Comparing Symbiotic Efficiency between Swollen versus Nonswollen Rhizobial Bacteroids1[C][W][OA]

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Symbiotic rhizobia differentiate physiologically and morphologically into nitrogen-fixing bacteroids inside legume host nodules. The differentiation is apparently terminal in some legume species, such as peas (Pisum sativum) and peanuts (Arachis hypogaea), likely due to extreme cell swelling induced by the host. In other legume species, such as beans (Phaseolus vulgaris) and cowpeas (Vigna unguiculata), differentiation into bacteroids, which are similar in size and shape to free-living rhizobia, is reversible. Bacteroid modification by plants may affect the effectiveness of the symbiosis. Here, we compare symbiotic efficiency of rhizobia in two different hosts where the rhizobia differentiate into swollen nonreproductive bacteroids in one host and remain nonswollen and reproductive in the other. Two such dual-host strains were tested: Rhizobium leguminosarum A34 in peas and beans and Bradyrhizobium sp. 32H1 in peanuts and cowpeas. In both comparisons, swollen bacteroids conferred more net host benefit by two measures: return on nodule construction cost (plant growth per gram nodule growth) and nitrogen fixation efficiency (H₂ production by nitrogenase per CO₂ respired). Terminal bacteroid differentiation among legume species has evolved independently multiple times, perhaps due to the increased host fitness benefits observed in this study.

Legume-rhizobia interactions vary widely across a diverse paraphyletic group of soil bacteria known for symbiotic nitrogen fixation inside root nodules of over 18,000 species of legumes throughout the world (Lewis et al., 2005). In several legume species, rhizobial cells are induced to swell during their differentiation into nitrogen-fixing bacteroids (Oono et al., 2010). These legume species belong to five different major papilionoid clades (inverted repeat-lacking clade, genistoids, dalbergioids, mirbelioids, and millettioids), a pattern suggestive of convergent evolution. Swelling apparently leads to terminal differentiation; swollen bacteroids no longer divide normally (Zhou et al., 2018). In other legume host species, bacteroid differentiation is less extreme, leading to nonswollen bacteroids. Nonswollen bacteroids are similar in shape and size to free-living rhizobia and divide normally once outside of their nodules. The proximate mechanisms for host-imposed bacteroid swelling have been investigated (Van de Velde et al., 2010), but what drove the repeated evolution of this trait? The multiple independent origins of host traits causing bacteroids to swell suggest that swollen bacteroids may provide more net benefit to legumes. Could the swelling of bacteroids improve nitrogen fixation efficiency (e.g., nitrogen fixed relative to carbon cost)? In this study, we compare symbiotic efficiencies of rhizobia in legume hosts that are evolutionarily diverged but share a common effective rhizobial strain, whose bacteroids are swollen in one host and nonswollen in the other.

Variations among host species in benefits and costs of symbiosis with rhizobia are not commonly explored (Thrall et al., 2000) because legume species typically nodulate with only one group of rhizobia (e.g. Sinorhizobium sp. in Medicago), although some legumes and some rhizobia are more promiscuous. Rhizobium sp. NGR234 has the largest known host range but does not fix nitrogen effectively with any legume species currently recognized to induce swelling of rhizobial bacteroids (Pueppke and Broughton, 1999). Some Sinorhizobium fredii strains apparently fix nitrogen in certain cultivars of soybean (Glycine max; hosting nonswollen bacteroids) and alfalfa (Medicago sativa; hosting swollen bacteroids; Hashem et al., 1997), but our efforts to replicate these results did not lead to successful nodulation. Therefore, we studied two strains, a transgenic strain thatnodulates beans (Phaseolus vulgaris) and peas (Pisum sativum) and a second wild strain harvested from cowpeas (Vigna unguiculata) that also nodulates peanuts (Arachis hypogaea). Beans and cowpeas are both within the Phaseoloid group and do not induce terminal differentiation of rhizobial bacteroids. Peas and peanuts both host ter-

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1 This work was supported by the National Science Foundation (grant no. 0918986).

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www.plantphysiol.org/cgi/doi/10.1104/pp.110.163436
can also be important. Rhizobia that fix more nitrogen (Minchin et al., 1981). Faster fixation rates (mol nitro-

grobation per bacteroid might be restricted such that the rate of oxidative phosphorylation, necessary for nitrogen fix-

gation, is reduced. Fixation rates per bacteroid may be dif-

teimum above 25% of a legume’s net photosynthate

Differences in symbiotic qualities between swollen

Rhizobial performances are often compared by mea-

Table I. t test on-linear model controlling for a constant effect of plant age with all strain: host

| Bean/Pea | Estimate | SE | t Value | Pr (>|t|) |
|----------|----------|----|---------|----------|
| Nodule weight | 1.59 | 0.31 | 5.15 | 2.1 x 10^-6 |
| Plant age | 0.01 | 0.002 | 6.97 | 1.0 x 10^-9 |
| Nodule weight x A34:bean | -1.44 | 0.35 | -4.14 | 9.1 x 10^-5 |
| Nodule weight x 4292:bean | -1.35 | 0.33 | -4.10 | 0.0001 |
| Nodule weight x 3841:pea | 0.67 | 0.54 | 1.24 | 0.22 |

minally differentiated bacteroids but are in distant clades and likely have different genetic origins for traits that induce terminal differentiation (Oono et al., 2010). Also, the swollen bacteroids in peas are branched while those in peanuts are spherical.

Differences in symbiotic qualities between swollen and nonswollen bacteroids have been previously explored in peanuts and cowpeas by Sen and Weaver (1980, 1981, 1984), who also hypothesized that swollen bacteroids are more beneficial to the host plant than nonswollen ones. They found 1.5 to 3 times greater acetylene reduction by nitrogenase (as well as plant nitrogen) per nodule mass in peanuts than in cowpeas at multiple nodule ages (Sen and Weaver, 1980). Acetylene reduction per bacteroid was also greater in peanuts than in cowpeas when measuring whole nod-
ules, but this difference disappeared when isolated bacteroids were assayed (Sen and Weaver, 1984). They concluded that swelling of peanut bacteroids per se was not responsible for the higher rate of nitrogen fixation per bacteroid. They suggested that in cowpea nodules, with greater numbers of smaller bacteroids per nodule volume, availability of oxygen to each bacteroid might be restricted such that the rate of oxidative phosphorylation, necessary for nitrogen fixation, is reduced. Fixation rates per bacteroid may be different between hosts due to nodule gas permeability or bacteroid crowding within nodules. However, fixation efficiency (nitrogen fixed per carbon respired) would not necessarily be affected by these and may be more important for the host than the rate of fixation.

Rhizobial performances are often compared by mea-
suring the symbiotic benefits, e.g. rates of acetylene reduction or plant growth (Sen and Weaver, 1984; Hashem et al., 1997; Lodwig et al., 2005), but rarely by measuring the symbiotic costs, e.g. carbon consumed or respired. Up to 25% of a legume’s net photosynthate may be required for nitrogen fixation by rhizobia (Minchin et al., 1981). Faster fixation rates (mol nitrogen per s) can be beneficial for hosts, but carbon costs can also be important. Rhizobia that fix more nitrogen per carbon respired could free more carbon for other functions, including the option of supporting more nodules with the same amount of photosynthate. If legumes are sometimes carbon limited, then improved carbon-use efficiency could enhance plant fitness. Measuring both benefits and costs is therefore key to an accurate understanding of the symbiotic performance of a rhizobial strain.

While we recognize the many physiological differences between peas and beans or peanuts and cow-
peas, the fact that terminal differentiation induced by host legumes evolved multiple times independently (Oono et al., 2010) suggests there may be some con-
sistent host symbiotic benefit, such as improved fixa-
tion efficiency. Here, we measured the efficiency of each of two strains as swollen bacteroids in one host and nonswollen bacteroids in another. We measured nitrogenase activity as hydrogen (H2) production in an N2-free atmosphere (Layzell et al., 1984; Witty and Minchin, 1998), and compared it to carbon dioxide (CO2) respiration to estimate return on nodule operation cost. We also compared host biomass growth per total nodule mass growth to estimate return on nodule construction cost. To further assess carbon allocation to the different types of bacteroids, we also measured the average amounts per bacteroid of polyhydroxybutyrate (PHB), an energy storage compound that can comprise up to 50% of bacteroid dry weight (Trainer and Charles, 2006). A greater PHB accumulation per bacteroid may require a decreased allocation of carbon for nitrogenase activity within the bacteroids, and hence, less plant growth per carbon invested in bacteroids. We demonstrate that peas and peanuts that host swollen bacteroids have higher fixation efficiency as well as greater plant return on nodule construction than beans and cowpeas, respectively, nodulated with the same rhizobial strains. PHB was not consistently correlated with plant:nodule growth efficiency with the tested strains. These findings show that swollen bacteroids can indeed provide greater benefits to their legume hosts.
RESULTS

Plant Return on Nodule Construction Cost in Pea versus Bean

Peas and beans nodulated with *Rhizobium leguminosarum* A34 were harvested periodically to obtain a range of plant sizes and their dry plant weights and nodule weights were measured. Since A34 is a transgenic strain transformed from a rhizobial strain that only nodulates beans, we compared its performance on each host with the performance of a natural strain. The relationship between nodule fresh weight and shoot dry weight was not significantly different between pea hosts nodulated by A34 versus *R. leguminosarum* 3841, a wild strain that only nodulates peas (Table I; Supplemental Fig. S1). The relationship was also not significantly different between bean hosts nodulated by A34 versus *R. leguminosarum* 4292, a wild strain that only nodulates beans (Table I; Supplemental Fig. S2). However, the relationship between nodule fresh weight and shoot dry weight was significantly different between pea and bean hosts, controlling for a constant linear effect of plant age, when each was nodulated by A34 (Table I). Dry weights of shoots and roots (g) per nodule dry weight (g) were about 5 times greater for pea than for bean (10.68 versus 2.64, Fig. 1A). During the time period in which the host plants grew the fastest, pea plants grew 1.27 g dry weight per day per g of nodule dry weight while bean plants grew only 0.27 g dry weight per day per g of nodule dry weight with A34 in each host. Since shoot nitrogen concentration did not differ significantly between peas and beans (2.7% and 2.5%, respectively, *P* = 0.18, *n* = 50), greater plant biomass indicates greater fixed nitrogen. Both pea and bean biomasses were lower than expected under field conditions since they were grown in plastic pouches in the growth chamber.

Plant Return on Nodule Construction Cost in Peanut and Cowpea

To compare plant growth per gram of nodule growth between peanuts (hosting swollen spherical bacteroids) and cowpeas (hosting nonswollen bacteroids), peanuts and cowpeas were nodulated with *Bradyrhizobium* sp. 32H1 and harvested intermittently across 50 d. The relationship between nodule dry weight and host dry weight was significantly different between peanuts and cowpeas, controlling for a constant linear effect of plant age (Table II). Peanuts grew about 3 times more than cowpeas in shoot and root dry weight (g) per nodule dry weight (g; 39.16 versus 12.62, Fig. 1B). This 3-fold difference between peanuts and cowpeas is consistent with results reported for multiple rhizobial strains by Sen and Weaver (1981; 5.15 versus 1.5, Fig. 1B inset, *t* = 5.83, degrees of freedom = 7, *P* < 0.001). During the harvest period in which the host plants grew the fastest, peanuts grew about 3.74 g per day per g of nodule dry weight while cowpeas grew about 2.01 g per day per g of nodule dry weight.

Nitrogen Fixation Efficiency (H\(_2\):CO\(_2\))

Nitrogen fixation efficiency was measured as marginal increase in H\(_2\) evolution by nitrogenase in nitrogen-free air (argon:O\(_2\)) with increasing CO\(_2\) production from nodule-interior respiration induced by incremental increases of external oxygen from 21% to 33% (Fig. 2). Pea nodules averaged 0.50 H\(_2\)/CO\(_2\) (SD ± 0.04) while bean nodules averaged 0.34 H\(_2\)/CO\(_2\) (SD ± 0.01) over three independent measurements each (Fig. 3A) when each contained the same rhizobia, *R. leguminosarum* A34. Nitrogen fixation efficiency of peanuts averaged 0.62 H\(_2\)/CO\(_2\) (SD ± 0.18) while efficiency in cowpeas averaged 0.28 H\(_2\)/CO\(_2\) (SD ± 0.04) when each was nodulated with *Bradyrhizobium* sp. 32H1 (Fig. 3B).

H\(_2\) evolution is a by-product of the nitrogenase reaction, with at least 25% of nitrogenase activity going...
to H₂ production in ambient air (Schubert and Evans, 1976). For _R. leguminosarum_ A34, there were detectable levels of H₂ produced in air (N₂:O₂), which increased 3-fold when switched to argon:O₂, indicating that, in air, approximately 33% of nitrogenase activity was used in H₂ production instead of nitrogen fixation. However, when sampling H₂ evolution from _Bradyrhizobium_ sp. 32H1 in either cowpea or peanut nodules, there were no detectable levels of H₂ until the atmosphere surrounding nodules was switched to argon:O₂. Hence, 32H1 may contain hydrogen uptake (hup) enzymes, commonly found among cowpea rhizobia (Martins et al., 1997). If 32H1 expresses hup genes, our measurements of hydrogen production may not be equally proportional to nitrogen fixation in cowpeas and peanuts because differences in nodule permeability, for example, could affect nodule-interior H₂ and therefore H₂ uptake. However, if H₂ uptake was saturated in both species by the greater H₂ production in argon:O₂, then presence of hydrogenase would not affect marginal increase in H₂ efflux with respiration (i.e. the slope of H₂ versus CO₂ line), which we used to calculate efficiency (Fig. 2C). Differences between host nodule physiology, such as the fraction of a nodule that contains bacteroids, could also affect total fixation/respiration, but again, such differences would only affect the baseline respiration rather than the slope of the efficiency line.

**PHB Accumulation per Bacteroid**

*R. leguminosarum_ A34 bacteroids lacked PHB inside pea nodules (Fig. 4), which is a similar result seen in natural pea rhizobia, such as 3184. However, A34 accumulated high levels of PHB in beans (Fig. 4), as did 4292 (Lodwig et al., 2005) and many other natural bean rhizobia. PHB in swollen bacteroids was analyzed separately from PHB in the undifferentiated cells by distinguishing large and small cells by forward scatter in flow cytometry.

**Table II. t test on-linear model controlling for a constant effect of plant age, using cowpea plants nodulated by *Bradyrhizobium* sp. 32H1 as baseline comparison**

| Peanut/Cowpea | Estimate | se | t Value | Pr (>|t|) |
|---------------|----------|----|---------|----------|
| Nodule weight | 11.12    | 3.71| 3.00    | 0.007    |
| Plant age     | 0.01     | 0.01| 1.09    | 0.29     |
| Nodule weight × peanut | 25.14    | 5.34| 4.71    | 0.0001   |

**Figure 2.** Method for measuring nitrogen fixation efficiency. A, Either N₂ or argon was directed via a two-way valve into one mass flow controller (MFC). O₂ was connected to another MFC. Tubes from the two MFC mixed O₂ with either N₂ or argon before entering the nodule-containing tube. The bottom of the tube contained water to humidify the gas mixture before affecting the nodules, which were suspended in the middle of the tube with tissues. Gas from the top of the nodules was directed to an H₂ analyzer and an infrared gas analyzer. B, Increasing external oxygen step wise from 21% in a nitrogen-free atmosphere (argon: oxygen mixture) raises respiration and nitrogenase activity. Nitrogen fixation is measured by hydrogen production. C, Slope of H₂:CO₂ regression line defines efficiency for nitrogen fixation while CO₂ intercept defines baseline respiration for nodules, root fragments, and rhizobia in the tube. [See online article for color version of this figure.]
Bradyrhizobium 32H1 bacteroids had low levels of PHB in both cowpeas (0.045 pg/cell ± 0.03 SD, n = 20 nodules) and peanuts (0.03 pg/cell ± 0.02 SD, n = 14 nodules) compared to beans (0.25 pg/cell ± 0.13 SD, n = 20 nodules). PHB in cowpea bacteroids was significantly lower than normally found in closely related legume species with nonswollen bacteroids, e.g., bean bacteroids can accumulate up to 0.72 pg/cell, siratro (Macroptilium atropurpureum) bacteroids accumulate on average 0.35 pg/cell (W.C. Ratcliff, unpublished data).

DISCUSSION

Peas and Peanuts with Swollen Bacteroids Can Invest Less in Nodule Construction Than Beans and Cowpeas with Nonswollen Bacteroids

Peas grew about 5 times more per nodule mass than beans when each was nodulated by R. leguminosarum A34. Peanuts grew about three times more per nodule mass than cowpeas when each was nodulated by Bradyrhizobium sp. 32H1. The greater efficiency of 32H1 in peanuts compared to cowpeas can be generalized to other strains that nodulate both species as seen by Sen and Weaver (1981; Fig. 1B, inset), who compared four different effective strains’ host to nodule mass ratio, on peanuts, cowpeas, and siratro (data not shown). The study of Sen and Weaver (1981) shows that the superiority of peanut symbiosis over cowpeas was not unique to the 32H1 strain.

Peas and Peanuts Have Greater Fixation Efficiency Than Beans and Cowpeas, Respectively, with the Same Rhizobial Strain

Peas and peanuts, both hosting swollen rhizobial bacteroids, had higher nitrogen fixation efficiency (H₂ production per CO₂ respiration) than beans and cowpeas, respectively. The possible presence of hup genes in Bradyrhizobium sp. 32H1 would lead to underestimates of nitrogen fixation rates via H₂ evolution, but it would not affect efficiency estimates based on marginal rates, so long as uptake was saturated over the range used for calculating efficiency.

Similar methods have not shown consistent effects of bacteroid differentiation in the past. Our efficiency calculations are based on the approach of the late John Witty (Witty et al., 1983), who used acetylene reduction rather than hydrogen evolution to estimate nitrogenase activity. They measured fixation efficiency in 12 different legume genera and found no consistent difference between those species with swollen bacteroids and those with nonswollen ones. For example, peas ranged from 2.25 to 4.52 CO₂:C₂H₄ moles whereas beans ranged from 2.65 to 3.29 CO₂:C₂H₄ moles, depending on host cultivar and rhizobial strain. They also found no clear difference between cowpeas and peanuts (1.97 CO₂:C₂H₄ moles in cowpeas and 2.08 CO₂:C₂H₄ moles in peanuts) that were nodulated by the same strain of rhizobia RCR 3824. These comparisons by Witty et al. (1983), among others (Hunt et al., 1989), gave similar values to our H₂:CO₂ ratios assuming 1 mol of H₂ for 1 mol of C₂H₄ conversion. Further comparisons of single strains nodulating both hosts
that do or do not impose bacteroid swelling would be
informative. Unfortunately, such dual-host strains are
rare. Even R. leguminosarum A34 did not effectively
nodulate a wide array of bean cultivars, so the exper-
iment could not be extended even within bean. Inter-
estingly, A34 could effectively nodulate other pea
cultivars, including Green Arrow, albeit with delayed
nodulation with even higher efficiency (0.70 H2/
cultivars, including Green Arrow, albeit with delayed
nodulation with even higher efficiency (0.70 H2/
0.18 SD, n = 3) than in our first tested cultivar,
Maestro.

Greater nodule operation efficiency (N2 fixed per
CO2 respired) often correlates with greater return on
nodule construction cost (gram shoot per gram nod-
ule). Higher fixation efficiency often correlates with
the production of more plant mass relative to nodule
mass. Legumes will typically continue to form nodules
until they have attained an adequate nitrogen source,
i.e. legumes will form many more nodules with a
single less-effective strain than with a more-effective
one. Hence, the per-plant construction cost of nodules
is much greater for legumes when they do not find
effective strains. Peas had fewer nodules per plant (157
nODULES per g of plant mass) than beans (414 nodules
per g of plant mass) while the average nodule for the
two hosts weighed about the same (1.6 mg). Peanuts
had slightly more nodules than cowpea per g plant (68
versus 53), but their much smaller size (0.3 versus 1.4
mg) still resulted in less nodule construction cost per
gram shoot.

PHB in Swollen and Nonswollen Bacteroids

We hypothesized that if bacteroid PHB accumula-
tion is less in swollen bacteroids than in nonswollen
one, this could affect differences we saw in plant:
nodule growth efficiencies. PHB tends to be absent
from bacteroids of peas, and other vicieous legumes
with swollen bacteroids, but abundant in nonswollen
bean bacteroids. But we did not see significantly less
PHB in swollen peanut bacteroids compared to non-
swollen cowpea bacteroids. Plant interference with
bacteroid PHB synthesis, if it exists at all, is apparently
neither universal nor essential for increased nitrogen
fixation efficiency.

Why Do at Least Some Bacteroids Have Higher
Symbiotic Efficiency?

We do not have any direct evidence for possible
mechanisms whereby swollen and terminally differ-
entiated bacteroids would have increased efficiency,
but there are some possibilities that might merit fur-
ther research. When bacteroids are reproductive, there
are multiple bacteroids per peribacteroid unit, which
may lead to some bacteroids not having any contact
with the peribacteroid membrane that separates them
from host cytoplasm. This might decrease nutrient
exchange with the host. Swollen Y-shaped bacteroids
may have polar localization allowing partitioning of
metabolic functions in different areas of the cytoplasm
(Young 2006), which might increase fixation efficiency.
Terminally differentiated bacteroids are also known to
have genomic endoreduplication and their genomic
size can be commonly observed as 4C (Oono et al.,
2010; Van de Velde et al., 2010). This could increase the
potential rate of nitrogen fixation per bacteroid. A
faster rate would not necessarily increase bacteroid-
level efficiency (nitrogen fixed per carbon respired by
bacteroids). But nodule-level efficiency also depends
on respiration by plant mitochondria. If endoredupli-
cation leads to more active bacteroids and their respi-
ration is therefore a larger fraction of total nodule
respiration, then nodules containing swollen bacte-
roids could perhaps have more of their respiration
contributing directly to nitrogen fixation, increasing
nodule-level efficiency. Just as Sen and Weaver (1984)
found that isolated swollen and nonswollen bacteroids
did not differ in the fixation rate per bacteroid, isolated
bacteroids might not differ in their fixation:respiration
efficiency.

The terminal differentiation of bacteroids has been
shown to be mediated by plant factors known as
nodule-specific Cys-rich peptides (Van de Velde et al.,
2010). These peptides interfere with normal rhizobial
cell division (cytokinesis) once inside the peribacte-
roid membrane, which leads to a single bacteroid per
peribacteroid unit. This can lead to increased rhizobial
genome copies per cell if DNA synthesis was already
occurring. The cells typically become larger because
daughter cells cannot split off from each other during
cytokinesis. This tightly links the three characteristics
of (1) genomic endoreduplication, (2) single bacteroid
per host cell, and (3) swelling, making it difficult to
assess which characteristic might be most beneficial
for the host. Furthermore, other unknown peptide
effects could be the cause of a higher symbiotic effi-
ciency.

CONCLUSION

Only a fraction of legume species host terminally
derdifferentiated rhizobial bacteroids (Oono et al.,
2010). This may be because of certain trade-offs depending
on environmental conditions, much like C4 photosyn-
thesis has a greater carbon fixation efficiency than the
dominant C3 systems mainly at high temperatures.
Investigating bacteroid differentiation may reveal how
swollen bacteroids could be more symbiotically effi-
cient than nonswollen ones, and allow us to modify
other host species to have higher nitrogen fixation
efficiency as well.

MATERIALS AND METHODS

Plant/Rhizobia Culture and Experimental Conditions

Two rhizobial strains were compared for their symbiotic efficiencies: (1) 
Rhizobium leguminosarum A34, a transgenic strain, previously studied
by Lodwig et al. (2005) and Mergaert et al. (2006) among others, that nodulates

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Beans (Phaseolus vulgaris) and peas (Pisum sativum) and (2) a wild strain, Bradyrhizobium sp. 32H1 (= USDA3384), that nodulates cowpeas (Vigna unguiculata) and peanuts (Arachis hypogaea; Sen and Weaver, 1980). Seeds of peas (cv Maestro) and beans (cv Royal Burgundy) were surface sterilized with 0.09% hypochlorite (3% commercial bleach) for 5 min, rinsed in deionized water, and inoculated with 1 ml (approximately 10^8 cells) of stationary phase R. leguminosarum 4292 (Johnston et al., 1982), A34 (Gottz et al., 1985), or 3841 (Johnston and Biringer, 1975), which were grown in tryptone yeast media (Somasegaran and Hoben, 1994). A34 and 4292 are both derived from Rhizobium phaseoli 8002 (Lamb et al., 1982) but contain different plasmids allowing nodulation in peas (PRLI1) or beans (PRL2), respectively. A34 retained ability to nodulate beans (albeit with delayed nodulation compared to 4292) and was used as the common strain to compare pea and bean host effects. 4292 only nodulated beans and 3841 only nodulated peas. Hence, these strains were used to measure natural (control) host effects on symbiotic efficiency to compare with that of the common (A34) strain. Peas and beans grew in plastic growth pouches with nitrogen-free Fahraeus nutrient media (Fahraeus, 1957) using growth conditions previously described (Ratcliff et al., 2008). Pea and bean plants were sampled at random, but their position in the growth chamber was not randomized by species. However, differences between species unrelated to bacteroid morphology were unavoidable and presumably larger than placement effects. Seeds of peanuts (cv Starr) and cowpeas (cv California Blackeye) were surface sterilized and planted in 15-inch deep cones with sterile vermiculite: perlite (1:1) mixture. Growth chambers were set at 29°C light 16 h and 22°C dark 8 h. Peanuts and cowpeas were inoculated with Bradyrhizobium sp. 32H1, grown in modified arabinose Glu media (Somasegaran and Hoben, 1994). Since these peanuts and cowpeas commonly share many strains, we did not include control strains to assess natural host effects on symbiotic efficiency. These plants were also watered with nitrogen-free Fahraeus media (Fahraeus, 1957) and mixed haphazardly throughout the growth chamber. Plant individuals were randomly chosen for harvest between days 50 and 100.

Harvesting Nodules

Peas and beans were grown for 74 d, and four to nine plants each were harvested at five different time intervals. Only plants from the first four time intervals were included in the data analysis because the plants grew older, some leaves were lost, which underestimated the plant dry weights. Cowpeas and peanuts were harvested intermittently between day 50 and 100. All nodules were harvested from each plant and their total fresh weights were recorded. The host shoots were dried in an oven overnight and weighed. Pea and bean shoots were further processed for nitrogen content using elemental combustion analysis. Pea and bean root weights were estimated from typical shoot/root ratios estimated from a separate set of peas and beans nodulated with A34 (pea root = 0.25 × shoot, r^2 = 0.47, n = 7, bean root = 0.64 × shoot, r^2 = 0.37, n = 11). For peanuts and cowpeas, actual root weights were used. Nodule fresh weight was measured and dry weights were estimated by regression based on a separate experiment (pea dry nodule weight = 0.17 × wet nodule weight, r^2 = 0.99, bean dry nodule = 0.18 × wet, r^2 = 0.99, peanut dry nodule = 0.21 × wet, r^2 = 0.99, cowpea dry nodule = 0.25, r^2 = 0.99).

Nodules for H2:CO2 Efficiency Measurements

Healthy, mature, pink nodules were harvested and used immediately for measuring H2 production and CO2 respiration in nitrogen-free air, using a method adapted from Witty and Minchin (1998)'s open-flow-through system. Witty and Minchin (1998) showed respiratory cost (mol CO2/mol ethylene from acetylene reduction by nitrogenase) remained relatively constant with plant age for detached nodules, so nodules of various ages were pooled as long as they looked healthy. These nodules were detached from plants but still connected to some root fragments to minimize wounding or introduction of ambient oxygen into nodule interior. Detached nodules have lower fixation and respiration rates but the relationship between them apparently does not change (Witty and Minchin, 1998). Detached nodules were pooled from two or more individual host plants to obtain detectable levels of H2 production. Peanut and cowpea plants were acclimated to a cooler growth chamber of 20°C for 24 h before harvesting at room temperature (20°C) to reduce the temperature shock for the nodules. Productions of H2 and of CO2 by nodules (total fresh weight between 0.5 g and 1 g) were assayed in a flow-through chamber (Fig. 2A). Argon:O2 flowed through the nodules from below at a controlled rate (100 ml/min). Due to the absence of NO, 100% of nitrogenase activity went to H2 production. A subsample of gas from above the nodules was pulled through an H2 analyzer (Witty and Minchin, 1998) and infrared gas analyzer for CO2 (Qubit Systems) at a lower flow rate than the supply rate, with excess gas vented (Fig. 2A). After equilibrating under 21% O2 in argon, oxygen percentage was increased in steps to 35%. Increments of oxygen increases varied from 1% (cv, i.e., 1 kPa) every 4 min to 3% every 10 min depending on nodule sensitivity to O2 (Fig. 2B). When external O2 partial pressure is increased gradually, internal O2 partial pressure will rise enough to reduce O2 limitation of nodule interior respiration (increasing nitrogenase activity as well as respiration) but not enough to damage nitrogenase irreversibly (Denison et al., 1992; Witty and Minchin, 1998). Efficiency was calculated from linear regression of nitrogenase activity (H2 production) on respiration (CO2 production; Fig. 2C; Witty et al., 1983). If nitrogenase was damaged at high external oxygen concentrations, then when external oxygen was returned back to 21% at the end of the experiment from 33%, there would be a definitive drop in H2 evolution from the initial 21% reading (Fig. 2C). Hence, CO2 and H2 values at some high oxygen percentages were not included in the regression (Witty and Minchin, 1998) unless they remained linear. The H2 sensor was calibrated at 0.5 ml L^-1 (ambient air) and at 50 ml L^-1 (H2 standard).

Comparing PHB in Bacteroids

We also measured the amount of a carbon storage polymer, PHB, per bacteroid in each host since PHB is known to compete with nitrogen fixation for photosynthates (Trainor and Charles, 2006, and refs. therein). We tested whether there is less PHB in the swollen bacteroids than in the unswollen bacteroids, which might help explain any differences in symbiotic efficiency between hosts, if they are present.

Five to eight random nodules from each host plant were weighed individually and saved for PHB analysis via flow cytometry. Procedures for PHB analysis are described by Ratcliff et al. (2008).

Statistics

To test if the effect of nodule weight on plant weight was significantly different between the two strains on the same host species or between two host species with the same strain, controlling for a constant effect of plant age, we compared linear models using a t test in R. Formula: plant weight = b + x (nodule weight) + l (strain/host species combination) + plant age + I (strain or host species) × m (nodule weight), where l designates indicators for the model, b is the intercept of the model, and m is the coefficient of the nodule weight variable or the slope of the linear regression that was estimated. Slopes indicate plant growth per nodule growth, and allow discounting differences in initial seed weights. Fixation efficiencies were compared with unpaired equal variance two-tailed t test.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Pea plants nodulated by R. leguminosarum A34 and 3841 across four harvest dates (four plants each for 3841 and four to nine plants each for A34).

Supplemental Figure S2. Bean plants nodulated by R. leguminosarum A34 and 4292 across four harvest dates (four to six plants each).

ACKNOWLEDGMENTS

We would like to thank Peter Mergaert for providing the R. leguminosarum strains, the U.S. Department of Agriculture for Bradyrhizobium sp. 32H1, and Mark Burrow (Texas AgriLife Research) for A. hypogaea ‘Starr’ seeds. We would like to acknowledge the assistance of the Flow Cytometry Core Facility of the University of Minnesota Cancer Center, a comprehensive cancer center designated by the National Cancer Institute, supported in part by P30 CA7759.

Received August 1, 2010; accepted September 9, 2010; published September 13, 2010.
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