SMc01553 is the sixth acyl carrier protein in *Sinorhizobium meliloti* 1021

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Acyl carrier proteins (ACPs) are required for the transfer of acyl intermediates during fatty acid and polyketide syntheses. In *Sinorhizobium meliloti* 1021 there are five known ACPs: AcpP, NodF, AcpXL, the ACP domain in RkpA and SMb20651. The genome sequence of *S. meliloti* 1021 also reveals the ORF SMc01553, annotated as a putative ACP. *smc01553* is part of a 6.6 kb DNA region that is duplicated in the chromosome and in the pSymb plasmid, the result of a recent duplication event. SMc01553 overexpressed in *Escherichia coli* was labelled *in vivo* with [³H]β-alanine, a biosynthetic building block of the 4'-phosphopantetheine prosthetic group of ACPs. The purified SMc01553 was modified with 4'-phosphopantetheine in the presence of *S. meliloti* holo-ACP synthase, and this modification resulted in a major conformational change of the protein structure, since the holo-form runs faster in native PAGE than the apo-form. SMc01553 could not be loaded with a malonyl group by malonyl-CoA-ACP transacylase from *S. meliloti*. Using RT-PCR we could show the presence of mRNA for SMc01553 and of the duplicated ORF SMb22007 in cultures of *S. meliloti*. However, a mutant in which the two duplicated regions were deleted did not show any different phenotype with respect to the wild-type in the free-living or symbiotic lifestyle.

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INTRODUCTION

Acyl carrier proteins (ACPs) are small acidic proteins containing the 4'-phosphopantetheine prosthetic group, to which fatty acyl chains are linked as thioesters during fatty acid and polyketide syntheses. ACPs act as carriers of acyl intermediates, which are shuttled from one enzyme to another. 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. ACPs exist as a domain within large multifunctional fatty acid or polyketide synthase (PKS) proteins (type I), or as a small monomeric protein in the system for fatty acid biosynthesis in bacteria and plastids [type II fatty acid synthase (FAS)] and in the dissociated PKSs (type II PKS). Acyl-ACPs in bacteria appear to interact with a great diversity of enzymes to provide a broad array of products containing fatty acids,

such as phospholipids, lipid A, acylhomoserine lactones and nodulation factors (reviewed by Byers & Gong, 2007).

The fatty acyl chains in primary metabolism and the scaffolds of polyketide secondary metabolites are assembled by elongation of a small number of carboxylic acid building blocks. Malonyl-CoA is the extender unit in fatty acid biosynthesis. In the type II FAS, the enzyme malonyl-CoA:ACP transacylase (FabD) transfers the malonyl group from malonyl-CoA to holo-ACP, resulting in the formation of malonyl-ACP, which enters each elongation step (Rock, 2008). The FabD enzyme of *Streptomyces glaucescens*, which catalyses the conversion of malonyl-CoA to malonyl-ACP for fatty acid biosynthesis, has been shown to be also active with the ACPs of type II PKS (Florova *et al.*, 2002). Polyketides comprise a large class of structurally diverse natural products and, although malonyl-CoA is a common extender unit used by all types of PKSs, one important source of the structural diversity of polyketides is the variety of extender units used. These extender units can be divided into two classes depending upon whether they are linked to CoA or to an ACP (reviewed by Chan *et al.*, 2009). The set of genes required for the biosynthesis of the ACP-linked extender units are usually found within or

Abbreviations: ACP, acyl carrier protein; DOC, sodium deoxycholate; FAS, fatty acid synthase; KPS, K-antigen; MBOAT, membrane-bound O-acyltransferase; PKS, polyketide synthase.

flanking the associated biosynthetic gene clusters (Chan *et al.*, 2009).

In *Escherichia coli* K-12 there is a single and absolutely essential ACP, which is produced constitutively from the *acpP* gene (Rawlings & Cronan, 1992). In rhizobia, besides the constitutive acyl carrier protein (AcpP) involved in the biosynthesis and transfer of common fatty acids, there are at least three specialized ACPs: (1) the flavonoid-inducible nodulation protein NodF (Geiger *et al.*, 1991); (2) the RkpF protein, which is required for the biosynthesis of rhizobial capsular polysaccharides (Epple *et al.*, 1998); and (3) AcpXL, which transfers 27-hydroxyoctacosanoic acid to a sugar backbone during lipid A biosynthesis (Brozek *et al.*, 1996). RkpF was first identified as the sixth gene in the chromosomal region *rpk-1* (*rpkABCDEF*) in the strain *Sinorhizobium meliloti* Rm41. However, in the available genome of *S. meliloti* 1021, *rpkABCDEF* are fused into a single ORF, now named *rkpA*, and in this case RkpF corresponds to an ACP domain in a large multifunctional protein with homology to type I PKSs (Geiger & López-Lara, 2002; Becker *et al.*, 2005). Furthermore, the genome sequence of *S. meliloti* 1021 (Galibert *et al.*, 2001) reveals the existence of two additional putative ACPs, SMb20651 and SMc01553 (Geiger & López-Lara, 2002). Recently, we have characterized SMb20651 as the fifth functional ACP in *S. meliloti* 1021. We showed that SMb20651 actively participates in an acyl-involving metabolic step in wild-type *S. meliloti*, but no phenotype was found in mutants with a deletion in *smb20651* (Ramos-Vega *et al.*, 2009). Mutants of *S. meliloti* in *nodF* produce Nod factors lacking α,β -unsaturated fatty acids and exhibit a significantly reduced capacity to elicit infection thread formation in alfalfa, although they can form nitrogen-fixing nodules (Ardourel *et al.*, 1994). Mutants of *S. meliloti* in *acpXL* have increased sensitivity to a number of different stresses compared with the wild-type (Ferguson *et al.*, 2005). These mutants can still form a successful nitrogen-fixing symbiosis with alfalfa, but are less competitive in symbiosis than the parent strain (Ferguson *et al.*, 2005; Sharypova *et al.*, 2003). Furthermore, *acpXL* mutants of *S. meliloti* are seriously affected in early and late stages of bacteroid development (Haag *et al.*, 2009).

The other ORF of *S. meliloti* 1021 that presumably encodes a novel ACP (SMc01553) is located on the chromosome. Interestingly, a very recent gene transfer event involving *smc01553* occurred in *S. meliloti* 1021, where a 6.6 kb segment of DNA, including *smc01553*, appears to have been duplicated between the chromosomal DNA and the pSymb megaplasmid, giving this organism two copies of the gene encoding the putative new ACP and its adjacent genes (Galibert *et al.*, 2001; Fig. 1a). SMc01553 is assigned in the genome project as a putative ACP, and presents all the typical characteristics of ACPs, such as small size (82 aa), acidic pI (4.6), and a phosphopantetheine-binding motif. In the pSymb fragment, the ORF encoding the ACP is SMb22007, which is 100% identical to SMc01553 but has an additional glutamine at the C terminus of the

protein. The genes belonging to the duplicated DNA region are schematically represented in Fig. 1(a). SMc01550 and SMc01552, as well as the respective paralogous SMb21013 and SMb20842, are hypothetical proteins. SMc01551 and SMb20843 have homology to membrane-bound O-acyl-transferases (MBOATs). All characterized members of the MBOAT superfamily encode enzymes that transfer organic acids, typically fatty acids, onto hydroxyl groups of membrane-embedded targets (Hofmann, 2000). SMc01551 has been named in the genome as AlgI, and in experiments on natural genomic design carried out in *S. meliloti* it has been found that the site of cointegration of the pSymA–pSymB replicon to the chromosome corresponds to the reiterated *algI* genes (Guo *et al.*, 2003). SMc01554 is a protein of 651 aa that shows homology along the whole amino acid sequence to the unusually long FkbH homologues Tmn16 and ChlD1. Recently, it has been shown *in vitro* that Tmn16 and ChlD1 covalently tether the substrate 1,3-bisphosphoglycerate to a dedicated ACP and dephosphorylate it, forming the corresponding glyceryl-ACP (Sun *et al.*, 2008). In the pSymb plasmid, the DNA with homology to *smc01554* encodes two ORFs that are probably pseudogenes (Fig. 1a).

In only a few phylogenetically unrelated bacteria have ORFs that encode homologues to SMc01553 and SMc01554 been found and, except for the case of *Frankia* sp. EAN1pec, a gene encoding an MBOAT is situated in the vicinity (Fig. 1b). The scattered distribution of the region in only certain species of the proteobacteria, verrucomicrobia, acidobacteria and actinobacteria (Fig. 1b) indicates that in most of these cases the DNA fragment was acquired by horizontal gene transfer.

Here, we present the characterization of the protein SMc01553 as an ACP. SMc01553 was overproduced as insoluble inclusion bodies and the renatured recombinant protein can be converted to the holo-form. SMc01553 could not be loaded with the usual extender unit malonyl-CoA using the enzyme FabD of *S. meliloti*. A mutant was created in which both duplicated regions were deleted, but no phenotype was found in relation to the wild-type in the free-living conditions tested or in symbiosis with alfalfa.

METHODS

Bacteria and growth conditions. Table 1 lists the bacterial strains and plasmids used in this work. *E. coli* was grown at 30 °C in Luria–Bertani (LB) medium (Sambrook & Russell, 2001), or in M9 minimal broth (Miller, 1972) in the case of [^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 CaCl_2 (Beringer, 1974) or in the following minimal media: M9 supplemented with 2 μg biotin ml^{-1} and with succinate (10 mM) replacing glucose as the carbon source, Sherwood medium (Sherwood, 1970) with succinate (8.3 mM) replacing mannitol as the carbon source, or Robertsen medium (Robertsen *et al.* 1981). Antibiotics were added, when required, at the following final concentrations (μg ml^{-1}): carbenicillin, 100; chloramphenicol, 20; kanamycin, 50; gentamicin, 20; spectinomycin, 200; tetracycline, 20 for *E. coli*, and neomycin, 200;

Fig. 1. Genetic organization around the gene encoding SMC01553 and selected SMC01553 homologues in different organisms. (a) Duplicated DNA regions in *S. meliloti* 1021. Coordinates of the fragments in the *S. meliloti* genome are indicated with dashed lines and percentages of identity (% Iden.) at the DNA level are indicated on the line between the two fragments. The DNA region located between oligonucleotides o53 and o54 was deleted in mutant YD101, the region located between oligonucleotides o75 and o77 was deleted in mutant YD102, and both regions were deleted in mutant YD103. (b) Representative homologues in the bacterial species indicated on the right. Nomenclature used in (a) and (b): ACP, acyl carrier protein; FkbH, FkbH-like protein; MBOAT, membrane bound O-acyltransferase. (c) RT-PCR expression of *smc01553* and *smb22007* analysed in a 2% agarose gel. Lanes: 1, 100 bp molecular mass marker; 2 and 6, DNA from *S. meliloti* 1021; 3, RT-PCR using RNA isolated from YD101 mutant; 4, RT-PCR using RNA isolated from YD103 (double mutant); 5, RNA from *S. meliloti* 1021 (negative control); 7, RT-PCR using RNA isolated from YD102; 8, RNA from YD101; 9, RNA from YD103; 10, RT-PCR using RNA from *S. meliloti* 1021. The oligonucleotide primers used were *smc01553F* and *smc01553R* (Table 2).

Table 1. Bacterial strains and plasmids used in this study

Sm^R, Kn^R, Cb^R, Cm^R, Gm^R and Spc^R: streptomycin, kanamycin, carbenicillin, chloramphenicol, gentamicin and spectinomycin resistance, respectively. Subscript *Sm* denotes genes from *S. meliloti* 1021. Plasmids used and made for construction of mutants are specified in Methods.

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i> strains		
DH5α	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)
YD101	Deletion of 6.6 kb chromosomal region, Spc ^R	This study
YD102	Deletion of 4 kb in pSymb, Gm ^R	This study
YD103	Double mutant, having deletions of both YD101 and YD102, Spc ^R , Gm ^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)
pBluescript SK+	Cloning vector, Cb ^R	Stratagene
pK18 <i>mobsacB</i>	Suicide vector, Kn ^R	Schäfer <i>et al.</i> (1994)
pTB5035	<i>acpP_{Sm}</i> in pET9a	López-Lara & Geiger (2000)
pTB1003	<i>rkpF</i> of Rm41 in pET9a	Epple <i>et al.</i> (1998)
pAL20651	<i>smb20651</i> in pET9a	Ramos-Vega <i>et al.</i> (2009)
pCSF07	<i>nodF_{Sm}</i> in pET9a	Ramos-Vega <i>et al.</i> (2009)
pAL01553	<i>smc01553</i> in pET9a, Kn ^R	This study
pAL07	<i>acpXL_{Sm}</i> in pET9a, Kn ^R	This study
pYDM13	<i>smb22007</i> in pET9a, Kn ^R	This study
pYDM29	<i>smc01553</i> in pET16b, Cb ^R	This study
pYDM19	<i>acp</i> domain of <i>rpkA_{Sm}</i> in pET9a, Kn ^R	This study

spectinomycin, 400; gentamicin, 70; streptomycin, 500; tetracycline, 4 for *S. meliloti*.

pK18*mobsacB* derivatives were mobilized into *S. meliloti* strains by diparental mating using the *E. coli* S17-1 donor strain as described by Simon *et al.* (1983).

DNA manipulations and RT-PCR analysis. Recombinant DNA techniques were carried out using standard procedures (Sambrook & Russell, 2001). 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 TY culture using the 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. Using specific oligonucleotides (Table 2), the genes *smc01553*, *smb22007* and *acpXL* (*smc04277*) and the fragment encoding the ACP domain in RkpA were amplified and, after restriction with *NdeI* and *BamHI*, fragments were cloned into pET9a or pET16b (Studier *et al.*, 1990) that had been digested with *NdeI* and *BamHI*. Names given to the respective expression plasmids are indicated in Table 1.

Construction of sinorhizobial mutants lacking SMc01553-type ACPs. First, the duplicated region of 6.6 kb present in the chromosome (coordinates 2490 073 to 2496 674) was exchanged with a spectinomycin resistance cassette (Fig. 1a). The oligonucleotide primers o52 and o53 (Table 2) were used in a PCR to amplify 1496 bp

of genomic DNA upstream of this region, introducing *BamHI* and *HindIII* sites (underlined in the table) into the PCR product. Similarly, the primers o54 and o55 (Table 2) were used to amplify 1594 bp of genomic DNA downstream of the region, introducing *HindIII* and *XhoI* sites (underlined in the table) into this PCR product. After digestion with the respective enzymes, the 1496 bp fragment was cloned into plasmid pBluescript SK+ that had been digested with *BamHI* and *HindIII*, yielding the plasmid pYDM02. Then, the 1594 bp fragment was cloned as *HindIII/XhoI* into plasmid pYDM02 that had been digested with *HindIII* and *XhoI*, yielding the plasmid pYDM03. A resistance cassette obtained as a *HindIII* fragment from plasmid pPHY109 (Østeras *et al.*, 1998) was inserted into pYDM03 that had been digested with *HindIII* to yield pYDM04. Plasmid pYDM04 was digested with *BamHI* and *XhoI* to reclone the regions usually flanking the 6.6 kb region, as well as the spectinomycin resistance gene located in between, as a *BamHI/XhoI* fragment into the suicide vector pK18*mobsacB* (Schäfer *et al.*, 1994) that had been digested with *BamHI* and *SalI*, yielding pYDM06. Plasmid pYDM06 was introduced into wild-type strain *S. meliloti* 1021, and double recombinants in which the spectinomycin resistance cassette replaced the 6.6 kb chromosomal region were obtained following the procedure described previously (Sohlenkamp *et al.*, 2004). The mutant strain was named YD101.

A second mutant was created in which the gene *smb22007*, encoding the putative ACP in the pSymb plasmid, and surrounding areas were replaced by a gentamicin resistance cassette (Fig. 1a). A 4 kb DNA fragment was deleted comprising coordinates 633 000–637 000 in pSymb. The oligonucleotides o75 and o76 (Table 2) were used in a PCR to amplify 1610 bp of genomic DNA upstream of the 4 kb fragment, introducing *XbaI* and *HindIII* sites (underlined in the

Table 2. Oligonucleotide primers used in this study

For construction of expression plasmids, the forward primers incorporated an *Nde*I restriction site (underlined) overlapping the start codon of the respective gene. The reverse primers incorporated a *Bam*HI restriction site (underlined) after the stop codon. For amplification of *smb22007* the primers smc01553F and smb22007R were used.

Primer	Sequence (5'–3')
Primers used for construction of expression plasmids	
smc01553F	AGGAATACATATGTCTGACCATCAACTTTATC
smc01553R	AAAGGATCCTCAGTTCTGCTTGCGCTTGAC
smb22007R	AAAGGATCCTATTGGTTCTGCTTGCGCTTGAC
acpXLF	AGGAATACATATGCGCGTGACAGCTACATTC
acpXLR	AAAGGATCCTCAACCCGCTTTTGCGGCC
domainrkpAF	AGGAATACATATGATCGAAGGCAAATCGCC
domainrkpAR	AAAGGATCCTCATTGACTCAGTGCTTTTTC
Primers used for mutagenesis	
o52	AAAGGATCCGCCGGTTCCTTGAATGC
o53	ATAGTAAGCTTGAAGCAGGAGCGGCAAACG
o54	ATAGTAAGCTTGCCCTCTCGCGATCACTGG
o55	ATAGTCTCGAGTCACGGCCACCGCCAGG
o75	AGCTCTAGACCCAATGACGGCGAGCGAGAC
o76	ACCCAAGCTTGCGTGGCTCCCTGCTGCGTCC
o77	ACCCAAGCTTGCCCGACTACGCCGCCACCAG
o78	ACCGCTCGAGCGGTGGTCAGGTTGAAGTGG

table) into the PCR product. Similarly, the primers o77 and o78 (Table 2) were used to amplify 1382 bp of genomic DNA downstream of the selected region (Fig. 1a), introducing *Hind*III and *Xho*I sites (underlined in the table) into the PCR product. After digestion with the respective enzymes, the 1610 bp fragment was cloned into plasmid pBluescript SK+ that had been digested with *Xba*I and *Hind*III, yielding the plasmid pYDM39. Then, the 1382 bp fragment was cloned as *Hind*III/*Xho*I into plasmid pYDM39 that had been digested with *Hind*III and *Xho*I, yielding the plasmid pYDM49. A gentamicin resistance cassette obtained as a *Hind*III fragment from plasmid pACΩ-Gm (Schweizer, 1993) was inserted into pYDM49 that had been digested with *Hind*III, yielding pYDM53. Plasmid pYDM53 was digested with *Xba*I and *Xho*I to reclone the regions usually flanking the deleted region and the gentamicin resistance gene located in between those regions as an *Xba*I/*Xho*I fragment into the suicide vector pK18*mobsacB* that had been digested with *Xba*II and *Sal*I, yielding pYDM55. Plasmid pYDM55 was introduced into wild-type strain *S. meliloti* 1021 and into mutant YD101 that lacks the chromosomal duplicated DNA region, and double recombinants in which the gentamicin resistance cassette replaced the selected region of pSymb were obtained following the procedure described previously (Sohlenkamp *et al.*, 2004). The single mutant was named YD102, and the double mutant YD103. The correct genetic organization of the three mutants obtained was corroborated by Southern blot analysis.

Analysis of proteins. Proteins were analysed by 20% native PAGE (Jackowski & Rock, 1983), by conformationally sensitive gels as previously described (Ramos-Vega *et al.*, 2009), or by denaturing SDS-PAGE according to Schagger and von Jagow (1987) using 16.8% acrylamide in the separating gel. Gels were stained with Coomassie blue. Protein concentrations were determined by the method of Duley & Grieve (1975) using BSA (Sigma) as standard.

In vivo labelling 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).

Overproduction of ACPs in *E. coli* cultures and purification of AcpP. Cell-free extracts of *E. coli* BL21(DE3) pLysS overproducing the respective ACPs were obtained as previously described (Ramos-Vega *et al.*, 2009). Purified AcpP of *S. meliloti* was prepared as reported previously (López-Lara & Geiger, 2000), with the exception that in the last step, AcpP was purified using PrepCell (model 491, Bio-Rad) containing a cylindrical 20% native polyacrylamide gel rather than by electroelution from preparative gels. Fractions containing AcpP were combined and concentrated using a Centricon YM-3 centrifugal concentrator (Amicon, Millipore).

Renaturation and purification of SMC01553. SMC01553 and His-SMC01553 were overproduced in *E. coli* mainly as insoluble proteins. After cells were disrupted by French press, extracts were centrifuged and pellets were resuspended in 10 volumes of washing buffer (50 mM Tris/HCl, pH 6.8, 100 mM KCl) and centrifuged for 10 min at 15 000 g at 4 °C. Solubilization of the inclusion body-containing pellet was achieved with 50 mM Tris/HCl, pH 8, 5 M guanidine HCl, 0.005% Tween 80, and the solution was maintained at 8 °C overnight. To remove insoluble material, the solution was spun at 19 000 g for 15 min. The supernatant was diluted to a final A₂₈₀ of 0.1 in refolding buffer (50 mM Tris/HCl, pH 8, 1 M guanidine HCl, 0.005% Tween 80, 0.35 M L-arginine, 2 mM GSH, 0.02 mM GSSG) and kept at 8 °C for 50–60 h. After that, the Tween 80 concentration was adjusted to 0.05% and the resulting solution was dialysed against 50 mM Tris/HCl, pH 8, 50 mM NaCl, 0.05% Tween 80. After centrifugation of the solution to remove precipitates, SMC01553 protein was concentrated using a Centricon YM-3.

In the case of His-SMC01553, after adjusting the Tween concentration to 0.05%, the protein solution was dialysed against start buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4) and afterwards further purified using a 1 ml Ni²⁺ agarose column

(Hi Trap; Pharmacia Biotech) following the recommended standard method.

Generation of polyclonal antiserum against the ACP SMc01553 and Western blot analysis. Antiserum against the purified His-SMc01553 was raised in a white New Zealand rabbit that had been injected four times, each with a 1 : 1 mixture of Freund's complete or incomplete adjuvant (Roche) together with 150 µg His-SMc01553 antigen protein in 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄. Western blot analysis was performed as described previously (Ramos-Vega *et al.*, 2009), using anti-His-SMc01553 serum diluted 1000 times.

Enzymatic assays and MS. *In vitro* modification of apo-ACPs to holo-ACPs and *in vitro* malonylation of holo-ACPs were performed as previously described (Ramos-Vega *et al.*, 2009). Quantitative conversion of apo-SMc01553 to holo-SMc01553 was performed as reported previously (Epple *et al.*, 1998). MALDI-TOF MS was performed using a Bruker Daltonics Autoflex operated in the linear mode. Protein mixtures were prepared and spectra were obtained as described previously (Ramos-Vega *et al.*, 2009).

Phenotypes of mutants. Zones of inhibition, using the filter-disk assay with the detergents SDS and sodium deoxycholate (DOC), were measured as previously described (Ferguson *et al.*, 2002; Ramos-Vega *et al.*, 2009). The profile of rhizobial K-antigens (KPSs) was analysed by 18 % DOC-PAGE following the procedure described by Reuhs *et al.* (1998). Alfalfa (*Medicago sativa* L.) plants were grown in a nitrogen-free medium as described previously (Olivares *et al.*, 1980). Nitrogen fixation was determined by examining acetylene reduction using GC (Burris, 1972).

RESULTS AND DISCUSSION

Overproduction of the six different ACPs of *S. meliloti* 1021

In order to have the possibility of purifying all ACPs present in *S. meliloti* 1021, plasmids for the overproduction of AcpXL, the ACP domain of RkpA and SMc01553 were constructed in this study. Although all ACPs were predicted to be soluble proteins with similar characteristics, the total amount of the respective ACP present in cell

extracts varied greatly (Fig. 2a). In particular, little soluble SMc01553 was detected in cell extracts (Fig. 2a, lane 6). Cell extracts of *E. coli* overproducing SMc01553 were whitish, indicating that SMc01553 was overproduced as inclusion bodies. The analysis of pellets after low-speed centrifugation showed indeed that SMc01553 was mainly produced as inclusion bodies (Fig. 2b), and only a minor proportion of the protein was obtained as soluble protein (Fig. 2a, lane 6). Several parameters of the culture for overproduction of SMc01553 were varied, but in all cases the protein was mainly obtained as inclusion bodies (data not shown). Therefore, SMc01553 was purified from the inclusion bodies through a process of denaturation and renaturation. To our knowledge, this is the first bacterial ACP that, when overproduced, forms inclusion bodies. Although we observed that RkpF was also overproduced in part as inclusion bodies (results not shown), the amount of soluble protein obtained was enough for purification from the soluble fraction (Epple *et al.*, 1998). We have also amplified *smb22007*, which encodes a protein identical to SMc01553 with only the addition of the amino acid glutamine at the carboxy-terminus. However, as overproduced SMb22007 also formed mainly inclusion bodies (results not shown), the rest of the work was continued with SMc01553.

SMc01553 is modified *in vivo* and *in vitro* with 4'-phosphopantetheine

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 allowed overproduction of NodF, RkpF or SMc01553, which had been grown in the presence of [³H]β-alanine were analysed by PAGE and subsequent autoradiography. A Coomassie-stained gel showed bands in cell crude extracts corresponding to the overproduction in *E. coli* of NodF from *S. meliloti* 1021 and of RkpF from *S. meliloti* Rm41 (Fig. 3a, lanes 1 and 2) that efficiently incorporated [³H]β-alanine (Fig. 3b). Although SMc01553

Fig. 2. Overproduction of *S. meliloti* 1021 ACPs in *E. coli* (*Ec*). Analysis of cell-free extracts from *E. coli* separated by 20 % native PAGE (a) or of insoluble inclusion bodies separated in a 16.8 % SDS Schagger and von Jagow gel (b) stained with Coomassie blue. In (a) the overproduced ACPs were: lane 1, none (*E. coli* containing the pET9a vector); 2, AcpP; 3, NodF; 4, AcpXL; 5, SMb20651; 6, SMc01553; 7, ACP domain of RkpA. (b) Pellets after obtaining the crude extract from *E. coli* overproducing SMc01553 (lane 1) or from *E. coli* carrying the vector pET9a (lane 2); lane 3, molecular mass marker. In each lane the equivalent of ~0.25 ml culture was loaded.

Fig. 3. Overproduction of *S. meliloti* ACPs in *E. coli* (*Ec*) OG7001 (pLysS). Analysis of cell extracts from *E. coli* strains overproducing NodF, RkpF and SMc01553. (a) Proteins separated by 20 % native PAGE and stained with Coomassie blue. (b) Autoradiogram after labelling with [^3H] β -alanine. In (a) and (b) gels were loaded with cell-free extracts of *E. coli* overproducing: NodF (lane 1), RkpF (lane 2), no ACPs (*E. coli* strain carrying pET9a) (lane 3) and SMc01553 (lane 4). Note that the amount of soluble SMc01553 is not enough to be seen in a Coomassie blue-stained gel [lane 4 in (a)] but the [^3H]-labelled protein could be observed in a parallel protein sample treated in the same way (b). The bands corresponding to *E. coli* AcpP, NodF, RkpF and SMc01553 are indicated.

was poorly overproduced as a soluble protein, it was efficiently labelled with [^3H] β -alanine (Fig. 3, lanes 4), indicating the formation of holo-SMc01553. An enzyme activity present in *E. coli*, presumably AcpS, can modify SMc01553 post-translationally by adding the prosthetic group.

In order to test whether we could quantitatively convert apo-SMc01553 to holo-SMc01553, we incubated the purified apo-SMc01553 protein, obtained after the process of denaturation and renaturation, in the presence of CoA and His-AcpS from *S. meliloti* (Ramos-Vega *et al.*, 2009). The time-course of a quantitative conversion of apo-SMc01553 into holo-SMc01553 could readily be followed (Fig. 4). If the AcpS activity is inhibited by the presence of the chelating agent EGTA, apo-SMc01553 is not converted to holo-SMc01553. During analysis with native PAGE, even apo-SMc01553 protein and holo-SMc01553 protein migrate differently. Holo-SMc01553 migrates faster on native 20 % PAGE than apo-SMc01553 (Fig. 4), suggesting a more compact structure for holo-SMc01553 than for apo-SMc01553. In a similar manner, holo-RkpF migrates faster than apo-RkpF in 20 % native PAGE (Epple *et al.*, 1998) and, as expected, the holo-form of the ACP domain of RkpA also ran faster than its apo-form in the same types of gels (results not shown).

Analysis of purified SMc01553 by MALDI-TOF MS showed a major peak at m/z 9221.19 and a second peak at m/z 9351.85 (Fig. 5a), in agreement with the expected

Fig. 4. Quantitative conversion of apo-SMc01553 into holo-SMc01553 protein. Apo-SMc01553 was incubated with CoA in the presence His-AcpS, and the reaction was stopped with EGTA after different incubation times. A Coomassie-stained gel after native 20 % PAGE is shown. The various lanes show reaction mixture lacking His-AcpS (lane 1), reaction mixture in which EGTA was added before the incubation was started (incubation time, 128 min) (lane 2), 0 min incubation (lane 3), 8 min incubation (lane 4), 16 min incubation (lane 5), 32 min incubation (lane 6), 64 min incubation (lane 7) and 128 min incubation (lane 8). The positions of apo-SMc01553 (apo) and holo-SMc01553 (holo) are indicated.

masses for species of SMc01553 lacking (9221.41) or retaining the N terminal methionine (9352.60). SMc01553 that had been incubated with His-AcpS yielded masses of 9560.40 and 9691.23 (Fig. 5b), which correspond to increases of 339.21 and 339.38 atomic mass units (amu) with respect to the peaks observed in the untreated samples. These differences are consistent with the expected 339 amu for the 4'-phosphopantetheine addition, indicating that His-AcpS-treated SMc01553 is carrying the prosthetic group typical for ACPs. Preliminary data also detected an increase of about 339 amu in samples of NodF, AcpXL and the ACP domain of RkpA that had been treated with His-AcpS (data not shown). Therefore, AcpS of *S. meliloti* is able to modify all ACPs present in this strain.

SMc01553 is not 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, resulting in the formation of malonyl-ACP. Although the ACP SMb20651 is specifically modified by the enzyme SMb20650, it could also be loaded *in vitro* with a malonyl group using malonyl-CoA and His-FadD from *S. meliloti* (Ramos-Vega *et al.*, 2009). We tested whether SMc01553 could be modified with His-FabD. As a positive control, the AcpP of *S. meliloti* was used. Conformationally sensitive PAGE showed a slower-migrating form of AcpP that appeared only when the protein was incubated with malonyl-CoA and His-FabD (Fig. 6a). However, holo-SMc01553 that had been treated in the same way did not show any change in mobility in native

Fig. 5. *In vitro* 4'-phosphopantetheinylation of SMc1553 observed by MALDI-TOF MS. Spectra of SMc1553 incubated with CoA in the absence (a) or presence (b) of purified *S. melliloti* His-AcpS. Intensity is given in arbitrary units (a.u.).

PAGE (Fig. 6b) or in conformationally sensitive PAGE containing concentrations of urea ranging from 0.5 to 3.2 M. Analysis by MS of holo-SMc1553 that had been treated with His-FabD in the presence of malonyl-CoA did not show any difference from the spectra of non-treated holo-SMc1553, and a peak corresponding to malonyl-

SMc1553 could not be detected. In samples of holo-AcpP treated in parallel with His-FabD and malonyl-CoA, a clear peak was observed that corresponded to the expected increase of 86 amu for the addition of the malonyl group to holo-AcpP (results not shown). These results suggest that SMc1553 is not a substrate of FabD and probably cannot be loaded with the malonyl group. The genes *smc01553* and *smc01554* are predicted to form an operon. Also, in genomes that contain homologues for SMc1553, the gene encoding such a homologue is always located next to a gene encoding a homologue of SMc1554 (Fig. 1b). SMc1554 and the homologous proteins derived from genomes shown in Fig. 1(b) are members of the FkbH family of enzymes, and for some of those members it has been shown *in vitro* that they modify a dedicated ACP with an unusual extender unit (Sun *et al.*, 2008; Wu *et al.*, 2000). It is likely that SMc1553 is loaded by SMc1554 with a specific moiety, and experiments are under way to determine whether SMc1553 can be modified by SMc1554 *in vivo*.

Fig. 6. Malonylation assay of sinorhizobial AcpP or SMc1553. AcpP was separated by conformationally sensitive PAGE in gels containing 3.2 M urea (a), whereas SMc1553 was analysed by 20% native PAGE (b). Gels were visualized by staining with Coomassie blue. Proteins in (a): apo-AcpP (lane 1), holo-AcpP (lane 2) and holo-AcpP assay with malonyl-CoA in the presence of FadD (lane 3). Proteins in (b): apo-SMc1553 (lane 1), holo-SMc1553 (lane 2) and holo-SMc1553 assay with malonyl-CoA in the presence of FadD (lane 3).

Phenotypic characterization of sinorhizobial mutants lacking SMc1553-type ACPs

None of the three mutants constructed in this study displayed growth differences with respect to wild-type *S. melliloti* 1021 when grown on TY medium or in any of the minimal media (M9, Robertsen or Sherwood). Using filter disk assays, no difference of sensitivity was found to the detergents SDS or DOC. The DNA region deleted in pSymb is in between two paralogues of *rkpZ* and next to

the gene cluster 2 of *S. meliloti* 1021 involved in the biosynthesis of KPS (Becker *et al.*, 2005). Analysis by DOC–PAGE did not show any difference in the profiles of the different mutants in relation to the wild-type (results not shown). However, a small change in the structure of such a polysaccharide might not be detected by this technique.

With the polyclonal antibody raised against His-SMc01553, SMc01553 could not be detected in cell extracts of the wild-type strain grown in any of the media mentioned above. However, RT-PCR did reveal the presence of mRNA of *smc01553* or *smb22007* in the case of the wild-type and each of the single mutants, while such a transcript was not present in RNA extracted from the double mutant YD103 (Fig. 1c). The RT-PCR results obtained from the analysis of the single mutants YD101 and YD102 showed, respectively, the expression of *smb22007* and *smc01553* (Fig. 1c, lanes 3 and 7). An Affimetrix GeneChip containing the complete *S. meliloti* genome has shown that in TY-grown cells, *smc01553* and *smc01554* are among the two-thirds of putative coding regions that are expressed at above background levels (Barnett *et al.*, 2004), confirming that these two genes are expressed under such growth conditions. SMc01553 and SMc01554, and perhaps SMc01551, seem to be part of a pathway for the synthesis of a secondary metabolite, but from the genome of *S. meliloti* 1021 it is not clear which other genes could be involved in such a pathway. Since these genes are the result of a recent event of horizontal gene transfer (Galibert *et al.*, 2001), it is possible that only part of the genes required for the synthesis of such a metabolite were transferred.

Alfalfa plants were inoculated with either the parent strain 1021, each one of the mutants lacking either the chromosomal region or the region in pSymb, or the double mutant lacking both regions. No significant differences in the number of nodules or in the course of nodule development were observed (data not shown). After 40 days, the nitrogen-fixing ability was measured and no significant differences were observed (data not shown).

We have not found any phenotype in free-living or symbiotic conditions for the *S. meliloti* mutant lacking the DNA fragments encoding SMc01553 and Smb22007. Although more experiments need to be carried out to find possible functions encoded by these regions, it is possible that they contain only accessory DNA because these regions are the result of the duplication of DNA acquired by horizontal gene transfer. At this point it is not clear whether the products of the DNA of either region are responsible for the formation of a functional metabolite.

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