

Role of Polyhydroxybutyrate and Glycogen as Carbon Storage Compounds in Pea and Bean Bacteroids

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Rhizobium leguminosarum synthesizes polyhydroxybutyrate and glycogen as its main carbon storage compounds. To examine the role of these compounds in bacteroid development and in symbiotic efficiency, single and double mutants of *R. leguminosarum* bv. *viciae* were made which lack polyhydroxybutyrate synthase (*phaC*), glycogen synthase (*glgA*), or both. For comparison, a single *phaC* mutant also was isolated in a bean-nodulating strain of *R. leguminosarum* bv. *phaseoli*. In one large glasshouse trial, the growth of pea plants inoculated with the *R. leguminosarum* bv. *viciae* *phaC* mutant were significantly reduced compared with wild-type-inoculated plants. However, in subsequent glasshouse and growth-room studies, the growth of pea plants inoculated with the mutant were similar to wild-type-inoculated plants. Bean plants were unaffected by the loss of polyhydroxybutyrate biosynthesis in bacteroids. Pea plants nodulated by a glycogen synthase mutant, or the *glgA/phaC* double mutant, grew as well as the wild type in growth-room experiments. Light and electron micrographs revealed that pea nodules infected with the *glgA* mutant accumulated large amounts of starch in the II/III interzone. This suggests that glycogen may be the dominant carbon storage compound in pea bacteroids. Polyhydroxybutyrate was present in bacteria in the infection thread of pea plants but was broken down during bacteroid formation. In nodules infected with a *phaC* mutant of *R. leguminosarum* bv. *viciae*, there was a drop in the amount of starch in the II/III interzone, where bacteroids form. Therefore, we propose a carbon burst hypothesis for bacteroid formation, where polyhydroxybutyrate accumulated by bacteria is degraded to fuel bacteroid differentiation.

Legumes form symbiotic interactions with various species of *Rhizobium*, resulting in the formation of root nodules. Within root nodules, the symbiotic form of *Rhizobium* (bacteroid) reduces N₂ to ammonium, which is secreted to the plant in return for a dicarboxylic acid as a carbon and energy source (Lodwig and Poole 2003). Furthermore, this exchange of carbon and nitrogen appears to be regulated by the cycling of amino acids (Lodwig et al. 2003). Bacteroids also can form the carbon storage compounds polyhydroxybutyrate (PHB) and glycogen. However, the roles these compounds play in the symbioses are unclear and may differ between plants forming determinate or

indeterminate nodules. For example, although PHB forms up to 70% of the dry weight of bacteroids from determinate nodules on bean and soybean, and forms distinct electron transparent granules, the polymer does not accumulate as granules in bacteroids from indeterminate nodules on alfalfa, pea, and chickpea (Kim and Copeland 1996; Tombolini and Nuti 1989). This is not due to an inability of these strains to make PHB because free-living cells accumulate the polymer under growth-limiting conditions (De Vries et al. 1984, 1986; Tombolini and Nuti 1989; Zevenhuizen 1981), and enzyme assays, gas chromatography, and labeling experiments have shown small amounts present in chickpea bacteroids (Kim and Copeland 1996). Mutation in the PHB synthase gene (*phaC*) of *Sinorhizobium meliloti* does not alter the efficiency of nitrogen fixation compared with the wild type when inoculated onto alfalfa (Cai et al. 2000; Povolito et al. 1994; Willis and Walker 1998). Likewise, mutation of the methylmalonyl-CoA mutase (*bhbA*), β-hydroxybutyrate dehydrogenase (*bhdA*), and acetoacetyl-CoA synthetase (*acsA*), which are involved in the degradation of PHB in *S. meliloti*, does not affect nitrogen fixation (Aneja and Charles 1999; Cai et al. 2000; Charles and Aneja 1999).

For determinate nodules, there has been a long debate over the relevance of the PHB reserves to bacteroid metabolism and nitrogen fixation (Lodwig and Poole 2003). In soybean, PHB content of bacteroids is lower after plants are stem girdled, thus preventing the flow of carbon to root systems, suggesting that PHB can be degraded by bacteroids to meet metabolic requirements (Bergersen et al. 1991). It also has been shown that PHB accumulated in bacteroids of *Bradyrhizobium japonicum* can be used to support nitrogen fixation in vitro (Bergersen and Turner 1990a,b, 1992; Bergersen and Turner 1993). However, although PHB breakdown might provide reductant for nitrogen fixation under conditions of carbon starvation, its synthesis normally may compete with nitrogenase for reductant. Consistent with this, a mutation in *phaC* of *Rhizobium etli* increases the efficiency of symbiosis (Cevallos et al. 1996; Peralta et al. 2004). The nitrogenase activity of mutant bacteroids is higher than the wild type, and the mutant has a prolonged capacity to fix nitrogen. Plants are increased in dry weight, and have more seed and higher nitrogen content, compared with plants inoculated with the wild type.

This picture may be complicated by the presence of glycogen in addition to PHB as a storage compound in bacteroids. Less is known about the factors that lead to the accumulation of glycogen in free-living cells but, as with PHB synthesis in free-living cells, glycogen accumulates under growth-limiting conditions such as nitrogen-limitation (Zevenhuizen 1981). This suggests that glycogen metabolism may fulfill a role similar to that of PHB metabolism. Free-living *B. japonicum*, *Rhizobium leguminosarum*, and *S. meliloti* can produce glycogen at

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*The e-Xtra logo stands for “electronic extra” and indicates the HTML abstract available on-line contains sequence data not included in the print edition.

the same time as PHB (Povolo and Casella 2000; Povolo et al. 1994; Tsien and Schmidt 1977; Zevenhuizen 1981). Indeed, preventing PHB synthesis by mutation of *phaC* results in a greater accumulation of glycogen (50-fold) when the TCA cycle is impaired by serial subculture on media without vitamins (Cevallos et al. 1996). This suggests that the control of carbon flux into these two pools is cross-regulated.

The presence of glycogen in bacteroids of *R. leguminosarum* bv. *trifolii* was first noted by Dixon (1967) and also has been observed in *Rhizobium* strains infecting lotus (Craig and Williamson 1972). However, a detailed study of the role of glycogen metabolism during symbiosis has been conducted only in *Rhizobium tropici* (Marroqui et al. 2001). A mutant in glycogen synthase (*glgA*) was isolated by screening Tn5-induced mutants for enhanced respiration via cytochrome oxidase activity. The mutation resulted in an increase in the symbiotic efficiency of *Phaseolus* bean plants as measured by an increase in dry weight of up to 38%. However, the reason for the increased symbiotic efficiency of the *glgA* mutant is uncertain. It most likely is due to increased nodulation rather than increased nitrogenase activity.

In this study, we decided that it was important to look at both PHB and glycogen metabolism at the same time in the *R. leguminosarum*–pea symbiosis. In particular, by studying mutants unable to make either PHB or glycogen, we could determine whether carbon storage compounds are essential for the symbiosis. By choosing *R. leguminosarum* strain A34, we also could compare the effect of preventing PHB biosynthesis in the bean-nodulating strain 4292. Strains A34 and 4292 differ only in their pSym plasmids. In particular, we wanted to see whether changes in the synthesis of carbon storage compounds would alter nitrogen fixation, plant growth, or carbon utilization by the plant.

RESULTS AND DISCUSSION

Identification of PHB synthase gene in *R. leguminosarum*.

A *R. leguminosarum* bv. *viciae* strain (RU137) with a Tn5 mutation in the PHB synthase gene (*phaC*) was previously isolated from the parental strain 3841 (Walshaw et al. 1997). The transposon and adjacent DNA was cloned from RU137 as a 9.2-kb *EcoRI* fragment in pSK⁻ (pRU99). A single-stranded DNA sequence including the junction between the transposon and *phaC* and a divergent transaminase was determined (3,717 bp) and used throughout this study for oligonucleotide design and for comparison to *phaC* from other organisms. The recently completed genome sequence of 3841 gives an identical sequence match for *phaC* (locus RL2094) (Sanger Institute). There is one copy of the gene and the protein has a predicted relative molecular mass of 70,878 and an isoelectric point of 5.43. From BLAST analysis, it was found that the putative *R. leguminosarum* PhaC has 89, 72, 62, and 55% identity to the proteins from *R. etli* (Cevallos et al. 1996), *S. meliloti* (locus SMc000296) (Capela et al. 2001), *M. loti* (locus mlr0937) (Kaneko et al. 2000), and *B. japonicum* (locus bli4360) (Kaneko et al. 2002), respectively. Divergently transcribed from *phaC* is an open reading frame (locus RL2096) (Sanger Institute) with 92, 91, 82, and 70% identity to putative aspartate aminotransferase proteins from *S. meliloti* (locus SMc00294) (Capela et al. 2001), *R. etli* (*aatC*) (Girard et al. 1991), *M. loti* (locus mlr0935) (Kaneko et al. 2000), and *B. japonicum* (locus blr4361) (Kaneko et al. 2002), respectively. The peptide showed weaker homology to the enzymatically characterized aspartate aminotransferase (*aatA*) of *S. meliloti* and *R. leguminosarum* (27 and 29% identity, respectively) (Lodwig et al. 2003; Rastogi and Watson 1991). Strains mutated in *aatA* form nodules that are unable to fix nitrogen, suggesting that aspartate transamination is essential for bacteroid nitrogen fixation. However, multiple aspartate aminotransferases are present in *S. meliloti* and not all of these are

required for nitrogen fixation (Alfano and Kahn 1993; Rastogi and Watson 1991). There is no published experimental evidence that the *aat* gene upstream of *phaC* has aspartate aminotransferase activity. However, the original *R. leguminosarum* *phaC* mutant, RU137, was isolated because it is able to grow on levels of aspartate that are toxic to the wild-type strain 3841, suggesting an enhanced level of detoxification (Walshaw et al. 1997). The location of the putative aminotransferase adjacent to *phaC* in different rhizobia, and the escape from aspartate toxicity in RU137, suggests a possible link between aspartate and PHB metabolism. However, proof of the substrate specificity of this transaminase will require its expression and purification.

Our principle aim in this study was to examine the in planta effects of mutating PHB and glycogen synthesis in a single strain of *Rhizobium*. A second aim was to compare the difference in preventing PHB synthesis in determinate and indeterminate symbioses, using isogenic strains that differ only in *sym*-plasmids. For this purpose, we switched from *R. leguminosarum* bv. *viciae* strain 3841 to *R. leguminosarum* strains A34 (bv. *viciae*) and 4292 (bv. *phaseoli*). Strains A34 and 4292 are derived from the same parent and only differ in their pSym. DNA hybridization showed that the *phaC* gene was present on the same *EcoRI* fragment in A34, 4292, and 8401 (lacks a *sym* plasmid), showing that it is located on either the chromosome or a plasmid other than pSym (data not shown). Recent genome sequence data has shown that, in strain, 3841 *phaC* is located on the chromosome.

Omega interposon mutants were made in *phaC* of both A34 (RU1328) and 4292 (RU1329). Neither RU1328 nor RU1329 had detectable PHB in cells grown on fructose-ammonia minimal medium, whereas strains A34 and 4292 had PHB at 65 and 50 µg/mg of protein, respectively.

Identification of the glycogen biosynthetic genes in *R. leguminosarum*.

To identify the glycogen synthase gene in *R. leguminosarum*, a cosmid library of A34 was used to complement the *R. tropici* *glgA* mutant (A656) for growth on glucose minimal medium plates. One complementing cosmid (pIJ9019) was mutagenized with TnB60, and the library of mutated cosmids conjugated into *R. tropici* A656. Cosmid pRU3146, carrying a TnB60 insertion in *glgA*, failed to complement A656. Sequencing out from the IS50 insertion sequence (primer P113) confirmed that the transposon was inserted in *glgA* with a 9-bp repeat of GCTCGTTAT and that the *tac* promoter in TnB60 acts downstream of *glgA*.

This partial sequence of *glgA* obtained from pRU3146 also was used to identify the entire *glg* operon in the genome sequence of *R. leguminosarum* strain 3841 (Sanger Institute). As in *R. tropici*, it consisted of the following operon: *glgP* (glycogen phosphorylase), *glgB* (glycogen branching enzyme), *glgC* (glucose-1-phosphate adenylyl transferase), *glgA* (glycogen synthetase), *pgm* (phosphoglucomutase), and *glgX* (glycogen debranching) (Fig. 1). *GlgP* (locus RL4114) from *R. leguminosarum* has 81% identity to the proteins from *Agrobacterium tumefaciens* and *S. meliloti* (locus SMc04460); *GlgB* (locus RL4115) has 80 and 79% identity to *A. tumefaciens* and *S. meliloti* (locus SMc03922) proteins, respectively; *GlgC* (locus RL4116) has 88% identity to *A. tumefaciens*, 87% to *R. tropici*, and 84% to *S. meliloti* (locus SMc03923) proteins; *GlgA* (locus RL4117) has 74% identity to *R. tropici*, 68% to *A. tumefaciens*, and 61% identity to *S. meliloti* (locus SMc03924) proteins; *Pgm* (locus RL4118) has 84% identity to *R. tropici*, 75% identity to *S. meliloti* (locus SMc03925), and 73% identity to *A. tumefaciens* proteins; and *GlgX* (locus RL4119) has 65% identity to *R. tropici*, 64% to *A. tumefaciens*, and 63% identity to *S. meliloti* (locus SMc03926) proteins (Capela et al. 2001; Marroqui et al.

2001; Ugalde et al. 1998). This confirms that this operon is highly conserved in the rhizobia.

A chromosomal mutant (RU1448) was made by recombining *glgA::TnB60* from pRU3146 into strain A34. There was no detectable glycogen in RU1448 compared with A34 (glycogen at 70 µg/mg of protein). A double *phaC glgA* mutant (RU1478) was made by recombining *glgA::TnB60* from pRU3146 into RU1328.

Growth of PHB and glycogen synthase mutants.

The growth of the mutants was determined by streaking onto agar plates (Table 1). The *phaC* mutants (RU1328 and RU1329) were severely inhibited for growth on tryptone yeast (TY) agar plates, taking 10 days to form single colonies compared with 4 days for the wild-type strains. On glucose, malate, and succinate, the *phaC* mutants grew slightly slower, forming smaller colonies after 4 days of growth compared with the wild type. However, the growth of RU1328 and RU1329 was reduced severely on pyruvate and alanine. This effect already has been seen with *R. etli*, confirming that synthesis of PHB is an important part of pyruvate and acetyl-CoA metabolism in free-living cells (Dunn et al. 2002). The principal effect of the *glgA* mutation in strain RU1448 was to impair growth on glucose-containing medium. This effect has been seen before in *R. tropici* and suggests that there may be a com-

plex relationship between storage of glucose as glycogen and its use as the sole carbon source (Marroqui et al. 2001). As expected from the growth of the single mutants, the double *phaC glgA* mutant (RU1478) grew poorly on pyruvate, alanine, and glucose (Table 1).

Symbiotic growth properties.

The first experiment was sown in a glasshouse in May, during spring, and was repeated the following year in July, during midsummer (Table 2). In the spring experiment, pea plants nodulated by the *phaC* mutant (RU1328) were significantly reduced in acetylene reduction at 35 days and the dry weight was 37% less at 53 days than plants inoculated with the wild type (A34) (Table 2). This lowered acetylene reduction is consistent with a decrease in nitrogen fixation causing the weight reduction. Such a dramatic reduction in acetylene reduction and dry weight in pea was not expected because, like bacteroids of other indeterminate nodules, bacteroids of *R. leguminosarum* bv. *viciae* do not make large granules of PHB (discussed below). It also contrasts with other indeterminate legumes, such as alfalfa, where plant growth was unaffected by inoculation with *phaC* mutants compared with the wild type (Cai et al. 2000; Povoletto et al. 1994; Willis and Walker 1998).

Because these results were so unexpected, a second large glasshouse trial was conducted in July of the following year. In

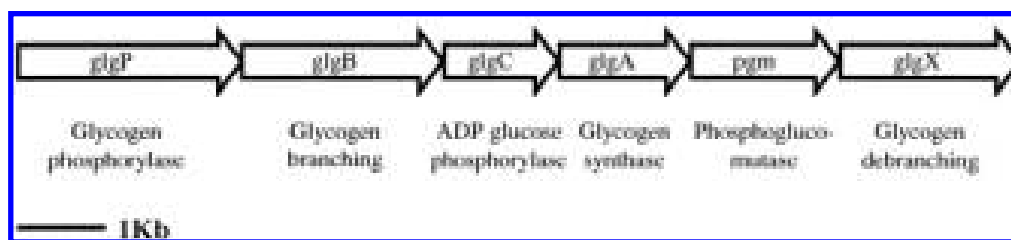


Fig. 1. Organization of glycogen biosynthetic genes in *Rhizobium leguminosarum*.

Table 1. Growth of various strains on minimal medium^a

Carbon source	A34 (wild-type <i>viciae</i>)	4292 (wild-type <i>phaseoli</i>)	RU1328 (<i>phaC viciae</i>)	RU1329 (<i>phaC phaseoli</i>)	RU1448 (<i>glgA viciae</i>)	RU1478 (<i>glgA phaC viciae</i>)
Malate	+++	+++	++	++	+++	++
Succinate	+++	+++	++	++	+++	++
Pyruvate	+++	+++	±	±	+++	-
Alanine	+++	+++	±	±	+++	-
Glucose	+++	+++	++	++	±	-
Sucrose	+++	ND	++	ND	+	++
Mannitol	+++	ND	++	ND	+++	+++
Fructose	+++	ND	++	ND	+++	++
Galactose	+++	ND	ND	ND	+++	ND

^a Scale: +++ = good growth, ++ = growth impaired, + = growth severely impaired, - = no growth, and ND = not determined.

Table 2. Effect of *phaC* mutants on acetylene reduction and the growth of pea and bean plants grown in a glasshouse and inoculated with various bacterial strains^a

Experiment time	Strain	Ethylene production (µmoles plant ⁻¹ h ⁻¹)	Dry weight (g plant ⁻¹)
May	A34 (wild-type <i>viciae</i>)	5.93 ± 0.59, n = 40	2.6450 ± 0.1326, n = 42
	RU1328 (<i>phaC viciae</i>)	2.99 ± 0.17, n = 40	1.6738 ± 0.0916, n = 45
	...	P < 0.001	P < 0.001
July	A34 (wild-type <i>viciae</i>)	4.52 ± 0.86, n = 15	2.5500 ± 0.0500, n = 40
	RU1328 (<i>phaC viciae</i>)	4.38 ± 0.61, n = 15	2.4700 ± 0.0600, n = 48
	...	P > 0.05	P > 0.05
May	4292 (wild-type <i>phaseoli</i>)	9.05 ± 0.92, n = 40	2.4166 ± 0.1520, n = 29
	RU1329 (<i>phaC phaseoli</i>)	10.97 ± 1.11, n = 40	2.1938 ± 0.1282, n = 32
	...	P > 0.05	P > 0.05

^a Plants were inoculated in early May or July and acetylene reduction measured at 35 days just as plants began to flower. Dry weights were taken at 53 days for pea plants and 54 days for bean plants. Values shown are means ± standard error. Each mutant is paired with a wild-type strain which was grown at the same time, and the P value for each pair is shown. In each experiment, approximately six plants were left uninoculated and these plants were stunted in growth and yellowing at dry weight harvest.

this experiment, there was no significant difference in acetylene reduction or the dry weight of pea plants inoculated with the *phaC* mutant versus the wild type (Table 2). The results of the first experiment may simply represent an uncontrolled element in a large glasshouse trial. However, it should be noted that, in both experiments, almost all the bacteria recovered from the nodules of plants inoculated with the *phaC* mutant had the expected spectinomycin and streptomycin resistance markers (98 and 94% for the May and July plantings, respectively) and the mean temperature and irradiance of the glasshouse for the two experiments were similar (19.1 and 21.6°C and 12.8 and 10.6 MJ m⁻² day⁻¹ for the May and July plantings, respectively). Given these glasshouse results, we believe that preventing PHB synthesis in rhizobia that infect pea does not necessarily lead to impaired plant growth. However, there may be some environmental conditions that can interact epistatically with the mutation in PHB synthesis.

In the May glasshouse experiment, the bean plants inoculated with the *phaC* mutant (RU1329) showed no significant difference in either acetylene reduction or dry weight (Table 2). This is particularly interesting because it has been reported that *Phaseolus vulgaris* plants inoculated with a *phaC* mutant of *R. etli* have an increased capacity for nitrogen fixation and enhanced growth (Cevallos et al. 1996). Our result shows that the absolute level of PHB formed in bacteroids may not be the only, or even the most important, factor affecting plant performance.

To determine the effect of preventing glycogen biosynthesis and both glycogen and PHB biosynthesis in *R. leguminosarum*, pea plants were inoculated with A34 (wild type), RU1448 (*glgA*), and RU1478 (*glgA phaC*) and grown in an environmentally controlled room. There was no difference in acetylene reduction or dry weight between mutants and the wild type (Table 3). The plant growth response to RU1328 (*phaC*) also was tested in the growth room but, as in the second glasshouse experiment, there was no difference in acetylene reduction or dry weight between pea plants inoculated with the mutant and the wild type (Table 3). However, the *phaC* control plants grown in the glasshouse were twice as heavy as those grown in the controlled environment. This shows that plants grown in small-scale growth-room experiments are significantly limited in growth.

Ultrastructural analysis of *phaC* and *glgA* mutants.

Electron micrographs show that, as expected, bean bacteroids synthesized large granules of PHB, and these were missing in the *phaC* mutant RU1329 (Fig. 2C and D). No PHB granules accumulated in the bacteroids of A34 or RU1328

(Fig. 2A and B). There were no other apparent differences between bacteroids of A34 and RU1328, as shown by the transmission electron micrographs. However, sections that cut through pea nodule infection threads show that wild-type bacteria contain granules of PHB. As would be expected for a mutant lacking PhaC, there were no PHB granules present in RU1328 bacteria inside infection threads (Fig. 2E and F). Previous ultrastructural studies have indicated that PHB granules are present in *S. meliloti* cells inside infection threads (Paau and Cowles 1978), but they disappear when cells are released from infection threads, and mature bacteroids do not contain visible granules of PHB (Hirsch et al. 1983; Vasse et al. 1990). The loss of electron-transparent granules in the infection threads of pea infected with *R. leguminosarum* bv. *viciae* RU1328 is the first clear evidence that the granules in A34 are PHB. It also confirms that the PHB reserves are degraded by wild-type (A34) bacteria when they leave the infection thread and differentiate into bacteroids.

Light micrographs of transverse sections of pea nodules infected by A34 showed characteristic zones of indeterminate nodules (Fig. 3). Particularly notable is interzone II-III, a 1- to 3-cell layer where plant cells are filled with starch granules (Vasse et al. 1990). This zone is highly significant to the development of the symbiosis because it represents the transition of bacterial cells (in zone II) into nitrogen-fixing bacteroids (occurring in zone III). Remarkably, interzone II-III is not apparent on the sections of nodules infected by RU1328, because there is no distinct histological zone of cells containing starch. Cutting a number of different nodule sections and then microscopically scanning up and down several cell layers to count starch granules confirmed this observation (data not shown). This depletion of starch, due to the loss of the ability to metabolize PHB in bacteria, suggests that the plant has had to compensate the developing bacteroids for their lack of PHB, and we propose the following hypothesis. Bacterial PHB reserves normally are mobilized during bacteroid differentiation, in the II/III interzone, to help fuel a burst of carbon metabolism that is required to convert a free-living bacterium into a much larger differentiated bacteroid. In a *phaC* mutant, the PHB reserve cannot be built up, and the carbon burst required for bacteroid differentiation must be met by the plant in the II/III interzone. This results in very little accumulated starch in the plant in the II/III interzone, because there is a much higher external carbon demand by the developing bacteroids. If this hypothesis is correct, it indicates a complex interaction between plant carbon supply and bacteroid carbon utilization. Various factors that stress the plant might reduce carbon supply to the nodule and disturb this complex interaction, and it is possible that the loss of symbiotic performance seen in the first large glasshouse trial of RU1328 may be due to such an interaction. Furthermore, this interdependence of plant and bacteroid carbon storage metabolism might explain the contradictory results found here and in the literature for mutants in either PHB or glycogen metabolism.

A second dramatic change is that pea nodules containing either strain RU1448 (*glgA*) or the double mutant RU1478 (*phaC glgA*) show a remarkable increase in the amount of starch through out the nodule (Fig. 3C and D). This implies that bacteria or bacteroids unable to synthesize glycogen require much less carbon from the plant. Such a reduction in carbon demand on the plant might lead to increased starch accumulation. Indeed, to our knowledge, the only other examples where pea nodules accumulated so much starch is when they were infected with a strain of *R. leguminosarum* that cannot fix nitrogen (Finan et al. 1983; Ludwig et al. 2003). Glycogen might be synthesized by bacteria in infection threads or by bacteroids. However, the peribacteroid membrane does not appear to be permeable to sugars and *R. leguminosarum* bac-

Table 3. Effect of *glgA* and *phaC* mutants on acetylene reduction and growth of peas grown in a constant environment growth room and inoculated with various bacterial strains^a

Strain	Ethylene production (μmoles plant ⁻¹ h ⁻¹)	Dry weight (g plant ⁻¹)
A34 (wild-type)	3.79 ± 0.40, n = 6	1.1667 ± 0.0458, n = 6
RU1328 (<i>phaC</i>)	4.48 ± 1.05, n = 6	1.1517 ± 0.1118, n = 6
	P > 0.05	P > 0.05
A34 (wild-type)	3.70 ± 0.60, n = 8	1.0518 ± 0.0979, n = 11
RU1448 (<i>glgA</i>)	3.38 ± 0.51, n = 8	1.0146 ± 0.0760, n = 13
	P > 0.05	P > 0.05
A34 (wild-type)	3.82 ± 0.46, n = 5	1.1600 ± 0.0700, n = 16
RU1478 (<i>phaC glgA</i>)	4.63 ± 0.33, n = 5	1.2800 ± 0.0900, n = 19
	P > 0.05	P > 0.05

^a Acetylene reduction measured at 28 days just as plants began to flower. Dry weights were taken at 35 days for the *phaC*, 50 days for the *glgA*, and 42 days for the *phaC glgA* mutants. Values shown are means ± standard error. Each mutant is paired with a wild-type strain which was grown at the same time, and the P value for each pair is shown. In each experiment, approximately three plants were left inoculated and these plants were stunted in growth and yellowing at dry weight harvest.

teroids have the gluconeogenic enzymes weakly induced (McKay et al. 1985; Udvardi et al. 1990). This suggests that glycogen synthesis may occur before bacteroid formation. Overall, the role of glycogen biosynthesis in the rhizobia-legume symbiosis may have been underestimated, but the exact role and site of glycogen accumulation or degradation remains to be elucidated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

The bacterial strains and plasmids used in this study are detailed in Table 4. *R. leguminosarum* strains were grown at 28°C on either TY extract (Beringer 1974), acid minimal salts medium (AMS), or acid minimal salts agar (Poole et al. 1994) with carbon and nitrogen sources added to 10 mM. Antibiotics were used at the following concentrations: ampicillin at 50 µg ml⁻¹, gentamycin at 20 µg ml⁻¹, kanamycin at 40 µg ml⁻¹, neomycin at 80 µg ml⁻¹, spectinomycin at 100 µg ml⁻¹, streptomycin at 500 µg ml⁻¹, and tetracycline at 2 µg ml⁻¹ in AMS and 5 µg ml⁻¹ in TY.

DNA manipulations.

Standard protocols were used for DNA manipulations (Sambrook and Russell 2001). Sequencing was carried out by MWG-Biotech AG or the Reading University AMSEQ service. The majority of sequence of *phaC* in strain 3841 was determined using the *phaC*::Tn5 clone, pRU99. The sequence was incomplete at the 3' end; therefore, a primer specific to the 3841 sequence (P139) and a primer (P234) that binds 69 bp outside the stop codon of *R. etli phaC* sequence (GenBank U30612) was used to amplify the remaining sequence. The 1.8-kb fragment obtained was cloned into pCR2.1-TOPO (pRU720) and the C-terminus was sequenced.

Using the sequence of *phaC* derived from strain 3841, the gene was amplified from strain 4292 using primers P138 and P166. The polymerase chain reaction (PCR) product was cloned into pCR2.1-TOPO (pRU566) and digested with *PshAI*. A *SmaI*-digested Ω-spectinomycin cassette from pPH45Ω (Fellay et al. 1989) was then ligated into the *PshI* site of *phaC* (pRU575). The *phaC*::Ω fragment was removed by *EcoRI* digestion of pRU575, and the cohesive ends filled in and then ligated into *SmaI*-digested pJQ200 (pRU577) (Quandt and

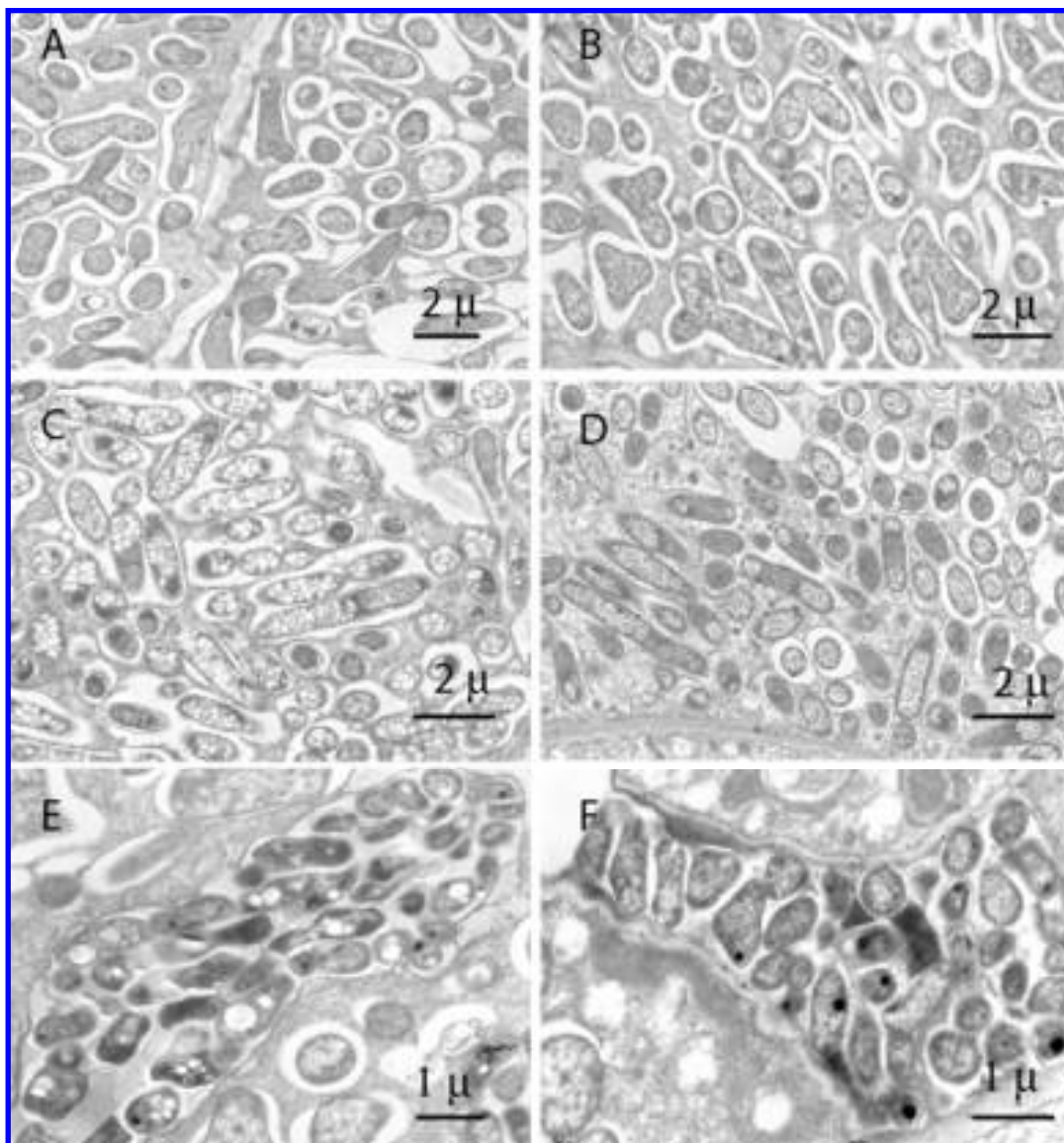


Fig. 2. Electron micrographs of pea and bean nodules infected with various *phaC* mutants. **A**, Pea nodule infected with A34; **B**, pea nodule infected with RU1328 (*phaC*); **C**, bean nodule infected with 4292; **D**, bean nodule infected with RU1329 (*phaC*); **E**, pea infection threads containing A34; **F**, pea infection threads containing RU1328 (*phaC*).

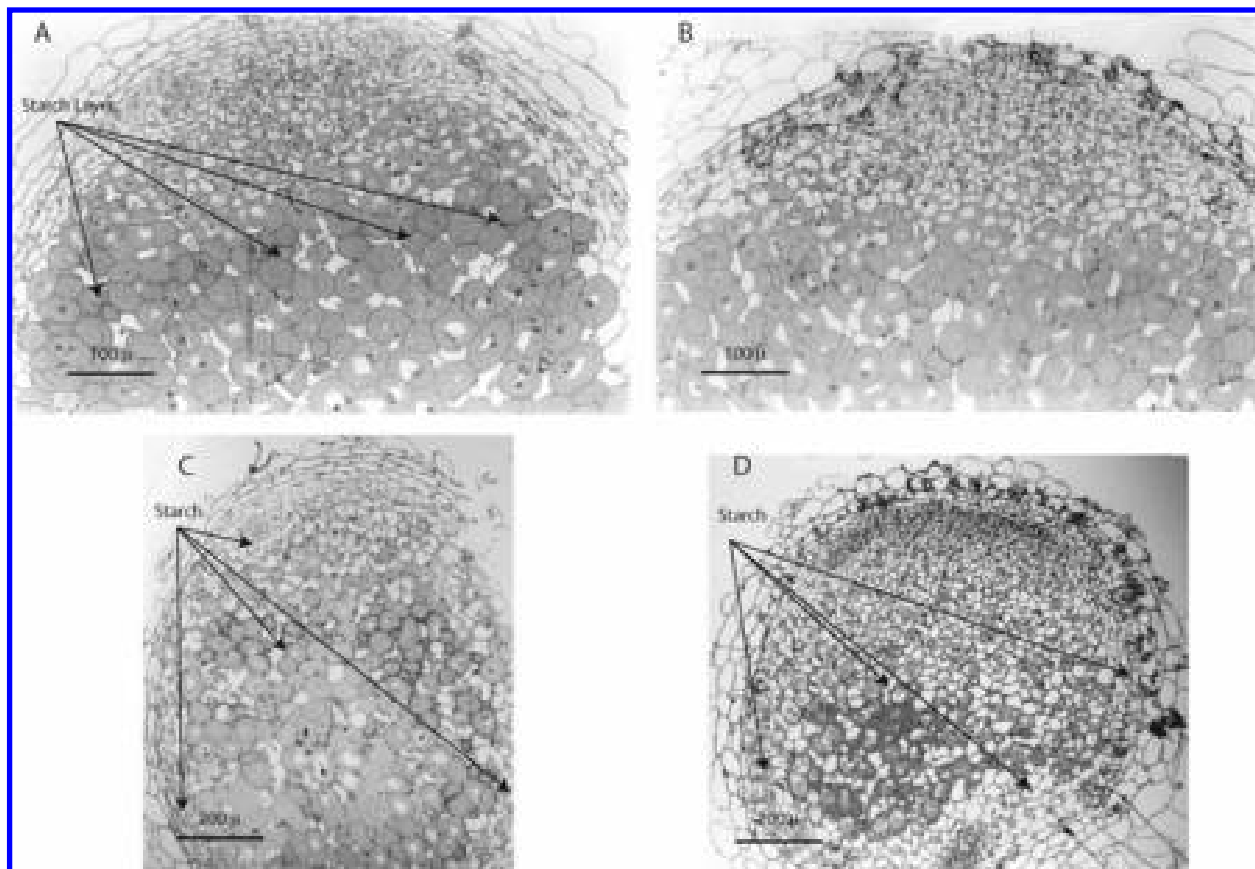


Fig. 3. Light micrographs of pea nodules infected with various *phaC* and *glgA* mutants. Nodules infected with **A**, A34; **B**, RU1328 (*phaC*); **C**, RU1448 (*glgA*); **D**, RU1478 (*glgA phaC*). The arrows point to a discrete (two- to three-cell-wide) starch layer in panel A. In panels C and D, starch is distributed throughout the nodule.

Hynes 1993). This was conjugated into 4292 and A34 and recombined by selecting for sucrose resistance. The mutants of 4292 (RU1329) and A34 (RU1328) were confirmed by Southern blotting using the *phaC* PCR clone (pRU566) as the probe.

Mutants in *glgA* were made by excising *glgA*::TnB60 and flanking DNA from pRU3146, as a 12-kb *SacI* fragment, and cloning this into pJQ200SK (pRU758). A *glgA* mutant of *R. leguminosarum* A34 was isolated by selecting for sucrose resistance. The mutant (RU1448) was confirmed by Southern blot and hybridization using a 0.7-kb *EcoRI/XhoI* fragment of *glgA* from *R. tropici* (pIJ7843) as probe. A double mutant in *phaC glgA* (RU1478) also was isolated by recombining pRU758 into RU1328 using sucrose selection. RU1478 was confirmed by PCR mapping using primers P471 and P472. Similarity searches were done using the Basic Local Alignment Search Tool on the National Centre for Biotechnology Information website.

Plant growth.

Seed of pea (*Pisum sativum* cv. Avola) were washed in 70% ethanol for 30 s, rinsed twice in sterile water, washed in 2% Na-hypochlorite for 8 min, and then rinsed in five changes of sterile water. Bean seed (*Phaseolus vulgaris* cv. Tendergreen) were treated as for pea seed, except the hypochlorite wash was reduced to 4 min. Bean seed also were washed rapidly because prolonged exposure to liquid causes imbibition shock.

Seed were sown in sterile vermiculite contained in 2-liter pots (one seed per pot) for studies in the plant growth room or in a sterile mixture of sand, gravel, and vermiculite (4:2:2) in 15-liter pots (seven seed per pot) for studies in the greenhouse. In each case, the growth medium was wetted and plants subsequently were watered with a nitrogen-free nutrient solution as described previously (Allaway et al. 2000). To inoculate seed, cultures of

Rhizobium were grown overnight in TY broth. Culture was harvested at an optical density at 600 nm (OD_{600}) of 0.5 to 0.7 and the cells were washed with sterile water to remove any remaining nitrogen compounds. Cells were resuspended to approximately OD_{600nm} 0.5 and seed were inoculated with 1 ml of cell suspension at the time of sowing. Plants in the growth room were grown under Sonti-agro lights at a constant temperature of 22°C with a 16-h light cycle. Plants were raised in the glasshouse at a constant temperature of 19 to 21°C under natural daylight. The aboveground biomass (shoots plus leaves) was dried to a constant weight in an oven at 75°C for 48 h before measuring dry weights. Acetylene reduction was measured as previously described (Trinick et al. 1976).

Quantification of storage polymers.

The concentration of PHB was determined from cells grown on 10 mM fructose–5 mM NH_4Cl as described previously (Law and Slepecky 1961). The concentration of glycogen was quantified from cells grown on sucrose–10 mM NH_4Cl by measuring the glucose formed after digestion of extracted glycogen with amyloglucosidase and amylase using oyster glycogen (Sigma, St. Louis) as a standard (0 to 200 μg), as described previously (Ernst et al. 1984).

Nodule sectioning, staining, and microscopy.

Nodules were cut in half longitudinally and placed in a solution of 2.5% (vol/vol) glutaraldehyde in 0.05 M sodium cacodylate (pH 7.3) and briefly vacuum infiltrated until they sank. The fixative was replaced with fresh and left 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% (wt/vol) OsO_4 in 0.05 M so-

Table 4. Strains, plasmids, and primers used

Strains, plasmid and primers	Genotype or characteristics ^a	Reference or source
Strain		
3841	<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>	Johnston and Beringer 1975
4292	Sym plasmid pRL2JI (<i>phaseoli</i>) derivative of 8401 bv. <i>phaseoli</i> , str ^r	Downie et al. 1983
8401	<i>R. leguminosarum</i> lacking a sym plasmid	Downie et al. 1983
A34	Sym plasmid pRL1JI (<i>viciae</i>) derivative of 8401 bv. <i>viciae</i> str ^r	Gotz et al. 1985
A656	<i>R. tropici</i> <i>glgA</i> Δ PstI	Marroqui et al., 2001
RU137	<i>R. leguminosarum</i> strain 3841 <i>phaC</i> ::Tn5	Walshaw et al. 1997
RU1328	A34 (bv. <i>viciae</i>)-derived <i>phaC</i> :: Ω , str ^r , spc ^r	This work
RU1329	4292 (bv. <i>phaseoli</i>) <i>phaC</i> :: Ω , spc ^r	This work
RU1448	A34 (bv. <i>viciae</i>)-derived <i>glgA</i> ::TnB60, str ^r , kan ^r	This work
RU1478	A34 (bv. <i>viciae</i>)-derived <i>phaC</i> :: Ω , <i>glgA</i> ::TnB60, str ^r , kan ^r , spc ^r	This work
Plasmid		
pCR2.1-TOPO	TA PCR cloning vector, f1 origin, ColE1 replicon, amp ^r , kan ^r , <i>lacZ</i>	Invitrogen
pHP45 Ω	pBR322 derivative carrying Ω , pHP45 replicon, amp ^r , spc ^r	Prentki and Krisch 1984
pIJ7843	0.7-kb <i>EcoRI/XhoI</i> fragment of <i>glgA</i> in pBluescript KS	This work
pIJ9019	Cosmid from gene bank of A34 which complements A656 (<i>glgA</i> -), tet ^r	This work
pJQ200SK	P15A origin from pACYC184, gen ^r , <i>lacZ</i> , <i>sacB</i> , <i>traJ</i>	Quandt and Hynes 1993
λ TnB60	λ Carrying transposable <i>tac</i> -promoter (Tn5 derivative)	Simon et al. 1989
pRU99	8.9-kb Tn5 bearing <i>EcoRI</i> fragment of RU137 in pSK ⁻ , kan ^r , amp ^r	Walshaw et al. 1997
pRU566	P138/P166 1.6-kb PCR fragment of <i>phaC</i> in pCR2.1-TOPO, amp ^r , kan ^r	This work
pRU575	Ω Cloned in pRU566 (<i>phaC</i>), amp ^r , kan ^r , spc ^r	This work
pRU577	<i>phaC</i> :: Ω in pJQ200SK, gen ^r , spc ^r	This work
pRU720	1.8-kb PCR fragment of 3' end of <i>glgA</i> in pCR2.1-TOPO	This work
pRU758	<i>glgA</i> ::TnB60 <i>SacI</i> fragment in pJQ200SK, gen ^r , kan ^r	This work
pRU3146	<i>glgA</i> ::TnB60 cosmid (parent pIJ9019), tet ^r , kan ^r	This work
Primer		
P113	AGGTCACATGGAAGTCAGATC	This work
P138	CTGCCGAAGAAGACGGAC	This work
P139	CGCTCGAAAATCTCGGAC	This work
P166	GGATAAAAAGAGTGGTGCTG	This work
P234	TCGCGAGGATGACGAATTAT	This work
P471	AAAGTTCTTTCCGGTTTCGTC	This work
P472	TGATAAAAATGCATCGCACG	This work

^a PCR = polymerase chain reaction. str = streptomycin, spc = spectinomycin, kan = kanamycin, amp = ampicillin, tet = tetracycline, and gen = gentamycin.

dium cacodylate for 1 h at room temperature. The osmium fixation was followed by three 10-min washes in distilled water before beginning the ethanol dehydration series (30, 50, 70, and 95% each for approximately 20 min, then 100% ethanol for 1 h). Once dehydrated, the samples gradually were infiltrated with LR White resin (London Resin Company, Theale, England) by successive changes of resin/ethanol mixes over approximately 24 h at room temperature (1:1 for 1 h, 2:1 for 1 h, 3:1 for 1 h, 100% resin for 1 h, then 100% resin for 16 h and a fresh change again for a further 6 h). Samples then were transferred into Beem capsules full of fresh LR White and placed at 60°C for 16 h to polymerize. The material was sectioned with a glass knife using a Reichert ultramicrotome (Leica, Ryswyk, The Netherlands).

For light microscopy, sections 0.5 μ m thick were dried onto glass slides and stained with 0.5% (wt/vol) Toluidine blue "O" in 0.5% (wt/vol) borax. Photographs were taken on a Nikon E800 light microscope with Kodak technical Pan film. For electron microscopy, ultrathin sections of approximately 90 nm were picked up on 200-mesh copper grids which had been pyroxylin and carbon coated. The sections were stained with 2% (wt/vol) uranyl acetate for 1 h and 1% (wt/vol) lead citrate for 1 min, washed in distilled water, and air dried. The grids were viewed in a Jeol 1200 EX transmission electron microscope at 80 kV and photographs were taken on Kodak electron image film.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Wellcome Trust Sanger Institute *Rhizobium leguminosarum* bv. *viciae* database: www.sanger.ac.uk/Projects/R_leguminosarum/
National Center for Biotechnology Information website: www.ncbi.nlm.nih.gov/BLAST/