Roles of poly-3-hydroxybutyrate (PHB) and glycogen in symbiosis of *Sinorhizobium meliloti* with *Medicago* sp.

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Poly-3-hydroxybutyrate (PHB) and glycogen are major carbon storage compounds in *Sinorhizobium meliloti*. The roles of PHB and glycogen in rhizobia–legume symbiosis are not fully understood. Glycogen synthase mutations were constructed by in-frame deletion (*glgA1*) or insertion (*glgA2*). These mutations were combined with a *phbC* mutation to make all combinations of double and triple mutants. PHB was not detectable in any of the mutants containing the *phbC* mutation; glycogen was not detectable in any of the mutants containing the *glgA1* mutation. PHB levels were significantly lower in the *glgA1* mutant, while glycogen levels were increased in the *phbC* mutant. Exopolysaccharide (EPS) was not detected in any of the *phbC* mutants, while the *glgA1* and *glgA2* mutants produced levels of EPS similar to the wild-type. Symbiotic properties of these strains were investigated on *Medicago truncatula* and *Medicago sativa*. The results indicated that the strains unable to synthesize PHB, or glycogen, were still able to form nodules and fix nitrogen. However, *phbC* mutations caused greater nodule formation delay on *M. truncatula* than on *M. sativa*. Time-course studies showed that (1) the ability to synthesize PHB is important for N₂ fixation in *M. truncatula* nodules and younger *M. sativa* nodules, and (2) the blocking of glycogen synthesis resulted in lower levels of N₂ fixation on *M. truncatula* and older nodules on *M. sativa*. These data have important implications for understanding how PHB and glycogen function in the interactions of *S. meliloti* with *Medicago* spp.

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INTRODUCTION

The Gram-negative soil bacterium *Sinorhizobium meliloti* fixes atmospheric nitrogen in symbiotic association with its host plants, including *Medicago truncatula* (barrel medic) and *Medicago sativa* (alfalfa). A successful symbiosis involves a complex series of interactions between the host and bacteria (Brewin, 1991; Long, 1989, 2001; van Rhijn & Vanderleyden, 1995). Initially, plants release flavonoids that attract *S. meliloti* cells from the surrounding environment to

the plant's roots, and induce the production of Nod factors, which are specific lipochitin oligosaccharides produced by the bacteria. The Nod factors then induce root hair curling and root nodule development. *S. meliloti* cells invade developing root nodules via extended invaginations of the root hair cell membrane, called infection threads, and root nodule invasion requires the action of additional signal molecules, such as the exopolysaccharides (EPSs) produced by *S. meliloti*. The infecting bacteria grow and divide as they progress through the infection thread (Gage *et al.*, 1996), which is then degraded and the bacteria are taken into the cells of the developing nodule (Udvardi & Day, 1997). Once released from the infection threads, the bacterial cells differentiate into bacteroids, able to convert atmospheric N₂ to ammonium (Schultze & Kondorosi, 1998).

Nitrogen fixation within legume nodules results from a complex metabolic exchange between the bacterium and its plant host. Rhizobia are strict aerobes; therefore dicarboxylates provided by plants are metabolized via the

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Abbreviations: ARA, acetylene reduction activity; d.a.i., days after inoculation; D-HB, D-3-hydroxybutyrate; EPS, exopolysaccharide; PGM, phosphoglucomutase; PHB, poly-3-hydroxybutyrate.

tricarboxylic acid (TCA) cycle in the bacteroid. Nitrogenase is the enzyme responsible for converting N₂ into NH₃ and its activity is sensitive to oxygen, bringing about the need for anoxic conditions within the nodule. Low levels of oxygen might be expected to limit the operation of the TCA cycle. It is possible that much of the carbon flow in the bacteroid may be linear, with the substrate diverted to storage pools such as polyhydroxybutyrate (PHB) and glycogen, and some of it may be secreted as amino acids (Lodwig & Poole, 2003). As a result of this complex situation, many of the bacterial metabolic processes that occur in the free-living state are not the same as in the nodule.

PHB and glycogen are major carbon storage compounds in rhizobia. The roles of PHB and glycogen in rhizobia-legume symbiosis are not fully understood (Lodwig & Poole, 2003; Mandon *et al.*, 1998; Marroqui *et al.*, 2001; Trainer & Charles, 2006). For instance, bacteroids of determinate nodules (e.g. bean nodules) often accumulate high levels of PHB, and the accumulation of PHB requires nitrogen fixation (Cermola *et al.*, 2000). However, bacteroids of indeterminate nodules (e.g. alfalfa nodules) do not accumulate PHB (Aneja & Charles, 1999; Hirsch *et al.*, 1983). In the symbiosis between *S. meliloti* and *M. sativa*, it has been reported that *phbC* mutants form bacteroids capable of fixing nitrogen as efficiently as the wild-type (Aneja & Charles, 1999; Aneja *et al.*, 2005; Cai *et al.*, 2000; Povolito *et al.*, 1994; Willis & Walker, 1998), but are less competitive than the wild-type (Aneja *et al.*, 2005; Willis & Walker, 1998). Recently, it has been shown that *S. meliloti phbC* mutants are not only unable to produce PHB but are also deficient in synthesis of the EPS succinoglycan (EPSI) (Aneja *et al.*, 2004). It has been hypothesized that PHB accumulated by the bacteria prior to infection serves to fuel infection and/or bacteroid differentiation (Lodwig *et al.*, 2005).

Glycogen is synthesized in bacteria through the action of glycogen synthase on ADP-glucose (Ugalde *et al.*, 2003), resulting in the formation of α -1,4-linked glucan. A common genetic organization of the glycogen metabolism genes is found in the closely related *Agrobacterium tumefaciens*, *Rhizobium tropici*, *Rhizobium leguminosarum* and *Mesorhizobium loti*, within the *glgPBCApgm* operon (Lepek *et al.*, 2002). Through analysis of a *glgA* mutant of *R. tropici*, it has been shown that there may be a link between glycogen synthase deficiency, decreased EPS, and increased symbiotic performance during symbiosis with *Phaseolus vulgaris* (Marroqui *et al.*, 2001). However, the reason for the increased symbiotic efficiency of the *glgA* mutant is uncertain (Lodwig & Poole, 2003). In *M. loti* and *R. leguminosarum* bv. *phaseoli*, *glgA* mutations were reported to have no effect on symbiosis (Lodwig *et al.*, 2005). There are two predicted glycogen-synthase-encoding genes in *S. meliloti* (Galibert *et al.*, 2001). The *glgA1* gene is located within the *glgPBCApgm* operon on the chromosome, while the *glgA2* gene is located on megaplasmid pSymB (Finan *et al.*, 2001; Galibert *et al.*, 2001). Under growth-limiting

conditions, free-living rhizobial cells produce glycogen simultaneously with PHB (Tsien & Schmidt, 1977; Zevenhuizen, 1981). This suggests that glycogen metabolism may fulfil a similar role to PHB, and the carbon flux between the two compounds is relatively plastic and appears subject to common control (Povolito & Casella, 2000; Encarnacion *et al.*, 2002; Dunn *et al.*, 2002). In *S. meliloti*, it has been reported that under oxygen-limiting conditions free-living cells can use intracellular glycogen to generate ATP while maintaining their PHB content (Povolito & Casella, 2000). However, the relationship between these two compounds in the bacteroid is unclear. To better understand the roles these carbon storage compounds play in the symbiotic process and in the overall metabolism of *S. meliloti*, in both the free-living and bacteroid states, we constructed strains containing different combinations of mutations affecting PHB and glycogen biosynthesis. The symbiotic performance of the mutants was then evaluated on *M. truncatula* and *M. sativa*.

METHODS

Bacterial strains, plasmids and growth conditions. Strains and plasmids used in this work are shown in Table 1. *S. meliloti* strains were grown in TY medium (Beringer, 1974), MOPS (Mendrygal & Gonzalez, 2000) or YMB (g l⁻¹: K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.2; NaCl, 0.1; mannitol, 10; yeast extract, 0.4) (Tombolini *et al.*, 1995) at 30 °C. *Escherichia coli* strains were grown at 37 °C in Luria-Bertani medium (Miller, 1972). Antibiotics were used at the following concentrations: 100 µg ampicillin ml⁻¹, 20 µg kanamycin ml⁻¹, 25 µg chloramphenicol ml⁻¹, 50 µg rifampicin ml⁻¹, 50 µg spectinomycin ml⁻¹ and 50 µg streptomycin ml⁻¹. M9 minimal medium with various carbon sources, each at a final concentration of 15 mM, was prepared as described previously (Charles & Finan, 1991; Charles *et al.*, 1997). Sucrose was added to the medium at 5 % (w/v) when required. Media were solidified by the addition of 1.5 % (w/v) agar. Liquid cultures were shaken at 220 r.p.m.

Genetics and molecular biology techniques. Bacterial conjugations and ϕ M12 transductions were carried out as described previously (Charles & Finan, 1990, 1991). DNA manipulations were performed using standard methods (Ausubel *et al.*, 1997). Oligonucleotide primers were purchased from Sigma-Aldrich. DNA amplification by PCR using *Pfu* DNA polymerase (Promega) was performed in an Eppendorf Mastercycler gradient (Brinkmann Instruments).

Construction of in-frame deletion of *glgA1*. The location of *glgA1* in a cluster of other glycogen pathway genes directly upstream of the *pgm* gene required that we construct a non-polar mutant of *glgA1*. Oligonucleotide primers 5'-GATCGACAAGCTGGGAATGT-3' and 5'-CACCGTCATATCCTCGAACCT-3' were designed to amplify a 2.0 kb *glgA1*-flanking region. After PCR using *Pfu* polymerase, the blunt-end amplification product was ligated into the *HincII* site of pUC7, and the resulting construct was designated pMS007. The cloned fragment was confirmed by DNA sequence analysis. Digestion of pMS007 with *PstI* followed by plasmid self-ligation resulted in the elimination of a 379 bp internal region of *glgA1*. This deletion construct was designated pMS008. The deletion fragment was subcloned into pK19mobsac using *EcoRI*, to make pMS010. Gene replacement in Rm1021 was carried out by first introducing pMS010 by triparental mating, selecting for Sm^rNm^r single-crossover cointegrants. Subsequent selection for double-crossover events was carried out on TY agar containing 5 %

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics*	Source or reference
<i>S. meliloti</i>		
Rm1021	Derived from wild-type SU47, Sm ^r	Meade <i>et al.</i> (1982)
Rm5000	SU47, Rif ^r	Finan <i>et al.</i> (1984)
Rm11105	Rm1021 <i>phbC</i> ::Tn5	Charles <i>et al.</i> (1997)
Rm11477	Rm5000 <i>glgA2</i> ::ΩSpSm	This study
Rm11478	Rm1021 <i>glgA2</i> ::ΩSpSm	This study
Rm11479	Rm1021 <i>glgA1ΔPstI</i>	This study
Rm11480	Rm1021 <i>phbC</i> ::Tn5, <i>glgA1ΔPstI</i>	This study
Rm11481	Rm1021 <i>phbC</i> ::Tn5, <i>glgA2</i> ::ΩSpSm	This study
Rm11482	Rm1021 <i>glgA1ΔPstI</i> , <i>glgA2</i> ::ΩSpSm	This study
Rm11483	Rm1021 <i>glgA1ΔPstI</i> , <i>glgA2</i> ::ΩSpSm, <i>phbC</i> ::Tn5	This study
<i>E. coli</i>		
DH5α	F [−] <i>endA1 hsdR17</i> (r _k [−] m _k ⁺) <i>supE44 thi-1 recA1 gyrA96 relA1</i> Δ(<i>argF-lacZYA</i>)U169 φ80 <i>dlacZ</i> ΔM15, λ [−]	Hanahan (1983)
MT616	MT607 (pRK600), mobilizer, Cm ^r	Finan <i>et al.</i> (1986)
Plasmids		
pGEM-T Easy	Amp ^r , for cloning PCR products	Promega
pK19mobsac	Suicide vector, Nm/Km ^r	Schafer <i>et al.</i> (1994)
pUC7	ColE1 cloning vector, Amp ^r	Vieira & Messing (1982)
pKS029	<i>glgA2</i> in pGEM-T Easy	This study
pKS031	<i>glgA2</i> ::ΩSp ^r Sm ^r in pGEM-T Easy	This study
pMS001	<i>glgA2</i> ::ΩSp ^r Sm ^r in <i>EcoRI</i> site of pK19mobsacB	This study
pMS003	<i>glgA1</i> in pGEM-T Easy	This study
pMS007	<i>glgA1</i> in <i>HincII</i> site of pUC7	This study
pMS008	<i>glgA1ΔPstI</i> in <i>HincII</i> site of pUC7	This study
pMS010	<i>glgA1ΔPstI</i> in <i>EcoRI</i> site of pK19mobsacB	This study

*Abbreviations for antibiotic resistance phenotypes: Amp, ampicillin; Sp, spectinomycin; Sm, streptomycin; Rif, rifampicin; Cm, chloramphenicol; Nm, neomycin; Km, kanamycin.

sucrose, followed by screening for Nm sensitivity. Presence of the deletion was confirmed by PCR, and the corresponding strain was designated Rm11479.

Construction of insertion in *glgA2*. In contrast to *glgA1*, the *glgA2* gene is not predicted to be in an operon with any other genes. Oligonucleotide primers 5'-GTCGCATTCAACACGTTTCC-3' and 5'-CCCAGCCCTTTTGCTTTC-3' were designed to amplify a 1.5 kb *glgA2*-flanking region. After PCR using *Taq* polymerase, the resulting fragment was cloned into pGEM-T Easy vector to make pKS029. The insert was confirmed by DNA sequence analysis. A unique *Bam*HI site was identified within the *glgA2* coding region 759 bp downstream from the translation start codon, and the ΩSpSm interposon (Prentki & Krisch, 1984) was ligated into this site to make pKS031. The insert fragment construct was then subcloned into pK19mobsac using *Eco*RI to make pMS001. Gene replacement in Rm5000 was carried out by first introducing pMS001 by triparental mating, selecting for Rf^rSp^rSm^r single-crossover cointegrants. Selection for double-crossover events was carried out on TY agar containing Sm, Sp and 5% sucrose. Presence of the insertion was confirmed by Southern blotting (data not shown), and the corresponding strain was designated Rm11477. The mutation was transferred to the Rm1021 background by transduction of Sp^r to make strain Rm11478.

Biochemical assays. Glycogen was extracted from cells and determined by anthrone, according to the procedure of Chun & Yin (1998). Cell number was determined by serial dilution and plating

on TY medium. PHB assays were performed using the spectrophotometric technique (Law & Slepecky, 1961) as modified by Peoples & Sinskey (1989). Cells were harvested from *S. meliloti* cultures grown to saturation in YMB medium (50 ml). Following a saline wash and resuspension of cells in 50 ml saline, PHB was extracted from a 2 ml fraction of culture and assayed. The remaining 48 ml of culture was used for dry weight determination after incubation of the wet pellet at 37 °C until no further decrease in weight was noted. Purified PHB was purchased from Sigma for use as a concentration standard. The EPS content was determined as previously described (Marroqui *et al.*, 2001) from cultures grown in MOPS, YMB or TY for 5 days at 30 °C. Phosphoglucumutase (PGM) activity was assayed according to the procedure of Buchanan *et al.* (2005) from bacteria in mid-exponential phase (OD₆₀₀ 0.4) grown in TY medium.

Symbiotic assays. TY-grown bacterial cells in the late-exponential phase were collected by centrifugation, washed twice in 10 mM MgSO₄, and resuspended in 10 mM MgSO₄. *Medicago truncatula* Jemalong (line A17) seeds were obtained from Dr S. Long (Stanford University). Prior to germination, seeds were scarified by treatment with concentrated H₂SO₄ for 5 min, rinsed thoroughly with sterile distilled water, and treated with 2.5% sodium hypochlorite for 3 min. *Medicago sativa* cv. Iroquois seeds were treated with 75% ethanol for 2 min followed by 2.5% sodium hypochlorite for 15 min with gentle shaking. Following thorough rinsing, the treated seeds were placed into sealed Petri plates containing 1% agar at room temperature in the dark for 24 h.

Nodulation kinetics were determined by the growth pouch method. Both *M. truncatula* and *M. sativa* were used as hosts to test the nodulation of the *S. meliloti* strains. Germinated seedlings were transferred to growth pouches (MEGA International). Each pouch contained 5 ml Fahraeus medium (Fahraeus, 1957) with six seedlings. Pouches were put in a growth chamber under the following conditions: 18 h day/6 h night cycle (22 °C light/18 °C dark), and 60 % relative humidity. After 4 days in the growth pouch, the roots were inoculated with 150 µl of appropriate bacterial suspension (OD₆₀₀ adjusted to 0.01) per plant. The pouches were wrapped with PVC film (Fisherbrand) and returned to the growth chamber. The plants were visually screened for nodule formation by observing the root system every second day for a minimum of 4 weeks. Bacteria were recovered from nodules as described by Doherty *et al.* (1988) and dilutions were plated on LB plates containing appropriate antibiotics.

For assays in the growth chamber, plants were grown in sterile pots (4 × 4 inches, 9 plantlets per pot) containing Jensen's N-free plant nutrient solution (Jensen, 1942) treated vermiculite (Sta-Green, St Louis, USA), as described previously (Aneja & Charles, 1999). The plants were inoculated 5 days after germination, with the appropriate *S. meliloti* strains. Each seedling was inoculated with 5 ml of the cell suspension (OD₆₀₀ adjusted to 0.12). The control seedlings were inoculated with 5 ml of 10 mM MgSO₄. Each treatment included three replicates, where each replicate consisted of one pot with nine plants. The pots were covered with PVC film and placed in randomized blocks in a growth chamber under the conditions previously described. Plants were harvested at 14, 21 and 49 days after inoculation (d.a.i.). Nitrogen fixation activity was determined by the acetylene reduction method as follows. Ten millilitres of acetylene gas (generated by the reaction of water and calcium carbide) was injected into stoppered 100 ml bottles containing plants inoculated with various *S. meliloti* strains. The bottles were incubated for 3 h before analysis. A 200 µl sample from each bottle was analysed for the presence of acetylene and ethylene using a gas chromatograph (ThermoFinnigan, Trace GC 2000) equipped with an HP-AL/M column (30 m, J&W Scientific, Agilent Technologies) and a flame-ionization detector. The flow rate of the carrier gas was 35 ml min⁻¹, air at 350 ml min⁻¹, and helium as the makeup gas at 30 ml min⁻¹. The oven programme was 100 °C isocratic for 6 min. Following the acetylene reduction assay, the nodules were stripped off the roots, counted, dried at 80 °C for 48 h, and weighed (nodule number corresponded to nodule dry weight). The shoot dry weight of the plants was also measured. Phenotypes of bacteria recovered from nodules were checked on the appropriate media. The presence of pink cylindrical nodules on healthy dark-green plants was taken as evidence that nitrogen fixation was occurring.

Nodule sectioning, staining and microscopy. Nodules were fixed in a solution of 2.5 % (v/v) glutaraldehyde in 0.05 M sodium cacodylate (pH 7.3) overnight to adequately fix all the cells. The fixative was washed out by three successive 10 min washes in 0.05 M sodium cacodylate and the nodules were postfixed in 1 % (w/v) osmium tetroxide (OsO₄) in 0.05 M sodium cacodylate for 1 h at room temperature. The osmium fixation was followed by three 10 min washes in distilled water before beginning the acetone dehydration series (10, 20, 30, 50, 70, 95 %, and 3 × 100 % acetone each for approximately 20 min). Once dehydrated, the samples were gradually infiltrated with Spurr's resin (Polysciences Co.) by successive changes of acetone/resin mixes over approximately 48 h at room temperature (3:1 for 1 h, 1:1 for overnight, 1:3 for 3–4 h, then 100 % resin overnight). Samples then were embedded into Beem capsules full of fresh Spurr's resin and placed at 60 °C for 24 h to polymerize. Sections (longitudinal) with a thickness of 90 nm were made with a Leica Ultracut UCT diamond knife and placed on a 200-mesh copper grid. Imaging was performed with a Zeiss 10CA transmission electron microscope at 60 kV accelerating voltage and at room temperature.

For light microscopy, sections (longitudinal) 0.9 µm thick were dried onto glass slides and stained with 1 % (w/v) periodic acid, followed by treatment with Schiff's reagent. Photographs were taken on a Nikon E600 light microscope.

Statistical analysis. All the data presented for PHB, glycogen, EPS, nodule numbers, acetylene reduction assays, shoot dry weight and nodule dry weight are given as means with standard errors. The significance of the results was assessed using Student's *t* test.

RESULTS

Construction of the mutant strains

There are two predicted glycogen-synthase-encoding genes in the *S. meliloti* genome (Galibert *et al.*, 2001). The *glgA1* gene (Smc03924) predicts a 480 amino acid product, while the *glgA2* gene (Smb20704) predicts a 486 amino acid product. While *glgA1* is found in a cluster with other glycogen-related genes located on the chromosome, *glgA2* is located on megaplasmid pSymB and is not associated with such genes. A *glgA1* mutation was constructed by in-frame deletion, preserving the expression of the downstream *pgm* gene, while a *glgA2* mutation was constructed by disruption of the gene with the ΩSpSm interposon. A pre-existing Tn5-generated mutation of the PHB-synthase-encoding gene *phbC* was combined with *glgA1* and *glgA2* mutations to make all combinations of double mutants, and the triple mutant by transduction (Table 1). The mutants were confirmed by Southern blot analysis (data not shown here).

S. meliloti strains lacking *phbC* function do not produce PHB

PHB production was assayed in *S. meliloti* strains, using the wild-type strain Rm1021 and a previously described *phbC* mutant strain as controls. As previously reported, no PHB production was detected in any of the tested strains grown in rich TY medium. However, PHB production can reach more than 20 % of cell dry weight when the strain (e.g. Rm1021) is grown in YMB medium (Table 2). PHB was not detectable in free-living cells of any mutants containing the *phbC* mutation (Rm11105, Rm11480, Rm11481 and Rm11483) (Table 2), indicating that strains containing the *phbC* mutation lack the ability to produce PHB. The production of PHB decreased significantly in the *glgA1* mutants (Rm11479 and Rm11482).

S. meliloti strains lacking *glgA1* function do not produce glycogen

Glycogen was not detectable in any of the mutants containing the *glgA1* mutation, but the *glgA2* mutation did not affect glycogen accumulation (Table 2). The *glgA1* mutation also resulted in significantly less PHB production than in the wild-type. The production of glycogen was significantly higher in the *phbC* single mutant (Rm11105) than it was in the wild-type or *glgA2* mutants in MOPS and YMB media (Table 2). PGM assays were performed to confirm that the *glgA1* mutant was a non-polar mutant. The

Table 2. Phenotypes of the wild-type and mutants

For all strains, PHB, glycogen and EPS were not detected in TY culture. ND, Not detectable due to poor growth in YMB medium. Values in the same column with different superscript letters are significantly different ($P < 0.05$).

Strain	Genotype	PHB (% cell dry wt)	Glycogen ($\mu\text{g per } 10^9 \text{ cells}$)		EPS per cell dry wt (g)	
		YMB	MOPS	YMB	MOPS	YMB
Rm1021	Wild-type	20.9 ^c	51.8 ^a	83.8 ^b	1.6 ^a	1.6
Rm11105	<i>phbC</i> ::Tn5	0	113.9 ^c	104.1 ^c	0	0
Rm11478	<i>glgA2</i> :: ΩSpSm	23.3 ^c	50.1 ^a	59.7 ^a	1.6 ^a	1.7
Rm11479	<i>glgA1</i> Δ <i>PstI</i>	8.3 ^a	0	0	1.3 ^a	1.7
Rm11480	<i>phbC</i> ::Tn5, <i>glgA1</i> Δ <i>PstI</i>	0	0	0	0	0
Rm11481	<i>phbC</i> ::Tn5, <i>glgA2</i> :: ΩSpSm	0	77.1 ^b	ND	0	0
Rm11482	<i>glgA1</i> Δ <i>PstI</i> , <i>glgA2</i> :: ΩSpSm	16.8 ^b	0	0	2.2 ^b	1.9
Rm11483	<i>glgA1</i> Δ <i>PstI</i> , <i>glgA2</i> :: ΩSpSm , <i>phbC</i> ::Tn5	0	0	0	0	0

results showed that PGM activity in the *glgA1* mutant (5.88 mU mg⁻¹) was similar to that in the wild-type strain (4.91 mU mg⁻¹).

EPS is not detectable in *phbC* mutants, but is increased in *glgA* mutants

All tested strains grew normally and formed similar-sized colonies on complete medium (TY). However, on MOPS and YMB plates, the wild-type strain Rm1021, the *glgA* single mutant strains (Rm11478 and Rm11479) and the double mutant (Rm11482) exhibited mucoid morphology (EPS production), while the colony size of the *phbC* mutants (e.g. Rm11105, Rm11480, Rm11481 and Rm11483) was reduced significantly. The colonies appeared to have severely reduced levels of EPS. The quantitative assay confirmed that the *glgA* double mutant (Rm11482) had significantly increased levels of EPS after growth in MOPS medium (0 mM phosphate), but levels were normal after growth in YMB medium (3 mM phosphate), while the *glgA1* or *glgA2* single mutant produced almost the same amount of EPS as the wild-type under the same condition. As previously reported (Aneja *et al.*, 2004), no EPS was detected in YMB cultures of any mutants containing the *phbC* mutation (Table 2), although succinoglycan production was detected in LB-agar-grown colonies stained with calcofluor (data not shown).

Growth of mutant strains in minimal medium

The growth of the various *S. meliloti* strains was determined in M9 containing different carbon sources at 30 °C (Table 3). The growth of the *glgA2* mutant, Rm11478, was similar to that of the wild-type, except for a slight reduction in growth rate in acetoacetate and D-3-hydroxybutyrate (D-HB). The *phbC* single mutant (Rm11105) grew similarly to the wild-type on glucose, sucrose, fructose and succinate. However, the growth of Rm11105 was reduced severely on acetoacetate and D-HB. In contrast, the *glgA1* mutant (Rm11479) grew similarly to the wild-type on acetoacetate

and D-HB, but grew much more slowly than the wild-type on glucose, fructose, succinate, and especially sucrose. As expected from the growth of the single mutant, *phbC* double and triple mutants (Rm11480, Rm11481 and Rm11483) grew poorly on acetoacetate and D-HB; the growth of *glgA1* double and triple mutants (Rm11480, Rm11482 and Rm11483) was reduced severely on glucose, sucrose, fructose and succinate compared with the wild-type (Table 3). Interestingly, although the *glgA1* mutant (Rm11479) grew very slowly on sucrose, when this mutation was combined with others, the corresponding strains (Rm11480, Rm11482, and Rm11483) grew much better than the *glgA1* single mutant.

Absence of carbon storage synthesis in *S. meliloti* results in delay of nodule formation

To address the question of whether glycogen or PHB production is required for effective symbiosis between legume and *S. meliloti*, we performed plant inoculation experiments using both *M. truncatula* and *M. sativa*. Plant seedlings were inoculated with *S. meliloti* strains and the appropriate controls. Nodule kinetics assays in growth pouches showed that all mutant strains, especially the *phbC* mutants, caused nodule formation delay compared with the wild-type strain, but the phenomenon was more clearly expressed on *M. truncatula* than *M. sativa* (Fig. 1). In all cases, nodules were cylindrical and pink.

PHB synthesis is important for N₂ fixation in *M. truncatula* and younger *M. sativa* nodules

For the wild-type Rm1021 strain, the nodulation assay demonstrated a severe reduction in acetylene reduction activity (ARA) at 49 d.a.i. compared to the level of ARA at 21 d.a.i. At 14 d.a.i., all treatments had similar shoot dry weight for both *M. truncatula* and *M. sativa*, reflecting the early stage of N₂ fixation. Inoculation with *phbC* mutants (Rm11105, Rm11480, Rm11481 and Rm11483) resulted in significantly lower nodule dry weight and ARA on both *M.*

Table 3. Doubling time (h) of the wild-type and mutants on different carbon sources

Each minimal medium contains 15 mM of carbon source in M9; the doubling time was calculated from the mean of three replicates based on the standard procedure.

Strain	Genotype	TY	Glucose	Sucrose	Fructose	Succinate	Acetoacetate	D-HB
Rm1021	Wild-type	4.1	5.8	5.5	5.2	6.3	15.7	12.1
Rm11105	<i>phbC</i> ::Tn5	4.1	5.1	6.0	4.6	6.3	30.1	26.4
Rm11478	<i>glgA2</i> :: Ω SpSm	4.1	5.1	4.8	4.9	6.1	16.8	13.6
Rm11479	<i>glgA1</i> Δ PstI	4.7	13.9	62.7	20.5	9.6	16.5	12.1
Rm11480	<i>phbC</i> ::Tn5, <i>glgA1</i> Δ PstI	4.5	13.9	23.5	19.8	9.5	35.4	31.4
Rm11481	<i>phbC</i> ::Tn5, <i>glgA2</i> :: Ω SpSm	3.9	5.1	5.1	4.3	7.1	31.4	29.5
Rm11482	<i>glgA1</i> Δ PstI, <i>glgA2</i> :: Ω SpSm	4.0	10.4	22.8	20.1	9.5	18.1	10.6
Rm11483	<i>glgA1</i> Δ PstI, <i>glgA2</i> :: Ω SpSm, <i>phbC</i> ::Tn5	4.4	20.2	27.9	20.8	16.2	33.8	34.2

truncatula and *M. sativa* (Fig. 2), but the effect was much more pronounced on *M. truncatula*. At 21 d.a.i., *phbC* mutants had significantly lower shoot dry weight than the wild-type on *M. truncatula*. However, on *M. sativa*, the *phbC* mutants performed differently. Some of them (Rm11105 and Rm11480) had significantly lower shoot dry weight than the wild-type, but some of them (Rm11481 and Rm11483,

both having *glgA2* mutation) were not as severely affected. As for the results observed from 14 d.a.i. plants, *M. truncatula* and *M. sativa* 21 d.a.i. plants inoculated with *phbC* mutants had significantly lower nodule dry weight than the wild-type. At 49 d.a.i., *phbC* mutants (Rm11105, Rm11480, Rm11481 and Rm11483) were still ineffective on *M. truncatula*, resulting in significantly lower nodule dry weight, shoot dry weight and ARA. On *M. sativa*, these mutants also resulted in lower shoot dry weight compared with the wild-type, but they had significantly higher shoot dry weight than the non-infected control. However, inoculation with the *phbC* single mutant (Rm11105) resulted in similar nodule dry weight and ARA to the wild-type in the older *M. sativa* plants (Fig. 2).

Block of glycogen synthesis results in low levels of N₂ fixation in *M. truncatula* nodules, but not in younger nodules of *M. sativa*

At 14 d.a.i., even though all treatments gave similar shoot dry weights on both *M. truncatula* and *M. sativa*, the glycogen mutants (Rm11478, Rm11479 and Rm11482) had significantly lower ARA than the wild-type strain on *M. truncatula*. On *M. sativa*, these mutants had significantly higher ARA than the wild-type strain. At 21 d.a.i., all the glycogen mutants gave lower shoot dry weights than the wild-type on *M. truncatula*, but similar shoot dry weights to the wild-type strain on *M. sativa*, while these mutants had lower nodule dry weight and ARA than the wild-type strain on both *M. truncatula* and *M. sativa* (Fig. 2). After 7 weeks (49 d.a.i.), plants inoculated with *glgA1* or *glgA2* mutants (Rm11478, Rm11479 and Rm11482) exhibited decreased shoot dry weight, nodule dry weight and ARA on both *M. truncatula* and *M. sativa*.

Ultrastructural analysis of *phbC* and *glgA* mutants

Electron micrographs of *M. truncatula* nodule (longitudinal) sections showed that bacteroids mutated in *glgA1*, *glgA2* or *phbC* were similar to the wild-type. Sections through nodule infection threads showed that wild-type bacteria contained PHB granules. As would be

Fig. 1. Kinetics of nodulation on *M. truncatula* (a) and *M. sativa* (b) in growth pouches. Strains used were Rm1021 (wild-type) (●), Rm11105 (*phbC*::Tn5) (■), Rm11478 (*glgA2*:: Ω SpSm) (▲), Rm11479 (*glgA1* Δ PstI) (×), Rm11480 (*phbC*::Tn5, *glgA1* Δ PstI) (□), Rm11481 (*phbC*::Tn5, *glgA2*:: Ω SpSm) (△), Rm11482 (*glgA1* Δ PstI, *glgA2*:: Ω SpSm) (+), and Rm11483 (*glgA1* Δ PstI, *glgA2*:: Ω SpSm, *phbC*::Tn5) (–). Values represent the mean of three replicates (six plants per replicate).

Fig. 2. Symbiotic properties of the wild-type and mutant strains on *M. truncatula* (a) and *M. sativa* (b). Strains used were Rm1021 (wild-type) (WT), Rm11105 (*phbC*::Tn5) (c), Rm11478 (*glgA2*:: Ω SpSm) (a2), Rm11479 (*glgA1* Δ PstI) (a1), Rm11480 (*phbC*::Tn5, *glgA1* Δ PstI) (a1/c), Rm11481 (*phbC*::Tn5, *glgA2*:: Ω SpSm) (a2/c), Rm11482 (*glgA1* Δ PstI, *glgA2*:: Ω SpSm) (a1/a2), and Rm11483 (*glgA1* Δ PstI, *glgA2*:: Ω SpSm, *phbC*::Tn5) (a1/a2/c). Plants were grown and inoculated under growth chamber conditions as described in Methods. Dry weights and acetylene reduction were measured at 14 d.a.i. (white bars), 21 d.a.i. (stippled bars) and 49 d.a.i. (black bars). The experiment included an uninoculated control (plants grown in absence of bacteria). Values represent the mean $\pm$ SD of three replicates (nine plants per replicate).

expected for a mutant lacking PHB, there were no PHB granules present in *phbC* mutant Rm11105 bacteria inside infection threads (Fig. 3).

Light micrographs of nodules (longitudinal sections) infected with Rm1021 and glycogen mutants (Rm11478, Rm11479) showed that the plant cells were filled with starch granules only in the defined region of the nodules (Fig. 4a–c). However, nodules formed by either the *phbC* mutant Rm11105 or the triple mutant Rm11483 (*glgA1 glgA2 phbC*) showed a remarkable increase in the amount of starch dispersed throughout the nodule, especially the *phbC* single mutant Rm11105 (Fig. 4d, e). In all cases, starch accumulation occurred mainly in non-infected interstitial host cells.

DISCUSSION

It is well established that PHB and glycogen both function as major carbon storage compounds in rhizobia. The study of their respective roles in the process of root nodulation is somewhat complicated by the potential overlap in their functions. The application of mutational analysis in the well-characterized *S. meliloti* Rm1021 was designed to address the roles of PHB and glycogen metabolism in the free-living state and in symbiosis with both *M. truncatula* and *M. sativa*. The differing results on the two hosts emphasize the potential for host-dependent effects that should not be ignored.

Fig. 3. Electron micrographs of *M. truncatula* nodules infected with the wild-type and *phbC* mutant. (a) Nodule infected with Rm1021; (b) nodule infected with Rm11105 (*phbC*); (c) infection threads containing Rm1021; (d) infection threads containing Rm11105 (*phbC*).

Fig. 4. Light micrographs of longitudinal sections of *M. truncatula* nodules infected with various *glgA* and *phbC* mutants: (a) Rm1021, (b) Rm11478 (*glgA2*), (c) Rm11479 (*glgA1*), (d) Rm11105 (*phbC*), (e) Rm11483 (*glgA1 glgA2 phbC*). The arrows point to starch layers in panels (a), (b) and (c). In panels (d) and (e), starch is distributed throughout the nodule.

The presence in the Rm1021 genome of two different genes predicted to encode glycogen synthase, the central enzyme of glycogen synthesis, needed to be clarified. Under the free-living growth conditions that we tested in this study, only *glgA1* is required for glycogen synthesis, while *glgA2* is not. Furthermore, the glucose growth deficiency of the *glgA1* deletion mutant is similar to what has been reported with *R. tropici* (Marroqui *et al.*, 2001) and *R. leguminosarum* bv. *viciae* (Lodwig *et al.*, 2005) *glgA* mutants. The inhibition of glucose utilization might be due to feedback events that occur when the glycogen synthesis pathway is not available to accommodate some of the glucose that is taken up by the cell. This suggests that glycogen synthesis is used to balance the metabolism of glucose within the cell.

Whether the *glgA2*-encoded enzyme contributes to glycogen synthesis in symbiosis remains to be determined. While *glgA2* does not appear to be directly involved in glycogen synthesis, it appears to influence nodulation and N₂ fixation by an unknown mechanism. On *M. sativa* especially, the decrease in symbiotic efficiency in older nodules resulting from the *glgA2* mutation is striking.

We have shown that plants inoculated with PHB synthase mutants exhibited significantly reduced symbiotic properties (e.g. nodule formation kinetics, nodule dry weight and shoot dry weight). In other words, the ability to synthesize PHB can result in increased infection and is important for

N₂ fixation. This may be partially due to the reduction of EPS in mutants containing an interrupted *phbC* gene. EPS is essential for the early infection process (Frayse *et al.*, 2003) and plays a signalling role during nodulation (Niehaus & Becker, 1998), and mutants of *S. meliloti* unable to produce EPS are unable to invade and thus form ineffective nodules (Leigh & Walker, 1994). *S. meliloti* has at least two types of EPS: succinoglycan (EPSI) (Leigh & Walker, 1994) and galactoglucan (EPSII) (Glazebrook & Walker, 1989). A correlation between PHB metabolism and EPSI synthesis has been demonstrated (Aneja *et al.*, 2004). However, mechanisms of regulation have not been characterized, and it is not known whether EPSI synthesis is affected by PHB metabolism during infection. Prior to symbiosis, *S. meliloti* maintains a store of carbon in the form of PHB when excess carbon is available, so that this polymer is presumably able to provide energy for cell division and invasion of the infection thread. EPS is synthesized during growth in high-carbon media (Mendrygal & Gonzalez, 2000); therefore this may occur in the rhizosphere as well. If PHB supplies extra carbon, the bacterium is better able to synthesize the required EPSs and initiate adherence, signalling, and infection of the nodule.

Previous ultrastructural studies have indicated that PHB granules are present in *S. meliloti* cells inside infection threads (Paau *et al.*, 1978), but they disappear when cells are

released from the infection threads, and mature bacteroids do not contain visible granules of PHB (Hirsch *et al.*, 1983; Vasse *et al.*, 1990). The absence of granules in the infection threads of *M. truncatula* infected with the *phbC* mutant Rm11105 reinforces the identification of the granules observed in Rm1021 during infection as PHB. It also confirmed that the PHB reserves are degraded by Rm1021 bacteria when they leave the infection thread and differentiate into bacteroids. Moreover, PHB-nonproducing mutants of *S. meliloti* have been described previously; these mutants are able to induce nitrogen-fixing nodules on *M. sativa* (Povolo *et al.*, 1994; Willis & Walker, 1998), but are less competitive than wild-type (Povolo *et al.*, 1994). Our results are consistent with these previous reports. We add the observation that PHB synthesis is important for N₂ fixation in older *M. truncatula* nodules.

Our light microscopy observations show that in *M. truncatula* nodules infected with the wild-type strain Rm1021 a large amount of intercellular starch accumulation occurs in the II/III interzone as described for the pea nodules and alfalfa nodules (Lodwig *et al.*, 2005; Vasse *et al.*, 1990). This suggests that starch is the important carbon storage compound in *M. truncatula* nodules. We also observed that *M. truncatula* nodules containing either Rm11478 (*glgA1* mutant) or Rm11479 (*glgA2* mutant) had similar nodule zonation to Rm1021. This implies that glycogen synthesized by bacteria or bacteroids may not significantly influence the amount of carbon from the plant. However, the exact role and site of glycogen accumulation or degradation in the symbiosis remains to be elucidated. The *phbC* mutant Rm11105 and the triple mutant Rm11483 (*glgA1 glgA2 phbC*) had a different nodule zonation compared with Rm1021. These mutants had a large accumulation of starch distributed throughout the nodule rather than being limited to the II/III interzone as is the case for the wild-type and *glgA* mutants. Cutting a number of the different nodule longitudinal sections and then microscopically counting starch granules confirmed this observation. This result is contrary to what has been described for pea nodules infected with PHB synthesis mutants in which starch is depleted (Lodwig *et al.*, 2005), and we believe that the starch accumulation that we observe is consistent with lower symbiotic nitrogen fixation and therefore reduced carbon demand on the plant.

Overall, we showed that neither the absence of PHB, glycogen, or both, completely abolishes the symbiotic capabilities of *S. meliloti*. However, our data suggest that both PHB and glycogen are influential in the nodulation and N₂ fixation capabilities of *S. meliloti*. It is now widely accepted that carbon is supplied to the bacteroids in the form of dicarboxylic acids by plants to fuel nitrogen fixation (Lodwig & Poole, 2003). However, it is remarkable how imprecise our knowledge of much of bacteroid metabolism remains (Lodwig & Poole, 2003). With the ongoing *S. meliloti* Rm1021 functional genomics efforts, the data generated here will help to contribute towards a more complete picture of carbon metabolism occurring within

this model microsymbiont during the transition from free-living to bacteroid states. Studies on carbon metabolism should enlighten us on the strategies that rhizobia use to survive and compete in the rhizosphere and in association with the host plant.

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