SMb20651 is another acyl carrier protein from *Sinorhizobium meliloti*

Ana Laura Ramos-Vega,¹ Yadira Dávila-Martínez,¹ Christian Sohlenkamp,¹ Sandra Contreras-Martínez,² Sergio Encarnación,² Otto Geiger¹ and Isabel M. López-Lara¹

Correspondence

Isabel M. López-Lara
isabel@ccg.unam.mx

¹Programa de Ecología Genómica, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Apdo. Postal 565-A, Cuernavaca, Morelos C. P. 62251, Mexico

²Programa de Genómica Funcional de Procariontes, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Apdo. Postal 565-A, Cuernavaca, Morelos C. P. 62251, Mexico

Acyl carrier proteins (ACPs) are small acidic proteins that carry growing acyl chains during fatty acid or polyketide synthesis. In rhizobia, there are four different and well-characterized ACPs: AcpP, NodF, AcpXL and RkpF. The genome sequence of *Sinorhizobium meliloti* 1021 reveals two additional ORFs that possibly encode additional ACPs. One of these, *smb20651*, is located on the plasmid pSymB as part of an operon. The genes of the operon encode a putative asparagine synthetase (*AsnB*), the predicted ACP (*SMb20651*), a putative long-chain fatty acyl-CoA ligase (*SMb20650*) and a putative ammonium-dependent NAD⁺ synthetase (*NadE1*). When *SMb20651* was overexpressed in *Escherichia coli*, [³H]β-alanine, a biosynthetic building block of 4'-phosphopantetheine, was incorporated into the protein *in vivo*. The purified *SMb20651* was modified with 4'-phosphopantetheine in the presence of *S. meliloti* holo-ACP synthase (*AcpS*). Also, holo-*SMb20651* was modified *in vitro* with a malonyl group by malonyl CoA-ACP transacylase. In *E. coli*, coexpression of *SMb20651* together with other proteins such as *AcpS* and *SMb20650* led to the formation of additional forms of *SMb20651*. In this bacterium, acylation of *SMb20651* with C12:0 or C18:0 fatty acids was detected, demonstrating that this protein is involved in fatty acid biosynthesis or transfer. Expression of *SMb20651* was detected in *S. meliloti* as holo-*SMb20651* and acyl-*SMb20651*.

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INTRODUCTION

Acyl carrier proteins (ACPs) are small acidic proteins that are key cofactors in fatty acid and polyketide syntheses. Acyl intermediates are covalently bound to an ACP, which shuttles them from one enzyme to another. The linkage between fatty acyl groups and ACP is achieved by a thioester bond in which the thiol of the ACP 4'-phosphopantetheine prosthetic group is condensed with the carboxy group of fatty acids. Apo-ACP is converted to the active protein by holo-ACP synthase (*AcpS*), which transfers the 4'-phosphopantetheine from coenzyme A (CoA) to apo-ACP (Flügel *et al.*, 2000; Lambalot *et al.*, 1996), thereby converting it to the functional holo-ACP.

Abbreviations: ACP, acyl carrier protein; *AcpS*, holo-ACP synthase; CTAB, cetyltrimethylammoniumbromide; DOC, sodium deoxycholate.

Two supplementary figures, showing results of RT-PCR analysis demonstrating that *asnB*, *smb20651*, *smb20650* and *nadE1* are encoded in an operon, the scheme of *asnB*, *smb20651*, *smb20650* and *nadE1* arrangement, and a multiple sequence alignment of the *S. meliloti* acyl carrier proteins and selected *SMb20651* homologues, are available with the online version of this paper.

In *Escherichia coli* K-12, the only ACP is *AcpP*. This essential and constitutive protein is involved not only in the synthesis of fatty acyl chains but also in their transfer during phospholipid, lipid A or haemolysin synthesis (reviewed by Byers & Gong, 2007). Acyl-ACP also functions as acyl donor during the synthesis of *N*-acylhomoserine lactones in Gram-negative bacteria (Fuqua & Greenberg, 2002) and of γ-butyrolactones in *Streptomyces* (Kato *et al.*, 2007). Bacteria with complex metabolism often have specialized ACPs and ACP homologues that are involved in the synthesis of secondary metabolites such as polyketides or non-ribosomal peptides (Geiger & López-Lara, 2002; Lai *et al.*, 2006), or in the synthesis of D-alanyl-lipoteichoic acid (Neuhaus & Baddiley, 2003).

Rhizobia have four well-characterized ACPs (Geiger & López-Lara, 2002): *AcpP* for general fatty acid synthesis (Platt *et al.*, 1990; López-Lara & Geiger, 2000), *NodF* for the synthesis of polyunsaturated fatty acids and for their transfer to nodulation factors (Ritsemá *et al.*, 1998), *AcpXL* for the synthesis and transfer of the 27-hydroxyoctacosanoic acid during rhizobial lipid A synthesis (Brozek *et al.*,

1996), and RkpF, which participates in the synthesis of rhizobial capsular polysaccharides (Epple *et al.*, 1998). The genome sequence of *Sinorhizobium meliloti* 1021 (Galibert *et al.*, 2001) reveals the existence of two additional putative ACPs (Geiger & López-Lara, 2002). One of these is encoded by the gene *smb20651* and is located in the plasmid pSymB, forming part of a possible operon of four genes (Fig. 1). The SMB20651 protein was assigned in the genome project as a hypothetical protein. However, it has all the characteristics of an ACP. SMB20651 is small (88 aa) and acidic (isoelectric point 3.8), and has a predicted 4'-phosphopantetheine binding motif. The genes of the operon code for a putative asparagine synthetase (AsnB), the predicted ACP (SMB20651), a putative long-chain (C12–C18) fatty acyl CoA ligase (SMB20650) and a putative ammonium-dependent NAD⁺ synthetase (NadE1).

Here, we present the characterization of the protein SMB20651 as an ACP; SMB20651 incorporates the 4'-phosphopantetheine prosthetic group, can be modified with a malonyl group and can carry C12:0 and C18:0 fatty acyl chains. Although no phenotype was associated with the absence of SMB20651 in *S. meliloti*, different modified forms of SMB20651 could be detected in the wild-type

strain, indicating that the protein is actively participating in an acyl-involving metabolic pathway.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this work and their relevant characteristics are listed in Table 1. *E. coli* strains were grown at 30 °C either in Luria–Bertani (LB) broth (Sambrook & Russell, 2001) or in M9 minimal broth (Miller, 1972), for [³H]β-alanine labelling assays. *S. meliloti* strains were grown at 30 °C either in complex tryptone yeast (TY) broth supplemented with 4.5 mM CaCl₂ (Beringer, 1974) or in one of the following minimal media: M9 supplemented with 2 µg biotin ml⁻¹ and succinate (10 mM) replacing glucose as the carbon source, Sherwood (1970) medium with succinate (8.3 mM) replacing mannitol as the carbon source, or Robertsen *et al.* (1981) medium. In experiments designed to evaluate the influence of the nitrogen source on growth, glutamate in Robertsen medium was replaced by NH₄Cl. In other experiments, glutamate and succinate were replaced by glutamine or asparagine as carbon and nitrogen sources, respectively. To study salt tolerance, cultures were grown in complex TY medium supplemented with 0.1, 0.25, 0.5 or 0.7 M NaCl. Antibiotics were added, when required, to the following final concentrations (µg ml⁻¹): carbenicillin, 100; chloramphenicol, 20; kanamycin, 50; gentamicin, 20; spectinomycin, 200; and tetracycline, 20 for *E. coli*; and neomycin, 200; spectinomycin, 400; streptomycin, 500; and tetracycline, 4 for *S. meliloti*.

Fig. 1. Genetic organization around the gene encoding SMB20651 and around selected SMB20651 homologues in different organisms. Nomenclature used for comparison: *acp*, acyl carrier protein homologous to SMB20651; *CoA-lig.*, long-chain CoA-ligase homologous to SMB20650; *asnB*, putative asparagine synthetase; *nadE1*, putative NAD synthetase; *ppt*, phosphopantetheinyl transferase.

Table 1. Bacterial strains and plasmids used in this study

Kn^R , Cb^R , Cm^R , Gm^R , Spc^R , Tc^R , kanamycin, carbenicillin, chloramphenicol, gentamicin, spectinomycin and tetracycline resistance, respectively. Sm and Ec denote genes from *S. meliloti* 1021 and *E. coli*, respectively. Plasmids pAL03 to pAL06 are described in Methods, Construction of *smb20651* mutants.

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i> strains		
DH5 α	Host used for cloning	Bethesda Research Laboratory
BL21(DE3)	Host used for protein expression	Studier <i>et al.</i> (1990)
OG7001	<i>panD</i> mutant of BL21(DE3), β -alanine auxotroph	Epple <i>et al.</i> (1998)
<i>S. meliloti</i> strains		
1021	Sm^R of SU47 wild-type	Galibert <i>et al.</i> (2001)
ALR30B1	<i>smb20651</i> deletion mutant of <i>S. meliloti</i> 1021, Spc^R	This study
Plasmids		
pET9a	Expression vector, Kn^R	Studier <i>et al.</i> (1990)
pET16b	Expression vector, Cb^R	Studier <i>et al.</i> (1990)
pLysS	Causes repression of T7 polymerase, Cm^R	Studier <i>et al.</i> (1990)
pBBR1MCS-5	Broad-host-range vector, Gm^R	Kovach <i>et al.</i> (1995)
pRK404	Broad-host-range vector, Tc^R	Ditta <i>et al.</i> (1985)
pBluescript SK	Cloning vector, Cb^R	Stratagene
pK18 <i>mobsacB</i>	Suicide vector, Kn^R	Schäfer <i>et al.</i> (1994)
pTB5035	<i>acpS_{Sm}</i> in pET9a	López-Lara & Geiger (2000)
pRJC005	<i>acpS_{Ec}</i> in pBluescript SK	Cox <i>et al.</i> (1997)
pTB1003	<i>rkpF</i> of Rm41 in pET9a	Epple <i>et al.</i> (1998)
pTB5086	6 kb <i>Bam</i> HI fragment carrying <i>S. meliloti</i> 1021 <i>fab</i> cluster, Cb^R	López-Lara & Geiger (2001)
pHY109	Broad-host-range vector containing Spc^R cassette	Østeras <i>et al.</i> (1998)
pAL20651	<i>smb20651</i> in pET9a	This study
pAL02	<i>smb20651</i> in pET16b	This study
pSBA01	<i>acpS_{Sm}</i> in pET16b	This study
pAL20	<i>acpS_{Sm}</i> in pET9a	This study
pAL41	<i>acpS_{Sm}</i> in pBBR1MCS-5	This study
pAL22	<i>smb20651</i> and <i>acpS_{Sm}</i> in pET9a	This study
pAL24	<i>smb20651</i> and <i>acpS_{Ec}</i> in pET9a	This study
pTB5001	<i>rkpF</i> and <i>acpS_{Ec}</i> in pET9a	This study
pAL26	<i>rkpF</i> and <i>acpS_{Sm}</i> in pET9a	This study
pAL30	<i>smb20651</i> (S40A) in pET9a	This study
pAL52	<i>smb20650</i> in pET16b	This study
pAL55	<i>smb20650</i> in pBBR1MCS-5	This study
pCSF07	<i>nodF_{Sm}</i> in pET9a	This study
pSBD02	<i>fabD_{Sm}</i> in pET16b	This study

Plasmids pRK404, pBBR1MCS-5 and pK18*mobsacB* and their derivatives were mobilized into *S. meliloti* strains by triparental mating using the mobilizing plasmid pRK2013, as described elsewhere (Figurski & Helsinki, 1979).

DNA manipulations and RT-PCR analysis. Recombinant DNA techniques were carried out using standard procedures (Sambrook & Russell, 2001). Commercial sequencing of amplified genes by Eurofins Medigenomix (Martinsried, Germany) corroborated the correct DNA sequence. DNA and derived protein sequences were analysed using the National Center for Biotechnology Information (NCBI) BLAST network centre (Altschul *et al.*, 1997). RNA was isolated from 2 ml of culture using TRIzol reagent (Invitrogen) and treated with DNase I (Fermentas) as indicated by the manufacturers. cDNA was synthesized using a RevertAid H Minus First Strand cDNA synthesis kit (Fermentas) as specified by the manufacturer.

Construction of expression plasmids. All genes were amplified from *S. meliloti* 1021 genomic DNA, with the exception of *smc00571* (*fabD*),

which was amplified from plasmid pTB5086 (López-Lara & Geiger, 2001). Using specific oligonucleotides (Table 2), the genes *smb20651*, *smb20650*, *fabD*, *nodF* (*smc0852*) and *acpS* (*smc02654*) were amplified and, after restriction with *Nde*I and *Bam*HI (or *Nde*I and *Bgl*II in the case of *smb20650*), the fragments were cloned into pET9a and/or pET16b (Studier *et al.*, 1990) that had been digested with *Nde*I and *Bam*HI. Names given to the respective expression plasmids are given in Table 1. Plasmid pAL20 was digested with *Bam*HI and *Bgl*II to reclone the *acpS* gene region into the *Bam*HI-digested broad-host-range vector pBBR1MCS-5 (Kovach *et al.*, 1995) to yield plasmid pAL41.

For coexpression of SMB20651 with AcpS from *S. meliloti*, plasmid pAL20 was digested with *Bam*HI and *Bgl*II and the 420 bp fragment carrying *S. meliloti acpS* was cloned into plasmid pAL20651 that had been digested with *Bam*HI, to yield pAL22. Similarly, the *E. coli acpS* gene (*acpS_{Ec}*) was obtained after digesting the plasmid pRJC005 (Cox *et al.*, 1997) with *Bgl*II and then cloning the fragment into *Bam*HI-digested pAL20651 to yield pAL24. Plasmids for coexpression of the protein RkpF from *S. meliloti* Rm41 with *S. meliloti* AcpS or *E. coli*

Table 2. Oligonucleotide primers used in this study

For construction of expression plasmids, the forward primers incorporated an *NdeI* restriction site (underlined) overlapping the start codon of the respective genes. The reverse primers incorporated a *BamHI* (or *BglII* in the case of *smb20650*) restriction site (underlined) after the stop codon.

Primer	Sequence (5'–3')
Primers used for construction of expression plasmids	
smb20651F	AGGAATACATATGACCCAAGCGATTAAGGAC
smb20651R	AAAGGATCCTTACGCCGAGACCGGCACG
acpSF	AGGAATACATATGATCATCGGTATCGGCAG
acpSR	AAGGATCCCTAAACCGTTCCTCGGC
smb20650F	AGGAATACATATGCGGTCGAGCAATTCCTC
smb20650R	AAATAAGATCTCATTCTGCGGGCTCCAATG
fabDF	AGGAATACATATGAGCATCGCATTACGTTCC
fabDR	AAAGGATCCTCAGGCCAAGAGGACGGCAAG
nodFF	AGGAATACATATGGTAGATCAACTCGAAAG CGAAATC
nodFR	AAAGGATCCTCAAGCCCCCTTAGTGAGCAA GCCTCG
Primers used for mutagenesis	
oAL3	GCGCCCGGGACATCGCCAGGGACTTTCCC
oAL4	TAAGGATCCCCTTTTTTCCAAGGGTTTTACGC
oAL5	GTCGGATCCGGCATCACCATCATGCGGTTCC
oAL6	CATCTAGACCGCGGTGTTGGAGAGATAACG
oAL100	ACGACATCATCGATGCGACCGCGGTTCTG
oAL101	CAGAACGCCGGTCGCATCGATGATGTC
Other	
pet16bup	AACTGCAGGGGAATTGTGAGCGGATAAC

AcpS, were constructed in an analogous way to that described for pAL22 and pAL24. The resulting plasmids were pAL26 and pTB5001. All coexpression plasmids had the genes for the ACP and for AcpS in the same orientation.

In order to clone *smb20650* into pBBR1MCS-5, the primers smb20650R and pet16bup (Table 2) were used to amplify the gene from pAL52. The PCR product was digested with *XbaI* and *BglII* and cloned into pBBR1MCS-5 that had been digested with *XbaI* and *BamHI*, resulting in plasmid pAL55.

Site-directed mutagenesis of SMb20651. The single-site mutant of SMb20651 S40A was generated by the PCR overlap extension method (Ho *et al.*, 1989). The oligonucleotide primers oAL3 and oAL101 (Table 2) were used to amplify 1112 bp of genomic DNA spanning from upstream of *smb20651* to 129 bp downstream of the *smb20651* start codon. Similarly, the oligonucleotide primers oAL100 and oAL6 (Table 2) were used to amplify 1003 bp of genomic DNA spanning from 101 bp downstream of the *smb20651* start codon to downstream of *smb20651*. The primers oAL101 and oAL100 overlap in their DNA binding region and introduce a point mutation (G to T) in nucleotide 118, causing a serine to alanine substitution in residue 40 of the SMb20651 protein. The two PCR products were used as a template for a new PCR and after heating them for 1 min at 92 °C, they were annealed by incubation for 4 min at 60 °C followed by 6 min at 72 °C. Then, the oligonucleotide primers smb20651F and smb20651R (Table 2) were added and the PCR was continued following standard procedures. After digestion with *NdeI* and *BamHI* enzymes, the 267 bp product was cloned into pET9a expression vector that had been digested with the same enzymes. The resulting plasmid was named pAL30.

Construction of *smb20651* mutants. The oligonucleotide primers oAL3 and oAL4 (Table 2) were used in a PCR to amplify 992 bp of genomic DNA upstream of the *smb20651* gene from *S. meliloti*, introducing *SmaI* and *BamHI* sites (underlined in the table) into the

PCR product. Similarly, the primers oAL5 and oAL6 (Table 2) were used to amplify 868 bp of genomic DNA downstream of *smb20651*, introducing *BamHI* and *XbaI* sites (underlined in the table) into this PCR product. After digestion with the respective enzymes, the 992 bp fragment was cloned into pBluescript SK plasmid that had been digested with *SmaI* and *BamHI*, yielding plasmid pAL03. Then, the 868 bp fragment was cloned as a *BamHI/XbaI* fragment into pAL03 plasmid that had been digested with *BamHI* and *XbaI*, yielding plasmid pAL04. A spectinomycin-resistance cassette obtained as a *BamHI* fragment from plasmid pHY109 (Østeras *et al.*, 1998) was inserted into pAL04 that had been digested with *BamHI* to yield pAL05. Plasmid pAL05 was digested with *SmaI* and *XbaI* to reclone the regions usually flanking *smb20651* and the spectinomycin-resistance gene located between those regions as a *SmaI/XbaI* fragment into the suicide vector pK18mobsacB (Schäfer *et al.*, 1994) that had been digested with *SmaI* and *XbaI* to yield pAL06. Plasmid pAL06 was introduced into wild-type *S. meliloti* 1021, and double recombinants in which the spectinomycin-resistance cassette replaced *smb20651* were obtained following a procedure described previously (Sohlenkamp *et al.*, 2004). The mutation was transduced back into *S. meliloti* 1021 using ϕ M12 as described by Finan *et al.* (1984), and the mutant strain was named ALR30B1.

Analysis of proteins. Proteins were analysed by 20 % native PAGE (Jackowski & Rock, 1983) or by conformation-sensitive gels (Post-Beittenmiller *et al.*, 1991) containing 16.8 % polyacrylamide in the separation gel and 3.2 M urea in both the separation and stacking gel. Samples were mixed with application buffer to give final concentrations of 7.9 % (v/v) glycerol before they were loaded onto polyacrylamide gels. Gels were stained with Coomassie blue. Protein concentrations were determined by the method of Duley & Grieve (1975) using BSA (Sigma) as standard. For hydroxylamine treatment, proteins were incubated with 100 mM hydroxylamine hydrochloride, pH 6.5, in the presence of 50 mM DTT at 25 °C for 2 h.

In vivo labelling of SMB20651 and RkpF with [^3H] β -alanine. *In vivo* labelling of ACPs with [^3H] β -alanine, the biosynthetic precursor of 4'-phosphopantetheine, was carried out essentially as described previously using the β -alanine auxotroph OG7001 (Epple *et al.*, 1998; López-Lara & Geiger, 2000).

Overexpression of proteins in *E. coli*. The strains were grown at 30 °C in 500 ml LB medium supplemented with the appropriate antibiotics. At OD₆₂₀ 0.3, IPTG was added to a final concentration of 100 μM . Cells were harvested by centrifugation and cell pellets were resuspended either in ice-cold buffer A (50 mM Tris/HCl, pH 6.8), or in ice-cold start buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4) when used for subsequent chromatography on Ni^{2+} agarose. The cells were broken by three passages through a French press at 138 MPa. Broken cell suspensions were centrifuged for 10 min at 15 550 *g* and the supernatants were recovered. His-AcpS was overproduced as inclusion bodies, in which case the pellet was recovered after centrifugation.

Purification of SMB20651 and NodF. Cold 2-propanol was added dropwise to the cell-free protein extracts until a final concentration of 50 % (v/v) was reached. After incubation for 90 min, the precipitate was removed by centrifugation at 15 550 *g* for 20 min and the final 2-propanol concentration was adjusted to 10 % with buffer A. The supernatant was then applied to a 5 ml DEAE-52 cellulose (Whatman) column, which had been equilibrated with buffer B (50 mM Bistris/HCl, pH 6.8). The column was washed with 25 ml buffer B and elution was performed with a step-wise gradient of 0.2, 0.5, 1 and 2 M NaCl in buffer B. Fractions (1 ml) were collected and aliquots were analysed by native or conformation-sensitive PAGE as required. The SMB20651, NodF and acylated SMB20651 proteins eluted with the addition of 0.5 M NaCl. Fractions containing the respective ACP were individually applied to a PrepCell (model 491, Bio-Rad) containing a cylindrical 20 % native polyacrylamide gel in the case of SMB20651 and NodF, and a 16.8 % polyacrylamide gel containing 3.2 M urea in the case of acylated SMB20651. Fractions (3 ml each) were collected and SMB20651- or NodF-containing fractions were concentrated by ultrafiltration at 4 °C. The buffer was changed to 10 mM Tris/HCl, pH 8.0, for enzymic preparation of holo-SMB20651 and holo-NodF. Samples containing acylated SMB20651 were dried and analysed for fatty acid content at the Bacterial Identification & Fatty Acid Analysis Laboratory, Plant Pathology Department, University of Florida, Gainesville.

For large-scale SMB20651 enrichment from *S. meliloti*, 10 l cultures were grown on TY at 30 °C to OD₆₂₀ 0.7. Purification steps were as described above with the following differences: cold 2-propanol was added to 30 % (v/v) final concentration and elution was performed with a linear gradient (0–2 M NaCl) from a DEAE-Sepharose column (Pharmacia Biotech). The fractions containing SMB20651 were pooled and passed through a YM-50 filter device with a molecular weight cutoff of 50 000 Da. Then, the filtrate was desalted and concentrated with a YM-3 filter device with a molecular weight cutoff of 3000 Da. A similar mock purification was carried out using *smb20651*-deficient-strain ALR30B1.

Purification of His-tagged proteins. The cell-free crude extract of the *E. coli* strain overproducing His-SMB20651 was first fractionated with 60 % 2-propanol, and the 2-propanol was then eliminated by rotatory evaporation. The inclusion bodies consisting of His-AcpS protein were washed twice in start buffer, then dissolved in start buffer containing 6 M guanidine hydrochloride and centrifuged at 6300 *g*. The samples containing His-SMB20651 or AcpS prepared as described above or the cell-free extract of the strain overproducing His-FabD were applied to a 1 ml Ni^{2+} agarose column (Hi Trap, Pharmacia Biotech). Ni^{2+} chromatography was performed according to the manufacturer's instructions. In the case of His-AcpS all buffers

contained 6 M guanidine hydrochloride. The three proteins were eluted with 500 mM imidazole in start buffer. After concentration with Amicon devices (Millipore), aliquots of purified His-AcpS and His-FabD were kept in sodium phosphate buffer containing 14.3 % (v/v) glycerol at –80 °C.

Generation of polyclonal antiserum against the ACP SMB20651 and Western blot analysis. Antiserum against purified His-SMB20651 was raised in a white New Zealand rabbit that had received four injections, each of a 1 : 1 mixture of Freud's complete or incomplete adjuvant (Roche) together with 150 μg His-SMB20651 antigen protein in 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4 .

For immunodetection of SMB20651, protein samples were separated by PAGE and electrophoretically transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences). Membranes were blocked with 5 % non-fat milk in PBS (150 mM NaCl, sodium phosphate, pH 7.2) and then incubated with anti-His-SMB20651 serum diluted either 1 : 1000 or 1 : 100 for *E. coli*- or *S. meliloti*-derived protein samples, respectively. Anti-rabbit immunoglobulin antibodies (alkaline phosphatase conjugate) were used as secondary antibodies. Reaction results were visualized with Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets according to the manufacturer's recommendations.

In vitro modification of apo-ACPs to holo-ACPs. For preparation of holo-SMB20651 and holo-NodF, the purified proteins were incubated with CoA in the presence of *S. meliloti* His-AcpS. Reactions were performed in 10 mM Tris/HCl, pH 8.0, containing 10 mM DTT, 5 mM MgCl_2 , 300 μM CoA, 40.6 μM ACP and 2.3 μM His-AcpS in a final volume of 50 μl . Reactions were incubated at 30 °C for 4 h. MALDI-TOF MS was performed using a Bruker Daltonics Autoflex mass spectrometer operated in the linear mode. Spectra were externally calibrated using a protein calibration standard (Bruker Daltonics 206295). Protein mixtures were desalted using a C18 ZipTip (Millipore) and analysed using a saturated solution of sinapinic acid in 50 % (v/v) acetonitrile and 0.1 % (v/v) trifluoroacetic acid.

In vitro malonylation of holo-SMB20651 and holo-NodF. Reactions were performed in 50 mM sodium phosphate buffer, pH 7.2, containing 2 mM DTT, 2 mM EDTA, 0.1 mM FeSO_4 , 25 mM MgSO_4 , 17 μM ACP, 6.2 μM His-FabD and 1 mM malonyl-CoA in a final volume of 30 μl . Reactions were incubated at 30 °C for 1 h. In assays involving radioactive malonyl-CoA, 6105 Bq [^{14}C]malonyl-CoA (specific activity 4.07×10^8 Bq mmol^{-1} ; American Radiolabelled Chemicals) were used per reaction in a final volume of 15 μl . Malonyl-ACP samples were separated by conformation-sensitive PAGE. Radiolabelled samples were also transferred to a nitrocellulose membrane, and the incorporation of the malonyl group was demonstrated by autoradiography.

Filter-disk assays. Zones-of-inhibition assays were performed as described elsewhere (Ferguson *et al.*, 2002). A 5 μl aliquot of the stock agents 10 % SDS, 70 mg gentamicin ml^{-1} , 2 % sodium deoxycholate (DOC), 10 % cetyltrimethylammoniumbromide (CTAB), 10 % Triton X-100 or 10 % Brij 58 was applied to the disk. For the polymyxin B assays, commercial test disks (300 units per disc, Oxoid) were used. At least three repetitions were made for each agent tested. Zones of inhibition were measured after 2 days of growth at 30 °C.

Plant assays. Alfalfa (*Medicago sativa* L.) plants were grown in a nitrogen-free medium as described elsewhere (Olivares *et al.*, 1980). Nitrogen fixation was determined by examining acetylene reduction using GC (Burris, 1972).

RESULTS

smb20651 is part of a four-gene operon

smb20651 is located on plasmid pSymB as part of a possible operon of four genes (Fig. 1). We designed primers to amplify the intergenic regions of the adjacent genes, using RT-PCR. Three intergenic regions between adjacent genes (*asnB*, *smb20651*, *smb20650* and *nadE1*) were amplified (Supplementary Fig. S1) from the RNA samples of *S. meliloti* 1021 cells grown on TY. These results indicate that the four genes (*asnB*, *smb20651*, *smb20650* and *nadE1*) form an operon.

Interestingly, while the same operon is conserved in the genomes of the rhizobial species *Sinorhizobium medicae* WSM419, *Mesorhizobium* sp. BNC1 and *Mesorhizobium huakuii* MAFF 303099 (Fig. 1), it is absent in the genomes of rhizobia from the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*. The four genes forming the operon are also present in the α -proteobacterium *Rhodopseudomonas palustris* BisA53. Remarkably, the operon is also found in phylogenetically unrelated bacteria such as *Planctomyces*, *Roseiflexus* and *Geobacter*, and even in the archaeon *Methanosarcina* (Fig. 1). Parts of the operon are conserved in other species, and the most frequent association is between the genes encoding the putative ACP and a potential long-chain fatty acyl-CoA ligase (Fig. 1), suggesting that SMb20650 homologues could be involved in loading the respective putative SMb20651 ACPs with acyl chains.

Efficient phosphopantetheinylation of SMb20651 occurs only in the presence of extra AcpS

ACPs are functional when substituted with the 4'-phosphopantetheine prosthetic group. Cell-free extracts of *E. coli* OG7001(pLysS) carrying either empty pET9a, or plasmids that allow overproduction of SMb20651 or RkpF that had been grown in the presence of [3 H] β -alanine, a biosynthetic precursor of 4'-phosphopantetheine, were analysed by PAGE and subsequent autoradiography. Although the SMb20651 protein was highly overproduced (Fig. 2a, lane 2), incorporation of [3 H] β -alanine was not detected (Fig. 2b, lane 2), indicating poor or lack of holo-SMb20651 production. In contrast, efficient production of holo-RkpF was detected (Fig. 2b, lane 6). ACPs are phosphopantetheinylated by enzymes of the phosphopantetheinyl transferase (PPT) family. To test whether SMb20651 requires *S. meliloti* PPT AcpS (SMc02654) for modification, we constructed plasmids that allow the coexpression *in cis* of SMb20651 together with either *E. coli* AcpS or *S. meliloti* AcpS. As controls, we made the same constructions with RkpF, which is an ACP from *S. meliloti* Rm41 (Epple *et al.*, 1998). Although under coexpression conditions we detected much less SMb20651 or RkpF protein (Fig. 2a, lanes 1, 3, 5 and 7), both proteins were efficiently labelled with [3 H] β -alanine (Fig. 2b, lanes 1, 3, 5 and 7). Surprisingly, the over-

Fig. 2. Overproduction of *S. meliloti* ACPs in *E. coli* OG7001 (pLysS). Analysis of cell-free extracts from *E. coli* strains overproducing SMb20651 and RkpF. (a) Proteins separated by 20 % native PAGE and stained with Coomassie blue. (b) Autoradiogram after labelling with [3 H] β -alanine. (a, b) Cell-free extracts of *E. coli* overproducing: SMb20651 and AcpS_{Ec} (lane 1), SMb20651 (lane 2), SMb20651 and AcpS_{Sm} (lane 3), nothing (*E. coli* strain carrying pET9a) (lane 4), RkpF and AcpS_{Ec} (lane 5), RkpF (lane 6), and RkpF and AcpS_{Sm} (lane 7). The bands corresponding to SMb20651, RkpF and the *E. coli* AcpP are indicated.

expression of either AcpS of *S. meliloti* or AcpS of *E. coli* can modify SMb20651, indicating that the level of AcpS in wild-type *E. coli* is not enough to modify SMb20651, or that *E. coli* AcpS does not work efficiently with SMb20651. The latter could be due to the presence of the sequence motif DST (where S is the 4'-phosphopantetheine binding site) instead of the DSL sequence present in *E. coli* and other sinorhizobial ACPs (Supplementary Fig. S2).

Serine 40 is necessary for 4'-phosphopantetheine binding to SMb20651

SMb20651 carrying the S40A change in the conserved serine of the predicted 4'-phosphopantetheine binding site was coexpressed *in trans* together with *S. meliloti* AcpS, and *in vivo* labelling with [3 H] β -alanine was performed. The amount of SMb20651 (S40A) present in the crude extract was similar to that found when SMb20651 is overexpressed alone (Fig. 3a, b, lane 4) but it could no longer be labelled with [3 H] β -alanine (Fig. 3c, lane 4), indicating that serine 40 is necessary for 4'-phosphopantetheine binding. SMb20651 (S40A) migrates similarly in native PAGE to

Fig. 3. Serine 40 is required for 4'-phosphopantetheinylation of SMB20651. (a) Analysis of cell-free extracts from *E. coli* strains overproducing SMB20651. Proteins were separated by 20 % native PAGE and stained with Coomassie blue. (b) Western blot detection of SMB20651 using anti-His-SMB20651 antibodies. (c) Autoradiogram of cell-free extracts of *E. coli* strains overproducing SMB20651 after labelling with [^3H]-alanine. In (a), (b) and (c), cell-free extracts of *E. coli* are shown overproducing SMB20651 and AcpS_{Sm} *in cis* (lane 1), SMB20651 (lane 2), SMB20651 and AcpS_{Sm} *in trans* (lane 3), and SMB20651(S40A) and AcpS_{Sm} *in trans* (lane 4). Overexpression *in trans* was achieved by expressing in *E. coli* derivatives of pET9a and pBBR1MCS-5 carrying *acpS_{Sm}* (pAL41). The bands corresponding to SMB20651 and *E. coli* AcpP are indicated.

wild-type SMB20651, and therefore the exchange S to A probably does not affect the overall folding and conformation of this protein (Fig. 3, lane 4).

***In vitro* 4'-phosphopantetheine transfer to SMB20651**

Analysis of purified SMB20651 by MALDI-TOF MS showed an *m/z* of 9307.3 and one of 9645.5 for

SMB20651 that had been incubated with His-AcpS (Fig. 4). These values are in close agreement with the predicted masses for apo-SMB20651 (9305.51) and holo-SMB20651 (9644.51) lacking the initial methionine. The difference of 338.2 atomic mass units (a.m.u.) is consistent with the expected 339 a.m.u. for the 4'-phosphopantetheine addition, indicating that His-AcpS-treated SMB20651 is carrying the prosthetic group typical of ACPs. Therefore, AcpS attaches 4'-phosphopantetheine to SMB20651.

Fig. 4. *In vitro* 4'-phosphopantetheinylation of SMB20651 observed by MALDI-TOF MS. Spectra of SMB20651 incubated with CoA in the absence (a) or presence (b) of purified *S. meliloti* His-AcpS. Intensity is given in arbitrary units (a.u.).

SMB20651 is malonylated by FabD

Malonyl CoA:ACP transacylase (FabD) is the enzyme responsible for the transfer of the malonyl group from malonyl-CoA to holo-ACP. The resulting product is an intermediate in the initiation and elongation steps during fatty acid and polyketide synthesis. We examined the *in vitro* transfer of the malonyl moiety from malonyl-CoA to SMB20651 by *S. meliloti* His-FabD. Conformation-sensitive electrophoresis of the reaction products shows a slower-migrating form of SMB20651 that appeared only when the protein was incubated with malonyl-CoA (Fig. 5a, lanes 1 and 2). It has been shown that NodF from *Rhizobium leguminosarum* bv. *viciae* can be malonylated *in vitro* using the enzyme FabD from *E. coli* (Ritsema *et al.*, 1998), and therefore we used *S. meliloti* NodF as a positive control in this study. Although no conformational change was observed when NodF_{Sm} was incubated with malonyl-CoA and FabD_{Sm} (Fig. 5b, lanes 1 and 2), the radioactive assays confirmed the incorporation of the malonyl group into both ACPs (Fig. 5, lanes 4).

Fig. 5. Malonylation of SMb20651 (a) or NodF (b) by FabD. Proteins were separated by conformation-sensitive PAGE. (a, b) Lanes 1 and 2, Coomassie blue-stained proteins; lanes 3 and 4, autoradiogram. (a, b) The proteins are: holo-ACP assayed without (lane 1) or with malonyl-CoA (lane 2), holo-ACP assayed without (lane 3) or with [^{14}C]malonyl-CoA (lane 4). The positions of malonyl-SMb20651 (malonyl) and holo-SMb20651 (holo) are indicated.

SMb20651 is modified in *E. coli* when it is coexpressed with *S. meliloti* enzymes

SMb20650 is a predicted long-chain fatty acyl-CoA ligase and its gene is cotranscribed with the gene for SMb20651. Although apo- and holo-SMb20651 show the same mobility in 20% native PAGE, they run significantly differently in conformation-sensitive gels (Fig. 6, lanes 1 and 2), and possible acylated forms are expected to have different mobilities in this type of gel (Post-Beittenmiller *et al.*, 1991). To test whether SMb20650 could be involved in the acylation of SMb20651, we coexpressed in *E. coli* the protein SMb20651 together with AcpS_{Sm} and SMb20650 (by means of the plasmids pAL22 and pAL55). As controls, we expressed SMb20651 alone or together with AcpS_{Sm} using plasmid pAL22. After DEAE-52 cellulose purification, the resulting proteins were analysed by conformation-sensitive PAGE. The *E. coli* strain carrying plasmid pAL20651 produces apo-SMb20651 (Fig. 6, lane 4). Surprisingly, besides the expected holo form (mainly migrating as a dimer), possible modified SMb20651 species were also detected when the protein was coexpressed with AcpS alone (Fig. 6, lane 5). When SMb20651 was coexpressed with AcpS and SMb20650, we detected two additional forms that are possibly acyl-SMb20651 (indicated by asterisks in Fig. 6, lane 6). The presence of the modification makes the proteins more compact so that they run significantly faster in these gels. After hydroxylamine treatment, which is known to cleave thioester linkages, all the possible modifications on SMb20651 disappeared, while holo-SMb20651 was increased (Fig. 6a, lanes 8 and 9), indicating that the hydroxylamine-sensitive forms of SMb20651 carry thioester-linked acylations. The identity of the proteins detected in *E. coli* as SMb20651 species was confirmed by Western blot analysis (Fig. 6b). These results suggest that *E. coli* enzymes can modify holo-SMb20651. GC analysis of CellPrep-purified SMb20651 obtained from *E. coli* BL21(DE3) (pLysS) (pAL22) (pAL55) revealed the presence of C12:0 and C18:0 fatty acids that were not detected in a similarly purified fraction obtained from the strain *E. coli* BL21(DE3) (pLysS) (pET9a) (data not shown).

Different forms of SMb20651 are detected in *S. meliloti* 1021

SMb20651 is expressed in *S. meliloti* 1021 growing in complex TY and in minimal M9 medium in the exponential and stationary phases of growth (data not shown). SMb20651 was partially purified by DEAE-Sepharose chromatography from cell-free extracts obtained from *S. meliloti* 1021 cultures, and a similar mock purification was carried out from cell-free extracts of *S. meliloti* ALR30B1 as a control. When the *S. meliloti* SMb20651-enriched fractions were analysed by conformation-sensitive PAGE and tested with anti-His-SMb20651, five different SMb20651 forms were detected (Fig. 7b, lane 4). Presumably these forms could be holo- and modified species of holo-SMb20651. Two of these SMb20651 forms migrate slower than the possible holo-SMb20651; the other two migrate faster than the possible holo-SMb20651. No signal was detected in the mock purification of the control ALR30B1 (Fig. 7b, lanes 6 and 7). After hydroxylamine treatment, the possible modified proteins disappeared, while holo-SMb20651 was increased (Fig. 7b, lane 5). These results strongly indicate that SMb20651 is acylated in *S. meliloti*. Interestingly, the protein band indicated as X in lanes 4 and 5 (Fig. 7a) was obtained only in the enrichment procedure with *S. meliloti* 1021. This protein is not recognized by anti-His-SMb20651, is not sensitive to hydroxylamine treatment and is detected only in the presence of SMb20651. After hydroxylamine treatment of enriched samples of either wild-type or SMb20651 mutant, a fast doublet migrating band appeared (Fig. 7a, lanes 5 and 7). This band is probably the result of deacylation of different AcpP forms, since these doublets migrate similarly to purified holo-AcpP from *S. meliloti* 1021 (Fig. 7a, lane 8). From the results shown in lane 5 (Fig. 7a), it can be concluded that SMb20651 is far less abundant than AcpP, which is known to be one of the most abundant soluble proteins in the bacterial cell.

Phenotypic characterization of SMb20651 mutants

The mutant ALR30B1 presented no growth differences with respect to the wild-type 1021 when grown on TY

Fig. 6. Analysis of DE52-enriched SMb20651 proteins from different *E. coli* BL21(DE3) (pLysS) host strains. Proteins were partially purified by anion exchange on a DE52-cellulose column and separated by conformation-sensitive PAGE. (a) Coomassie blue staining of proteins. (b) Western blot detection using anti His-SMb20651 antibodies. The proteins analysed are: purified apo-SMb20651 (lane 1), purified holo-SMb20651 (lane 2); enrichment from *E. coli* BL21(DE3) (pLysS) overexpressing: nothing (empty pET9a plasmid) (lane 3), SMb20651 (lane 4), SMb20651 and AcpP_{Sm} (lane 5), SMb20651, AcpP_{Sm} and SMb20650 (lane 6); lanes 7, 8 and 9 contain the same samples as in lanes 4, 5 and 6, respectively, after hydroxylamine treatment. The two asterisks in lane 6 indicate the bands specifically formed after coexpression of the three proteins in *E. coli*, while the diamonds in lanes 5 and 6 indicate the band formed after coexpression of SMb20651 and AcpS. The positions of the dimer, apo and holo forms of SMb20651 are indicated.

medium or in any of the minimal media (M9, Robertsen or Sherwood). In addition, no differences were found when using different nitrogen sources for growth or TY medium with different concentrations of NaCl. Using filter-disk assays, no difference was found in sensitivity to the detergents SDS, DOC, CTAB, Triton X-100 and Brij 58, nor to the antibiotics gentamicin and polymyxin.

Alfalfa plants were inoculated with either ALR30B1 or the parental 1021 strain, and no significant differences in the number of nodules or in the course of nodule development

Fig. 7. Analysis of DEAE-enriched proteins from *S. meliloti* 1021 and ALR30B1. Proteins were separated by conformation-sensitive PAGE. (a) Coomassie blue staining of proteins. (b) Western blot detection using anti His-SMb20651 antibodies. The proteins analysed are: purified apo-SMb20651 (lane 1), purified holo-SMb20651 (lane 2), enrichment from *E. coli* BL21(DE3) pLysS overexpressing SMb20651, AcpP_{Sm} and SMb20650 (lane 3), enrichment from *S. meliloti* 1021 (lane 4), enrichment from *S. meliloti* 1021 followed by hydroxylamine treatment (lane 5), enrichment from *S. meliloti* ALR30B1 (lane 6), enrichment from *S. meliloti* ALR30B1 followed by hydroxylamine treatment (lane 7), purified holo-AcpP_{Sm} (lane 8). X indicates a protein band in lanes 4 and 5 that is present only in the enrichment from wild-type *S. meliloti*. The running positions of the dimer, apo and holo forms of SMb20651 as well as *S. meliloti* AcpP (AcpP_{Sm}) are indicated. The brackets in lanes 3 and 4 enclose different forms of SMb20651 after overexpression of three proteins in *E. coli* and those observed in wild-type *S. meliloti*, respectively.

were observed. After 40 days, the nitrogen-fixing ability was measured and no significant differences were observed.

DISCUSSION

S. meliloti has four well-characterized ACPs, one for the synthesis and transfer of essential fatty acids, and three

specialized ACPs whose functions are related to symbiotic interaction between rhizobia and their host plants (Geiger & López-Lara, 2002). Here, we present the characterization of SMb20651 as the fifth functional ACP of *S. meliloti*. Importantly, SMb20651 can be loaded with a malonyl residue, and the malonyl-ACP product is the building block in fatty acid synthesis and in the synthesis of some polyketides. In cultures of *S. meliloti*, holo and acylated forms of SMb20651 were detected, indicating that SMb20651 is metabolically active, probably in the formation and transfer of a fatty acid for which the function or final target molecule is not yet known. So far, the relevance of this pathway cannot be established, since we could not find any phenotype associated with the deletion of SMb20651, either in free-living bacteria or in symbiosis with alfalfa plants. In earlier expression profile experiments (Becker *et al.*, 2004), it has been found that both *smb20651* and *smb20650* are considerably repressed during symbiosis. Also, *nadE1*, *smb20650* and *smb20651* are repressed under iron-limiting conditions (Chao *et al.*, 2005). In another study, Domínguez-Ferreras *et al.* (2006) showed repression of *smb20651* and *smb20650* after osmotic shock with NaCl, although in RT-PCR experiments *smb20651* was induced twofold. While all these data confirm that *smb20651* and *smb20650* are part of an operon and that their products might function together, they offer no clue to the possible function of SMb20651. *smb20651* is part of an operon with *asnB* (encoding a putative asparagine synthetase) and *nadE1* (encoding a putative ammonium-dependent NAD⁺ synthetase). However, the operon is not essential for asparagine or NAD synthesis, since the mutant in *smb20651* can grow in minimal media.

The coexpression of SMb20651 with either *E. coli* AcpS or *S. meliloti* AcpS (SMc02654) leads to efficient *in vivo* labelling with [³H]-alanine, but formation of holo-SMb20651 goes in parallel with reduced growth of the expressing strain and a remarkable reduction of the total amount of ACP present. The source of this seemingly toxic effect is probably the products formed by the coexpression, because expression of either SMb20651 alone or of AcpS together with SMb20651 (S40A), which cannot be phosphopantetheinylated, does not cause growth inhibition. Furthermore, we have observed that after coexpression of SMb20651 and AcpS in *E. coli*, a significant proportion of SMb20651 is modified not only to the holo form but also to acylated forms (Fig. 6). It is possible that by means of this modification the *E. coli* strain is being depleted of some essential substrates. The toxic effect is also observed after coexpression of RkpF and AcpS, and it would be interesting to study the molecular mechanism that causes this toxicity.

The overall amino acid sequence identity of SMb20651 to the other ACPs of *S. meliloti* ranges from 10 to 31 %, and a conserved amino acid region can only be observed around the phosphopantetheine binding site (Supplementary Fig. S2). With 31 % identity, AcpP is the most similar of the *S. meliloti* ACPs to SMb20651. However, among the ACP

homologues to SMb20651, regions of conserved amino acids are observed along the whole sequence of the proteins and cluster mainly in three regions (Supplementary Fig. S2). If SMb20651 is most similar to AcpP, it might replace AcpP in some of its functions.

SMb20650 is predicted to be a long-chain fatty acyl-CoA ligase (FadD), which activates acyl groups to acyl-CoAs. However, our results indicate that SMb20650 plays a role in the transfer of a specific product, presumably a long-chain fatty acid, to the ACP SMb20651. The soluble acyl-ACP synthetase (AasS) of *Vibrio harveyi* B392 links fatty acids to ACP and is a member of the medium-chain acyl-CoA synthetase family (Jiang *et al.*, 2006). AasS of *V. harveyi* shows only 25 % identity to SMb20650 and therefore they are only distantly related.

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