RESEARCH ARTICLE

Poly-3-hydroxybutyrate (PHB) supports survival and reproduction in starving rhizobia

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Abstract

The carbon that rhizobia in root nodules receive from their host powers both N_2 fixation, which mainly benefits the host, and rhizobium reproduction. Rhizobia also store energy in the lipid poly-3-hydroxybutyrate (PHB), which may enhance rhizobium survival when they are carbon limited, either in nodules or in the soil between hosts. There can be a conflict of interest between rhizobia and legumes over the rate of PHB accumulation, due to a metabolic tradeoff between N_2 fixation and PHB accumulation. To quantify the benefits of PHB to carbon-limited rhizobia, populations of genetically uniform rhizobia with high vs. low PHB (confirmed by flow cytometry) were generated by fractionating *Sinorhizobium meliloti* via density gradient centrifugation, and also by harvesting cells at early vs. late stationary phase. These rhizobia were starved for 165 days. PHB use during starvation was highly predictive of both initial reproduction and long-term population maintenance. Cultured *S. meliloti* accumulated enough PHB to triple their initial population size when starved, and to persist for *c.* 150 days before the population fell below its initial value. During the first 21 days of nodule growth, undifferentiated *S. meliloti* within alfalfa nodules accumulated enough PHB to support significant increases in reproduction and survival during starvation.

Introduction

Rhizobia (*Sinorhizobium*, *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Azorhizobium* species) are *alphaproteobacteria* capable of symbiosis with legume roots. Legumes supply rhizobia in nodules with reduced carbon (Minchin & Pate, 1973), which rhizobia respire to power fixation of atmospheric N_2 into NH_3. Symbiotic rhizobia may sequester some of this carbon in bacterial storage polymers, such as the lipid poly-3-hydroxybutyrate (PHB). PHB accumulation often exceeds 50% of rhizobium cell dry weight (Bergersen & Turner, 1990; Tavernier *et al.*, 1997).

Various ways in which rhizobia might use PHB to benefit their plant hosts have been proposed, including respiration to protect nitrogenase from O_2-inactivation 'until the last stages of seed development' (Bergersen *et al.*, 1991) and fueling the differentiation of some rhizobia into larger N_2-fixing bacteroids (Lodwig *et al.*, 2005), which thereby lose the ability to reproduce (Denison, 2000). Alternatively, rhizobia might use PHB in ways that enhance their own fitness. PHB could be used to provide the energy and carbon required for bacterial reproduction or stress tolerance, perhaps within senescing nodules or after symbiotic rhizobia escape into the soil. PHB is synthesized by many species of bacteria, where it has been shown to improve survival during starvation (Tal & Okon, 1985; James *et al.*, 1999; Kadouri *et al.*, 2002), as well as improve tolerance to high temperatures, H_2O_2 exposure (Kadouri *et al.*, 2003; Ruiz *et al.*, 2004), UV-irradiation, desiccation, and osmotic stress (Kadouri *et al.*, 2003).

Biochemically, PHB synthesis directly competes with N_2 fixation for reductant (Anderson & Dawes, 1990). Thus, N_2-fixing rhizobium bacteroids should face a tradeoff between nitrogen fixation and PHB accumulation. Physiological and mutagenesis studies support this hypothesis. Romanov and colleagues (Kretovich *et al.*, 1977; Romanov *et al.*, 1980) found a negative correlation between the rate of nitrogen fixation and PHB accumulation. Cevallos *et al.* (1996) and Peralta *et al.* (2004) demonstrated that a PHB (−) mutant of *Rhizobium etli* fixed significantly more nitrogen than the isogenic PHB (+) wild type. Nonfixing *nifH* mutants of *R. etli* (Cermola *et al.*, 2000) and *Bradyrhizobium*

japonicum (Hahn & Studer, 1986) have been shown to accumulate more PHB than their isogenic nitrogen-fixing parental strains.

The quantitative tradeoff between N_2 fixation and PHB accumulation is most direct in rhizobia where the N_2-fixing bacteroids themselves accumulate PHB, as in soybean nodules. In some legume species, including alfalfa, nodules contain both highly differentiated N_2-fixing bacteroids, which have lost the ability to reproduce, and also undifferentiated reproductive rhizobia. Only the latter typically accumulate PHB (Denison, 2000). In these nodules, increasing N_2 fixation by bacteroids would potentially compete with PHB hoarding by reproductive rhizobia in the same nodule, but the tradeoff is less direct than when individual bacteroids must allocate available carbon between N_2 fixation and PHB accumulation. If PHB increases rhizobium fitness, increasing survival or reproduction inside senescing nodules or later in the soil, then PHB accumulation during symbiosis would serve as a pathway for rhizobia to divert host carbon from nitrogen fixation into their own reproductive success. This would set up a conflict of interest between host and symbiont that could influence the coevolution of legumes and rhizobia.

Rhizobia with disrupted PHB synthesis genes have been shown to be less competitive for nodulation (Willis & Walker, 1998; Aneja *et al.*, 2005) and reproduce less under starvation (Cai *et al.*, 2000; Povolo & Casella, 2004) than wild-type cells. A complete absence of external carbon sources may not be required for PHB to provide a reproductive benefit. Cai *et al.* (2000) found that *Sinorhizobium meliloti* str. 1021 mutants unable to synthesize or degrade PHB reproduced 7.5-, and 5.1-fold when starved, respectively, while wild-type PHB (+) rhizobia reproduced to 9.7-fold their inoculum population size. The overall high reproduction in their experiments suggests that some exogenous carbon was present, but the increased reproduction of the wild-type rhizobia reflects the reproductive benefit of PHB accumulation.

While informative, these studies may confound the fitness consequences of energy and carbon storage in PHB with other effects of a functional PHB metabolism. The ability to synthesize and degrade PHB may improve rhizobium fitness by stabilizing cellular redox conditions and relieving TCA cycle inhibition under low oxygen conditions (Anderson & Dawes, 1990; Dunn, 1998; Poole & Allaway, 2000). Furthermore, PHB synthase (*phbC*) knockout mutants are pleiotropic, exhibiting reduced growth on a variety of carbon sources (Cevallos *et al.*, 1996; Cai *et al.*, 2000; Lodwig *et al.*, 2005; Wang *et al.*, 2007a), and can lack production of the extracellular polysaccharide (EPS) succinoglycan (Aneja *et al.*, 2004). These factors may reduce the fitness of rhizobia entering symbiosis (Leigh *et al.*, 1985) or living saprophytically. As a result we do not know if PHB accumulation, *per se*, is responsible for the superior fitness of wild-type rhizobia. In the context of the legume–rhizobium symbiosis, it is the quantitative tradeoff between nitrogen fixation and PHB accumulation, not the qualitative ability to make PHB that would result in evolutionary conflict. The existence of this conflict assumes that the relationship between PHB per cell and rhizobium fitness is positive, a hypothesis tested in this paper.

The objective of this study was to determine the quantitative relationship between accumulated PHB and rhizobium fitness during starvation. Phenotypic differences in PHB accumulation (measured by flow cytometry) were generated among five genetically identical populations of *S. meliloti* using two independent methods. These rhizobia were starved, and the effect of differences in cellular PHB on reproduction and long-term survival was measured. To link these results to PHB levels in symbiotic rhizobia, a flow-cytometric protocol for measuring PHB per cell in the reproductively viable (nonbacteroid) rhizobium fraction from alfalfa nodules was developed, and PHB accumulation by symbiotic *S. meliloti* measured during nodule development. Finally, rhizobium reproduction and PHB content of rhizobia in senescing nodules was measured, as a preliminary indication of whether rhizobia consume PHB inside senescing nodules or conserve it for use after escaping into the soil.

Materials and methods

Starvation

Sinorhizobium meliloti 1021 populations with either high- or low-PHB content per cell were starved for 165 days to determine if PHB confers a fitness advantage when carbon is limiting. High- and low-PHB cells were generated by fractionating a single population by buoyant density and by growing rhizobia to late (7-day incubation) and early (3-day incubation) stationary phase (Patel & Gerson, 1974) in M9 minimal media (Miller, 1992) using $20\,g\,L^{-1}$ mannitol as the carbon source.

PHB accumulation increases bacterial buoyant density (Pedrós-Alió *et al.*, 1985). To exploit this effect, late-stationary phase *S. meliloti* grown in M9 were suspended in 0.5 mL of 85% Percoll and 15% phosphate-buffered saline (PBS) in sterile 6×50 mm glass test tubes, sealed with Parafilm and centrifuged at 15 000 ***g*** for 30 min in an Eppendorf 5415 D microcentrifuge. When centrifuged, Percoll forms a density gradient. Rhizobia separated on the density gradient were divided into three fractions of equal volume containing cells with high (1.138–$1.119\,g\,mL^{-1}$), medium (1.119–$1.109\,g\,mL^{-1}$), and low ($\leq 1.109\,g\,mL^{-1}$) density. Marker beads (Amersham Biosciences, Sweden) were used to determine density.

Before starvation, all cells were double washed in carbon-free M9 media (containing no mannitol, $0.1\,g\,L^{-1}$ thiamine,

and HPLC-grade water). All glassware used for making carbon-free M9 was rendered carbon-free before use by acid washing in 0.6 M HCl for 1 h, then pyrolizing at 550 °C overnight. The initial population size (rhizobia mL^{-1}) was determined by dilution plating. Cells were diluted to 5×10^5 cells mL^{-1} in carbon-free M9; 1-mL aliquots were placed in sterile 2-mL microcentrifuge tubes and inverted daily to resuspend. Three replicate tubes were harvested at each time point and the number of viable rhizobia was determined by plating. Rhizobia were fixed, resuspended in PBS +20% glycerol, and stored at −80 °C until the end of the experiment, when PHB per cell of all samples was determined by flow cytometry.

Plant growth

Alfalfa (*Medicago sativa* L. cv. Rebound 5.0, from C. Scheaffer, University of Minnesota) was grown in hydroponic growth pouches (Mega International, Minneapolis, MN). Plants were grown in a Conviron E7/2 growth chamber with a 16-h photoperiod, light intensity of 500 μE m^{-2} s^{-1} photosynthetically active radiation, and a day/night temperature of 22/15 °C. Plants grown for the PHB accumulation experiment were inoculated as seedlings with 5×10^7 cells per plant of *S. meliloti* strain 1021 (from M. Sadowsky, University of Minnesota). Five nodules from the same cohort (same day of emergence) were harvested from three plants after 7, 14, 21, and 28 days of nodule growth; each plant was sampled only once. Plants grown for the nodule senescence experiment were inoculated with 10^5 cells per plant of *S. meliloti* strain 20MP6, an *S. meliloti* 2011 mutant with a highly stable, chromosomally integrated *gfp* (from A. Lagares, Universidad Nacional de La Plata, Argentina) (Pistorio *et al.*, 2002). Plants were grown as above for 5 weeks before senescence was initiated by either the complete removal of aboveground biomass (regrowth was removed every 2–3 days), or shading, achieved by placing a foil-covered cardboard box over the plants. Forty-two days after the induction of nodule senescence, four nodules were harvested from three plants per treatment.

Developing nodules were weighed and surface sterilized by a 10-s dip in 95% ethanol and a 5-min submersion in 3% bleach (0.18% NaOCl), then washed five times in sterile deionized (DI) water. Senescent nodules were not surface sterilized, to avoid killing rhizobia inside. All nodules were crushed in a sodium ascorbate extraction buffer (Arrese-Igor *et al.*, 1992), and then centrifuged at 100 ***g*** for 10 min to separate the supernatant containing rhizobia from nodule debris. Extracted rhizobia were pelleted at 5000 ***g*** for 5 min, resuspended in starvation buffer (Wei & Bauer, 1998), and stored at 4 °C.

Flow cytometry

To quantify PHB accumulation flow cytometrically a standard curve was generated. High- and low-PHB-containing *S. meliloti* were grown by culturing cells for 4, 8, 12, or 16 days with high or low gas exchange. Cells (5×10^7) were inoculated into five replicate 125-mL Erlenmeyer flasks per treatment combination containing 50 mL yeast mannitol broth with 20 g mannitol L^{-1} (Somasegaran & Hoben, 1994). Low gas exchange was imposed by covering the flask mouth with stretched sterile Parafilm and aluminum foil; Parafilm was omitted for high gas exchange. All flasks were harvested simultaneously. Forty milliliters of each sample was removed and centrifuged at 2600 ***g*** for 10 min. Pellets were double washed in DI water and dried for 2 days at 70 °C in 13 × 100 mm screw cap glass test tubes. Cellular PHB was determined by propanolysis and analysis following the protocol of Riis & Mai (1988) on an Agilent 6890N gas chromatograph with autosampler and HP-5 column (from J&W Scientific, 30 m, 0.32 mm ID, 0.25 μm film size). The remaining 10 mL of cells were fixed in 30% ethanol for 30 min and diluted to 10^7 cells 10 μL^{-1} aliquot and stored at −80 °C in PBS+20% glycerol. Five samples were discarded due to contamination or PHB extraction errors.

To prepare cells for PHB analysis, rhizobia were fixed and diluted to either 5×10^5 or 5×10^6 cells mL^{-1} (all cells in a run were diluted to the same concentration) in PBS and stained with Nile Red (NR), a fluorescent probe that binds PHB (Gorenflo *et al.*, 1999). Diluted rhizobia were stained with 1% NR stock solution [100 μg mL^{-1} in dimethyl sulfoxide (DMSO)] for 1 h, then pelleted at 5000 ***g*** for 5 min and resuspended in PBS to remove excess stain. Cells were analyzed on a Benton Dickson FACSCalibur, exciting with a 15 mW 488 nm argon laser. NR fluorescence was measured in channel FL2 (585 ± 42 nm bandpass filter). Background fluorescence from NR-stained bacteria is minimal (Kacmar *et al.*, 2005). Thus, 10 samples of *S. meliloti* cells of known PHB accumulation (by GC above) were used to generate a NR vs. PHB standard curve, by regressing geometric-mean NR fluorescence on mean PHB per cell (pg). These were stained and sampled under the same conditions as the cells run for PHB determination. PHB per cell was then determined by converting a sample's mean NR fluorescence to PHB per cell through the standard curve regression function. A new standard curve was fit for each cytometry session that PHB was quantified. Senescing nodules contained large number of nonrhizobium contaminating bacteria. To exclude these cells from PHB analysis, and to count the number of rhizobia in senescing nodules, all cells without GFP fluorescence were excluded by gating on FL1.

To obtain cell counts, fixed rhizobia were diluted to between 5×10^5 and 10^7 cells mL^{-1} and data were acquired for 15 s on the flow cytometer. If the population of cells on the FSC × SSC (forward by side scattering) plot was overlapped by instrument noise, rhizobia were stained with 1% propodium iodide stock solution (1 mg mL^{-1}, in water) and

incubated at least 5 min to label cells (specifically, DNA) before being rerun. Cell counts mL^{-1} (C) of the original samples was determined by

$$C = F/(t \times R \times D)$$

where F is the number of cells acquired (after removing noise by gating using the FSC × SSC plot, or alternatively the number of cells fluorescing above background in channel FL3, if stained with PI), t is the time in seconds of data acquisition, R is the flow rate in $mL\,s^{-1}$ of the cytometer, and D is the dilution performed before staining. The flow rate (R) of the cytometer was determined by regression: four 12 × 75 mm FACS tubes were filled with *c.* 1 mL DI water and weighed with 0.1 mg precision. These were run for 1, 2, 3 and 4 min on the flow cytometer, and then weighed again to determine the volume removed. The slope of the linear regression for volume removed vs. time in seconds was used for R. The flow rate was recalculated each session to correct for any variation. To determine if the flow cytometer accurately counted cells, 60 samples of cultured *S. meliloti* were diluted to between 5×10^5 and 3.5×10^7 cells mL^{-1}; then rhizobium density in each culture was determined by both drop plating on TY agar (Somasegaran & Hoben, 1994) and flow cytometry. For flow analysis all cultures were diluted to 5×10^5–1×10^7 cells mL^{-1}.

Statistical analyses

JMP 7.0 was used for all statistical analyses. One-way ANOVA and *t*-tests were used to determine if means varied significantly. Linear regression was used to describe the relationship between PHB accumulation and reproduction or survival. Assumptions of these parametric tests were checked and met for all data with the exception of PHB accumulation in alfalfa nodules, where the variance in PHB per cell increased with time. Before analysis, these data were power transformed using the Box–Cox algorithm to homogenize the variance. Tukey's HSD was used to determine if rhizobium populations fractionated by density contained significantly different amounts of PHB, and if fresh vs. senescing nodules differed in the number of rhizobia per nodule and PHB per rhizobium. In calculating the SE of PHB used during starvation, we conservatively assume there was no covariance between initial PHB accumulation and PHB content after starvation.

Results

Flow cytometric measurement of PHB

PHB per cell by GC was highly correlated with NR fluorescence ($P < 0.0001$, $r^2 = 0.95$, Fig. 1). The intercept for this regression was not significantly different from 0 ($t = 0.04$, d.f. = 35, $P = 0.96$. two-sided *t*-test), indicating that *S. meliloti* cells without PHB possess no detectable background fluorescence when stained with NR.

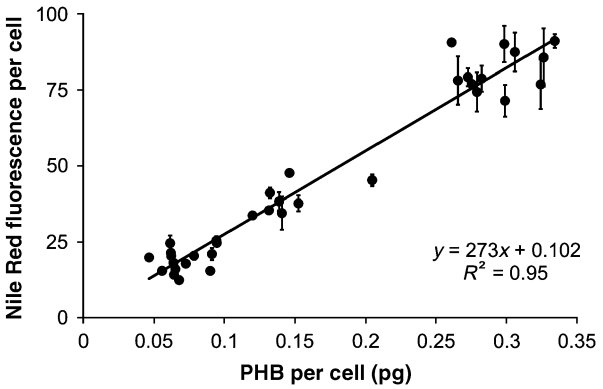

Fig. 1. Standard curve relating Nile Red fluorescence to gas chromatographically determined PHB per cell (in pg) in *Sinorhizobium meliloti*. Lines shown are least squares linear regressions. Error bars are the SE of three replicates.

Manipulation of cellular PHB content

Sinorhizobium meliloti cultured to late stationary phase accumulated an average of 0.50 pg PHB per cell, significantly more than the 0.30 pg PHB per cell accumulated by early stationary phase cells ($t = 10.43$, d.f. = 4, $P = 0.0004$; two-sample *t*-test of NR fluorescence). When split by buoyant density, cells from the dense, medium, and buoyant fractions accumulated 0.40, 0.30, and 0.18 pg PHB per cell, respectively ($F_{2,8} = 120.9$, $P < 0.0001$, one-way ANOVA, all means significantly different at $\alpha = 0.01$).

Effect of PHB on the fitness of starving rhizobia

We examined the extent to which PHB use during starvation correlated with the ability of *S. meliloti* to reproduce and persist during long-term starvation. When starved, cells from all treatments initially reproduced, with population sizes peaking after 29–36 days of starvation (Fig. 2). Reproduction was measured as the difference between the maximum population size attained during starvation and the initial population size. Rhizobia using more PHB during starvation (a direct consequence of starting with more PHB) reproduced significantly more ($P = 0.003$, linear regression, Fig. 3a).

Sinorhizobium meliloti consumed the majority of stored PHB in the first 29–36 days of starvation; after which all *S. meliloti* populations steadily decreased in numbers (Fig. 2). Linear regressions of relative population size (viable cells on day n, divided by cells inoculated) vs. days of starvation, during this population decline (days 29–36 through 165, $P < 0.0001$ for all treatments) were used to calculate the time elapsed before each treatment crossed the line

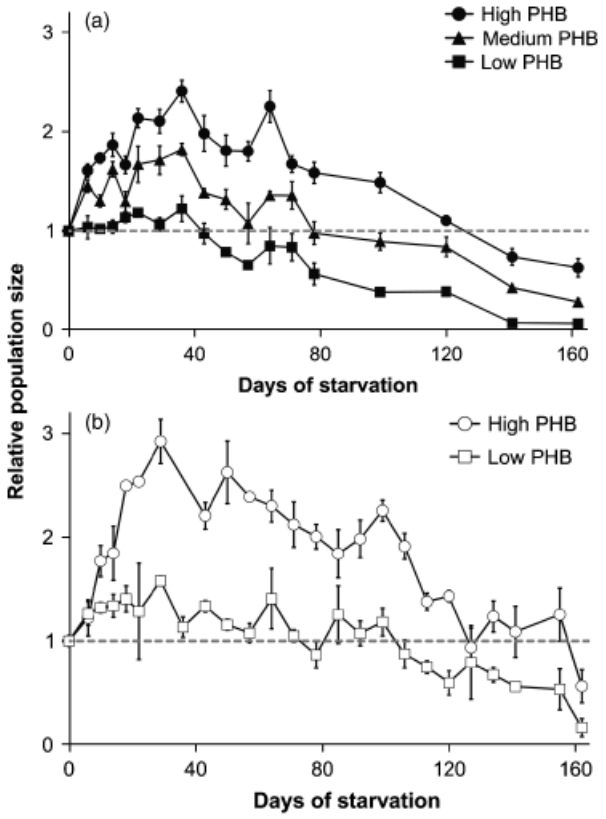

Fig. 2. Starving *Sinorhizobium meliloti*. (a) Rhizobia with high (0.4 pg per cell), medium (0.3 pg per cell) and low (0.18 pg per cell) mean PHB accumulation were obtained by separating stationary phase cells into high, medium, and low buoyant density fractions, respectively. (b) High (0.5 pg per cell) and low (0.3 pg per cell) mean PHB accumulation were generated by harvesting cells at late or early stationary phase culture, respectively. Relative population size, the number of viable cells divided by the initial population size, was determined by plating. Dashed gray lines show time-zero population size. Error bars are the SE of three replicates.

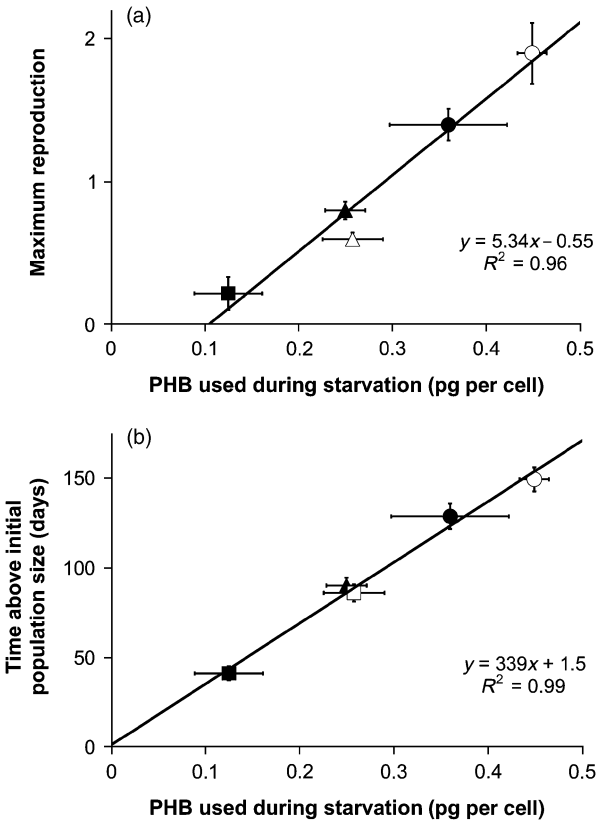

Fig. 3. PHB linearly increases rhizobium fitness under starvation. PHB was used as a source of energy and carbon for reproduction (a); and increased the ability of cells entering starvation with more PHB to maintain larger population sizes (b). PHB used during starvation was determined by subtracting mean PHB per cell at the population size peak (at this point PHB per cell was constant for the rest of the experiment) from initial PHB per cell for each treatment. PHB per cell was determined by flow cytometry. Maximum reproduction (a) was measured as the difference between the maximum number of viable cells measured and the number of inoculated cells. Population maintenance over long-term starvation (b) was determined by linearly regressing viable population size on time for all points after the population size peak to calculate the time taken for the population to return to its initial size, by interpolation. Treatment symbols used are the same as in Fig. 2. Error bars are 1 SE.

corresponding to its initial inoculum population size. Populations of *S. meliloti* with initially higher PHB persisted significantly longer than those with less PHB ($P = 0.0005$, linear regression, Fig. 3b), with a threefold range in survival time over the 2.8-fold PHB range tested.

Flow cytometric analysis of symbiotic rhizobia

Alfalfa nodules typically contained two *S. meliloti* populations that could be distinguished by flow cytometry because they varied >10-fold in forward scatter (Fig. 4), indicating a large size difference (Shapiro, 2003). A bimodal distribution in cell size was confirmed by microscopy (data not shown). Only the small-celled population (R_1 in Fig. 4) accumulated PHB, as measured by NR fluorescence. PHB accumulation and small size are both consistent with these being the undifferentiated, reproductive rhizobia (Denison, 2000; Lodwig *et al.*, 2003). An observed correlation of viable cells per nodule with PHB-containing cells further supports this conclusion. Linear regression of flow-cytometric counts onto plate counts had a slope near one and an intercept near zero for both cultured *S. meliloti* and PHB-containing *S. meliloti* from alfalfa nodules (Table 1). This contrasts with cytometric counts of total rhizobia (including those without PHB, assumed to be bacteroids), which significantly overestimated viable rhizobia numbers (Table 1).

Undifferentiated *S. meliloti* cells in developing nodules accumulated a within-nodule mean of 0.04–0.25 pg PHB per cell during the first 3 weeks of nodule growth (Fig. 5). Mean

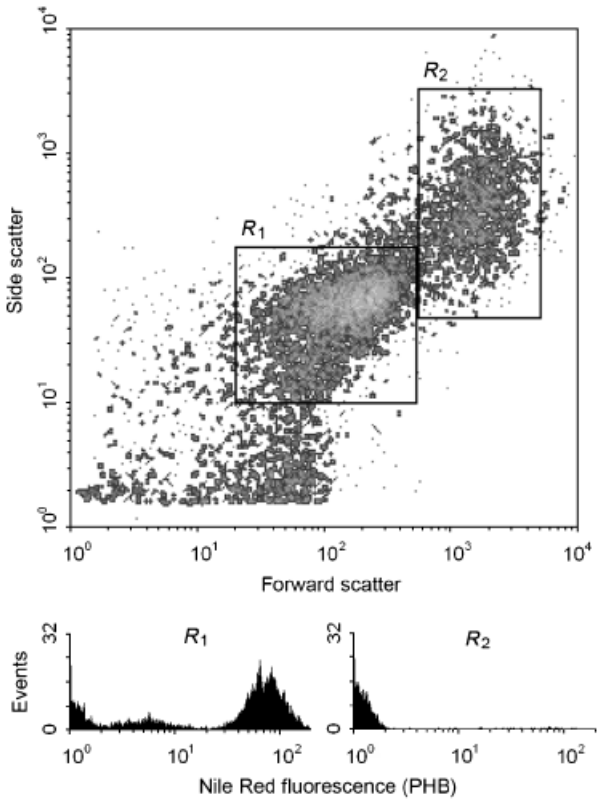

Fig. 4. Flow cytometry of *Sinorhizobium meliloti* from an alfalfa nodule. Bacteroids (R_2) possess a higher forward scatter (because they are larger) than undifferentiated cells in R_1, and did not contain detectable levels of PHB, unlike undifferentiated cells.

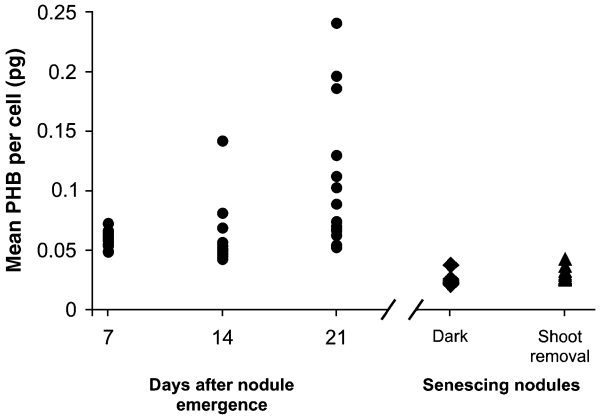

Fig. 5. Mean PHB content of undifferentiated *Sinorhizobium meliloti* in symbiosis with alfalfa. Each point represents the average of thousands of rhizobia from a single nodule. At 7, 14, and 21 days from nodule emergence, five healthy nodules were harvested from three plants. Nodules from day 28 were senescent and contaminated with other bacteria so were excluded. To quantify PHB accumulation by rhizobia in senescing nodules, plants were inoculated with GFP-marked *S. meliloti*, allowing the flow cytometer to exclude contaminant bacteria when measuring cellular PHB. These plants were grown for 5 weeks, then senescence was initiated by shading or shoot removal. After 42 days four nodules were harvested from three plants and PHB accumulation was determined by flow cytometry.

PHB per cell within a nodule increased over this period ($P = 0.0011$, $n = 43$, linear regression). In senescing nodules, 42 days after alfalfa biomass was removed or shaded, nodules had changed color from pink to gray, but were still physically intact and firm to the touch. Developing nodules contained an average of 1.6×10^6 undifferentiated rhizobia per nodule after 21 days of growth, whereas nodules induced to senesce by dark and detopping had an average of 5.6×10^6 and 7.8×10^5 undifferentiated rhizobia per nodule, respectively. Nodules in the dark treatment contained significantly more rhizobia than either the detopped or healthy nodule treatments, which were not significantly different ($F_{2,36} = 15.1$, $P < 0.0001$, one-way ANOVA, differences assessed at $\alpha = 0.05$). Senescing nodules contained rhizobia with 0.021–0.043 pg PHB per cell (Fig. 5), significantly less PHB than rhizobia in developing 21-day-old nodules ($F_{2,36} = 134.3$, $P < 0.0001$, one-way ANOVA, differences assessed at $\alpha = 0.01$).

Table 1. Linear regression of the viable *Sinorhizobium meliloti* nodule population size (determined by plate counts) on the flow cytometric determination of cells per nodule

Source	Slope	Intercept as a percentage of plate count range	r^2
Culture	1.06	5.7***	0.84
Nodule (all rhizobia)	0.30****	.92	0.83
Nodule (cells with PHB)	1.17	2.3	0.71

Two-way *t*-tests that the slope $\neq 1$ and the intercept $\neq 0$.
Significant at the ***$P < 0.001$ and ****$P < 0.0001$ level. $n > 40$ for all treatments. All regressions were highly significant ($P < 0.0001$).

Discussion

PHB can serve as a reproductive currency for starving *S. meliloti*, increasing short-term fitness by providing the carbon and energy required for reproduction. PHB linearly increased long-term population maintenance of starving *S. meliloti*, mainly because the population increased more before declining. Early and late stationary phase cells doubtless vary in traits other than PHB accumulation (Kolter *et al.*, 1993), which may also be the case for cells varying in buoyant density. Nevertheless, variation in the amount of PHB used during starvation explained the great majority of variation in reproduction and population persistence (96% and 99%, respectively), with all five populations falling on the same lines. This suggests that the relationship between PHB catabolism and reproduction and survival during starvation is robust to variation in other phenotypic traits

in *S. meliloti*. In addition to PHB, *S. meliloti* can store energy in glycogen and EPSs (Zevenhuizen, 1981; Leigh *et al.*, 1985; Wang *et al.*, 2007a). The tight correlation between PHB used during starvation and reproduction and persistence suggests that energy storage in other polymers, at least in cultured cells, is either exceptionally well correlated with PHB accumulation or is of negligible importance under our conditions.

In developing alfalfa nodules, we found that *S. meliloti* 1021 accumulated a maximum of 0.25 pg PHB per cell; about half of the maximum PHB accumulation found in cultured cells. Most nodules contained *S. meliloti* with little PHB, but these rhizobia may accumulate more PHB as the nodule matures. Undifferentiated rhizobia in alfalfa nodules are released to the soil when nodules senesce (Vance *et al.*, 1980). Rhizobia within senescing nodules face a potential tradeoff: stored PHB can be conserved for later use in the soil, or PHB can be used to fuel growth and survival within the nodule, increasing the number of rhizobia that are eventually released to the soil. *Sinorhizobium meliloti* appears to have adopted the latter strategy. After 42 days in senescing alfalfa nodules, undifferentiated *S. meliloti* contained almost no PHB. Nodules induced to senesce by shoot removal contained similar numbers of undifferentiated rhizobia as developing nodules after 21 days of growth, while nodules induced to senesce by shading contained 3.5-fold as many undifferentiated rhizobia. This relatively limited reproduction suggests that senescing nodules are a poor food source for *S. meliloti*, and that energy storage in PHB during nodule development may significantly increase rhizobium reproduction and survival within senescing nodules. Not all rhizobia in nodules will endure prolonged nodule senescence, however. Nodules can be attacked by herbivores (Quinn & Hall, 1992). Any rhizobia released by herbivores from nonsenescent nodules may benefit from stored PHB. Although *S. meliloti* accumulated PHB relatively slowly in alfalfa, rhizobia associated with legumes that form determinate-type nodules may accumulate PHB faster. For example, Wong & Evans (1971) found that *B. japonicum* in soybean nodules accumulated 40% PHB by cell dry weight over a 10-day period.

Many genes have been shown to affect PHB accumulation in rhizobia, suggesting that PHB accumulation is a quantitative trait. Mutants show that genetic variation can exist for PHB accumulation (Cai *et al.*, 2000; Cermola *et al.*, 2000; Encarnacion *et al.*, 2002; Aneja *et al.*, 2005; Wang *et al.*, 2007a, b), but the extent to which indigenous rhizobia vary in these genes (and their phenotypic consequences) remains to be determined. Past estimates of symbiotic rhizobium fitness typically considered only the number of viable rhizobia per nodule (Kiers *et al.*, 2003) or nodule size, which is correlated with viable rhizobia per nodule (Simms *et al.*, 2006; Heath & Tiffin, 2007). We found that rhizobia can accumulate enough PHB in symbiosis to double their numbers subsequently (perhaps within senescing nodules or after escaping from a damaged nodule), so rhizobium fitness estimates would be improved by including information on cellular PHB.

The results of this study suggest that, all else being equal, rhizobia should be under strong directional selection for increased PHB accumulation, which would tend to decrease nitrogen fixation. Yet field studies examining rhizobium mutualism have found that rhizobium populations are highly polymorphic for host benefit (Erdman, 1950; Burdon *et al.*, 1999; Heath & Tiffin, 2007). Host sanctions imposed on cheating rhizobia (Denison, 2000) may counter the selective advantage rhizobia would obtain by maximizing PHB accumulation. Kiers *et al.* (2003) showed that sanctions reduced the number of viable rhizobia inside soybean nodules by up to a factor of 3, but PHB was not measured. Given that the potential reproductive advantage high-PHB cells obtained over low-PHB cells in this study is also on the order of threefold, a comparison of PHB accumulation between sanctioned and nonsanctioned rhizobia would improve our understanding of the efficacy of sanctions in selecting against cheating rhizobia.

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