipid classes are represented by different symbol shapes and the fatty acid compositions of individual lipids are represented by colours. PC1 accounts for 85 % and PC2 for 13 % of the variance in the dataset. Approximately half of the dataset clustered very tightly and therefore did not contribute to the differences observed in the loadings plots (Fig. 4b). The data points that did not cluster were grouped by colour (fatty acid composition) and not by symbol shape (lipid class). These data points corresponded to lipids with fatty acid compositions of 36:2, 34:1, 37:2 and

38:2. Lipids with fatty acid compositions of 37:2 and 38:2 contained CFAs while lipids 36:2 and 34:1 contained the biochemical precursors of CFAs. Since data points clustered for certain fatty acid compositions (colours) and not for a particular lipid class (symbol shapes), the dataset was interpreted as indicating that cyclopropanation occurred independent of lipid classes. PC1 was correlated with percentage composition of lipids while PC2 represented the extent of cyclopropanation in lipids. In the loadings plots, samples with the highest percentage of cyclopropane-containing lipids were located in the top quadrant relative to PC2 (Fig. 4b), correspond-

Table 2. Relative percentage composition of PCs in *S. meliloti* wild-type and *cfa1* and *cfa2* mutants with 2 mM P_i relative to 0 mM P_i and at pH 7.0 relative to pH 5.5

ND, Not detected. Mean values in bold presented errors of less than 10 %.

Lipid	Fatty acids in lipids (<i>sn</i> -1/ <i>sn</i> -2) [#]	Relative percentage composition of phosphatidylcholines, mean values (<i>n</i> =3)											
		Wild-type				<i>cfa1</i> mutant				<i>cfa2</i> mutant			
		P _i		pH		P _i		pH		P _i		pH	
		2 mM	0 mM	7.0	5.5	2 mM	0 mM	7.0	5.5	2 mM	0 mM	7.0	5.5
PC-34:2	16:1/18:1	0.7	0.8	0.7	0.5	1.9	0.8	0.8	0.4	3.3*	3.6†	5.0*	4.7†
PC-34:1	18:1/16:0	9.1	6.5‡	7.5	7.0	9.0	5.8§	8.5	6.0§	12.2	10.3†	9.4	13.7†
PC-35:2	18:1/17:0cyclo; 16:1-19:0cyclo	2.9	3.1	2.7	2.0	3.0	3.2	3.2	2.4	1.9	2.0	ND*	ND†
PC-35:1	19:0cyclo/16:0	3.5	4.9‡	3.4	7.8‡	4.2	4.6	3.8	7.3§	1.4*	1.7†	ND*	ND†
PC-36:2	18:1/18:1	43.7	26.3‡	35.4	19.4‡	42.3	30.9§	37.3	22.5§	78.4*	79.5†	85.6*	81.6†
PC-37:2	19:0cyclo/18:1	36.2	48.3‡	44.8	46.7	35.7	46.7§	40.3	46.8§	1.7*	2.2†	ND*	ND†
PC-38:2	19:0cyclo/19:0cyclo	3.9	10.1‡	5.6	16.5‡	4.0	8.0§	6.2	14.5§*	1.1*	0.8†	ND*	ND†

[#]When the assignment of fatty acids to the *sn*-1 and *sn*-2 positions in lipids was ambiguous, the fatty acids are separated by a hyphen instead of a forward slash.

*Under normal growth conditions (2 mM P_i or pH 7.0), *cfa2* mutant is significantly different from wild-type (*P*≤0.05).

†Under stressed conditions (0 mM P_i or pH 5.5), *cfa2* mutant is significantly different from wild-type (*P*≤0.05).

‡Wild-type under stressed conditions (0 mM P_i or pH 5.5) is significantly different (*P*≤0.05) from wild-type under normal growth conditions (2 mM P_i or pH 7.0).

§*cfa1* mutant under stressed conditions (0 mM P_i or pH 5.5) is significantly different (*P*≤0.05) from *cfa1* mutant under normal growth conditions (2 mM P_i or pH 7.0).

||*cfa2* mutant under stressed conditions (0 mM P_i or pH 5.5) is significantly different (*P*≤0.05) from *cfa2* mutant under normal growth conditions (2 mM P_i or pH 7.0).

ing to samples under acidic conditions (wild-type and *cfa1* mutant), while samples with the lowest content of cyclopropane-containing lipids were located at the bottom (*cfa2* mutants).

Cyclopropanation of lipids was more accentuated with acidity than with P_i starvation since samples at pH 5.5 were preceded by samples at 0 mM P_i along PC2. A third principal component (PC3) that contributed 2% of the variance differentiated samples at pH 5.5 (wild-type and *cfa1*) given by an increase of 38:2 lipid species in PCs and SLs rather than an increase of 37:2 species (Fig. 4c, d). Two main conclusions can be made from PCA analysis: (i) acidity caused more extensive cyclopropanation of lipids than P_i starvation and (ii) cyclopropanation of lipids did not occur preferentially for a given lipid class.

Expression of the *cfa* genes and examination of the Cfa1 and Cfa2 proteins

Since disruption of the *cfa1* and *cfa2* genes also created a transcriptional fusion to the *uidA* gene encoding β-glucuronidase (GusA), it was possible to measure promoter activities of those genes under various growth conditions. These data showed that *cfa1* and *cfa2* were expressed at

similar levels in cells grown in M9 or MOPS-buffered minimal media at neutral pH or in rich medium LBmc (Fig. 5). However, *cfa1* and *cfa2* expression increased 3.2- and 3.5-fold respectively, for cells grown in acidic conditions (pH 5.5) compared to cells grown under normal growth conditions (pH 7.0). All assays were conducted using cultures grown to the early stationary phase; assays performed on mid-exponential-phase cultures showed approximately half the activities for both *cfa1* and *cfa2* (data not shown), suggesting a twofold increase in promoter activities during stationary phase. To eliminate the possibility that the *cfa1* or *cfa2* mutations may influence their transcription, we also measured *cfa1* and *cfa2* transcription using *cfa1*- and *cfa2*-*gusA* transcriptional fusions (6448 and 869; Cowie *et al.*, 2006) which do not disrupt the respective *cfa* genes. Data from these strains were similar to those in Fig. 5; hence the disruption of these genes did not alter their transcription (data not shown).

DISCUSSION

To date, studies of CFA synthases have focused only on phospholipids, and the nature of the polar head group of phospholipids is thought to influence enzyme-substrate

Table 3. Relative percentage compositions of PGs in *S. meliloti* wild-type and *cfa1* and *cfa2* mutants grown with 2 mM P_i relative to 0 mM P_i and at pH 7.0 relative to pH 5.5

ND, Not detected. Mean values in bold presented errors of less than 10%.

Lipid	Fatty acids in lipids (<i>sn</i> -1/ <i>sn</i> -2) [#]	Relative percentage composition of phosphatidylglycerols, mean values (<i>n</i> =3)											
		Wild-type				<i>cfa1</i> mutant				<i>cfa2</i> mutant			
		P _i		pH		P _i		pH		P _i		pH	
		2 mM	0 mM	7.0	5.5	2 mM	0 mM	7.0	5.5	2 mM	0 mM	7.0	5.5
PG-32:1	16:1-16:0	0.7	ND*	0.4	0.2	0.6	ND†	0.3	0.4	1.0	ND‡	1.0§	1.0
PG-32:0	16:0/16:0	1.6	ND*	1.2	1.7	1.8	ND†	1.3	1.4	2.0	ND‡	1.5	1.9
PG-33:1	16:0/17:0cyclo	ND	ND	0.8	1.1	ND	ND	0.6	1.2†	ND	ND	ND§	0.2
PG-33:0	17:0-16:0	ND	ND	0.3	0.3	ND	ND	0.2	0.4	ND	ND	ND§	0.1
PG-34:2	16:1/18:1	1.9	1.5	1.5	1.0	2.2	1.5	1.4	1.2	3.9§	5.4‡ 	4.3§	6.6‡
PG-34:1	18:1/16:0	31.3	19.7*	28.6	21.7*	30.3	20.4	27.9	22.6†	33.6	27.2‡ 	29.0	27.2
PG-35:2	18:1/17:0cyclo; 16:1-19:0cyclo	1.9	2.9	2.5	3.0	2.1	3.3†	2.1	3.0	0.6§	ND	0.2§	0.2
PG-35:1	19:0cyclo/16:0	3.0	4.2	3.4	10.0*	2.6	3.7	3.3	6.6†	0.5§	ND	0.3§	0.6
PG-36:2	18:1/18:1	53.2	55.8	51.5	36.3*	54.1	59.1	53.5	42.4†	58.1	67.4‡ 	63.3§	61.6
PG-37:2	19:0cyclo/18:1	6.3	15.2*	9.9	24.7*	6.3	12.0†	9.4	20.8†	0.3§	ND	0.4§	0.5
PG-38:2	19:0cyclo/19:0cyclo	ND	0.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[#]When the assignment of fatty acids to the *sn*-1 and *sn*-2 positions in lipids was ambiguous, the fatty acids were separated by a hyphen instead of a forward slash.*Wild-type under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) from wild-type under normal growth conditions (2 mM P_i or pH 7.0).†*cfa1* mutant under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) from *cfa1* mutant under normal growth conditions (2 mM P_i or pH 7.0).‡*cfa2* mutant under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) from *cfa2* mutant under normal growth conditions (2 mM P_i or pH 7.0).§Under normal growth conditions (2 mM P_i or pH 7.0), *cfa2* mutant is significantly different from wild-type ($P \leq 0.05$).||Under stressed conditions (0 mM P_i or pH 5.5), *cfa2* mutant is significantly different from wild-type ($P \leq 0.05$).

interactions (Grogan & Cronan, 1997). Here we investigated cyclopropanation of *S. meliloti* phospholipids (PCs, PGs, PEs) and two non-phosphorus-containing lipid classes (SLs, TMHSs). We showed that PCs and PGs with fatty acid compositions corresponding to 35:2, 35:1, 37:2 and 38:2 contained primarily *cis*-11,12-methyleneoctadecanoic acid. Lipids with these fatty acid compositions were also observed in non-phosphorus-containing lipid classes (SLs, TMHSs). The preferred substrates of most CFA synthases are lipids containing *cis*-9-hexadecenoic acid and not *cis*-11-octadecenoic acid (Grogan & Cronan, 1997). However, since *cis*-11-octadecenoic acid is the major fatty acid in *S. meliloti*, it is not surprising that lipids containing this fatty acid were the principal CFA synthase substrate. MS/MS analyses of 37:2 lipids in *S. meliloti* showed that *cis*-11,12-methyleneoctadecanoic acid was mostly located in the *sn*-2 position in PCs while in PGs it was located at the *sn*-1 position. PC-37:2 most likely corresponds to a mixture of both isomers, where the 18:1/19:0cyclo isomer is more abundant. Therefore, in *S. meliloti* cyclopropana-

tion of lipids occurred in both the *sn*-1 and *sn*-2 positions. CFA synthases have been reported to cyclopropanate preferentially fatty acyl chains located in the *sn*-2 position over *sn*-1 (Grogan & Cronan, 1997).

The finding that cyclopropanation is drastically reduced in the *S. meliloti cfa2* mutant, together with the sequence similarity of Cfa2 to known cyclopropane fatty acyl synthases, is strong evidence that *cfa2* encodes a cyclopropane fatty acyl synthase. As cyclopropanation of both phospholipids and non-phosphorus-containing lipids was absent in the *cfa2* mutant, we infer that the nature of the polar head group had little effect on Cfa2-mediated cyclopropanation.

Both P_i starvation and acidic conditions resulted in increased cyclopropanation of lipids (10% and 15%, respectively) in *S. meliloti* in all lipid classes examined. In general, higher rates of cyclopropanation in PCs and SLs were reflected by the transformation of 37:2 species to 38:2 species; in PGs and PEs conversion of 37:2 lipids to

Fig. 4. PCA of fatty acid distributions in four lipid classes in *S. meliloti* wild-type and *cfa1* and *cfa2* mutants with P_i starvation and acidic conditions. (a, c) In the scores plots, lipid classes are represented by different-shaped symbols (◆, PCs; ■, PEs; ●, PGs; ▲, SLs). Lipid species are represented by colours (see key above figure). (b, d) In the loadings plots, the P_i starvation study is represented by triangles and the acidity study by circles. Filled symbols represent control conditions (2 mM P_i , pH 7.0) and hollow symbols represent stressed conditions (0 mM P_i , pH 5.5).

38:2 lipids was not observed. To our knowledge, P_i starvation has not previously been linked to increased cyclopropanation; however, acidity has been shown to increase cyclopropanation of lipids by 17% in *E. coli* (Brown *et al.*, 1997), and increased *cfa* transcription under acidic conditions has been reported for *Lactococcus lactis* (Budin-Verneuil *et al.*, 2005). Recently, Rosenthal *et al.* (2008) reported an induction in transcription from the *E. coli* *cfa* promoter in response to acetate. They suggested that accumulation of protons that build up upon acid stress, and other small molecules such as acetate and glutamate that accumulate upon osmotic shock, can influence the transcription complexes at promoters, and as a result can alter the transcription profiles from those promoters. Since we observed increased transcription of both *cfa1* and *cfa2* in *S. meliloti* under acidic conditions but not under P_i starvation conditions, the increased transcription would not seem to be part of a general stress

response. Perhaps the increased cyclopropanation observed in P_i -starved cells is related to the shift in the lipid species composition that occurs in P_i -starved cells.

The role of Cfa1 in *S. meliloti* remains unclear, as it shares considerable sequence similarity with Cfa2 and other bacterial Cfa proteins. Since the *cfa1* and *cfa2* genes were transcribed at similar levels under all growth conditions (Fig. 5), and assuming each is translated, we infer that the Cfa1 protein either has a low activity (<0.1% of Cfa2's activity based on fatty acid composition data) or has a different specificity from Cfa2. *Pseudomonas putida* also has two *cfa*-like genes and the gene designated *cfaB* appears to encode the major CFA synthase activity, as a *cfaB* mutant (containing only *cfaA*) produced less than 2% of the 17:cyclopropane produced by the wild-type strain (Munoz-Rojas *et al.*, 2006). The *S. meliloti* Cfa1 or Cfa2 proteins showed ~42% and ~62% amino acid sequence

Fig. 5. Expression from *cfa1* and *cfa2* promoters in various growth media. Wild-type along with *cfa1* and *cfa2* mutant strains of *S. meliloti* were grown in LBmc rich medium, MOPS-buffered minimal medium with excess and without P_i and M9 minimal medium at pH 7.0 and pH 5.5. Means $\pm$ SD, $n=3$.

identities, respectively, to CFA synthase orthologues in *Agrobacterium tumefaciens*, *Rhizobium etli* and *Rhizobium leguminosarum*, each of which carries only one CFA synthase. Putative orthologues of Cfa have been found in all groups of bacteria; however, the presence of two or more CFA synthases has only been found in a few members of the proteobacteria, including α - (*Sinorhizobium medicae*, *Mesorhizobium* sp. BNC1, *Acidiphilum cryptum*, *Xanthobacter autotrophicus* and *Roseovarius* sp.), β - (*Ralstonia solanacearum*), γ - (*Serratia proteamaculans* and *Pseudomonas putida*), and ϵ -proteobacteria (*Campylobacter jejuni*), as well as among members of the Gram-positive Actinobacteria and Firmicutes (a few *Mycobacterium* species, *Bacillus cereus*, *Clostridium beijerinckii* and *Lactobacillus reuteri*).

The CFA synthase of *E. coli* has been well characterized (Huang *et al.*, 2002; Wang *et al.*, 1992) and the crystal structures of *M. tuberculosis* CFA synthases have been solved (Huang *et al.*, 2002). In amino acid sequence alignments, Cfa1 shared 45 % amino acid sequence identity with Cfa2, and both these proteins shared 35–40 % amino acid identities with the Cfa proteins from *E. coli*, *P. putida* and *M. tuberculosis* proteins (Huang *et al.*, 2002; Munoz-Rojas *et al.*, 2006; Wang *et al.*, 1992). There were several regions that are conserved across all the Cfa proteins. These include amino acids 171–179 (*E. coli* Cfa numbering) corresponding to the motif (V/L)L(E/D)XGXGXG, which has been proposed to play a role in binding of the enzyme cofactor S-adenosylmethionine (Ingrosso *et al.*, 1989). Similarly, residues 130–142 and particularly C-139, which is thought to be involved in interactions with bicarbonate, are conserved (Courtois *et al.*, 2004) (see Supplementary Fig. S1).

The biological functions of CFAs are not clear; however, they are involved in host–pathogen interactions in some

bacteria such as *M. tuberculosis* (Glickman *et al.*, 2000; Rao *et al.*, 2005). In alfalfa plant nodulation assays, the *cfa1* and *cfa2* mutants of *S. meliloti* did not show impaired nodulation or nitrogen fixation (data not shown). These results suggest that the CFAs of *S. meliloti* do not play a significant role in plant–bacteria interactions.

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