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Rhizobium free-living nitrogen fixation occurs in specialized nongrowing cells

(prokaryotic differentiation/syntrophism)

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ABSTRACT **A model for free-living N_2 fixation by *Rhizobium* sp. RC3200 is presented that asserts that this process occurs in nongrowing cells. Cultures containing mixed populations of cell types, N_2-fixing and vegetative, grow cooperatively. In nitrogen-limited liquid suspension cultures, cooperative growth occurs by means of ammonium that is produced and exported by nongrowing, N_2-fixing cells and transported to vegetative cells. This model implies prokaryotic differentiation: the creation of metabolically specialized cells, terminally nonviable, that functionally cooperate in a higher cell order. Here, the switch to a Rhizobium N_2-fixing cell state is regulated by both O_2 and utilizable nitrogen.**

Although rhizobia primarily conduct N_2 fixation as legume root nodule endosymbionts ("bacteroids"), many strains exhibit N_2 fixation in free-living culture (1–5). The metabolic significance of this process has remained obscure because such strains fail to grow on N_2 as sole nitrogen source. Here, evidence will be presented that suggests *Rhizobium* free-living N_2 fixation occurs in specialized, nongrowing cells. This would allow ordered cell populations to grow syntrophically—i.e., by a mechanism of cooperative cross-feeding. The model can be summarized as follows: under conditions of low O_2 and limiting utilizable nitrogen (exclusive of N_2), *Rhizobium* sp. 32H1 (RC3200) can switch its metabolic cell state to one of nongrowth in which it is able to fix N_2. This switch in cell state is a stochastic process—i.e., it occurs in some fraction of cells of the cell population. Although N_2 is reduced to ammonium in these cells, nitrogen assimilation is blocked; instead, ammonium export is activated (6, 7). The exported ammonium can then be imported by the unmodified vegetative, non-N_2-fixing members of the population still active in nitrogen assimilation (Fig. 1). Thus, N_2 fixation may allow cooperative growth of *Rhizobium* sp. strains *ex planta*. In effect, this model implies true prokaryotic differentiation: the programmed creation of metabolically and morphologically specialized nonviable cells within a functioning cell population of higher order.

Biochemical physiology experiments have elucidated optimal conditions for *Rhizobium* free-living N_2 fixation in chemically defined liquid suspension culture. Such conditions require: (*i*) the use of organic acids such as succinate or L-malate as carbon and energy sources, (*ii*) the presence of a utilizable (i.e., growth-promoting) nitrogen source such as L-glutamate, and (*iii*) low O_2 (i.e., microaerobiosis). O_2 is essential because rhizobia are obligate aerobes. Furthermore, the large ATP requirement for N_2 fixation necessitates substantial rates of oxidative phosphorylation. Microaerobic conditions are required because intracellular O_2 inactivates nitrogenase and ancillary electron transport proteins during N_2 fixation.

When wild-type *Rhizobium* sp. strain RC3200 is aerobically cultured in a chemically defined liquid suspension medium fulfilling prerequisites for N_2 fixation and such a culture is then shifted to lower O_2, a growth discontinuity is observed. These cultures exit the exponential growth phase and enter a "stationary" phase, once a new, lower O_2 steady state has been reached (8). However, aerobic cultures continue to grow past this point, suggesting that nutrients in low O_2 cultures are not growth-limiting. Concomitant with the growth discontinuity, N_2 fixation activity commences, but cultures fail to assimilate and instead export the bulk of the ammonium so produced (6). This block results from rapid turnover of glutamine synthetases (GlnSase) I and II, adenylylation of GlnSase I, and repression of *gln* gene expression after onset of N_2 fixation (6).

It was hypothesized that the inability to assimilate ammonium during N_2 fixation precluded growth. A mutant derivative of RC3200, strain RC3205, overproduces nitrogenase in culture and possesses a constitutively unadenylylated (biosynthetically active) GlnSase I but remains GlnSase II defective, like its parent Gln^- strain (7). As a result, RC3205 cultures can assimilate ammonium during N_2 fixation; wild-type RC3200 only exports ammonium during N_2 fixation (6).

Given these abilities, it seemed possible that RC3205 might grow with N_2 as the sole nitrogen source. However, under all conditions tested, both RC3200 and RC3205 fail to grow when N_2 is the sole nitrogen source (9). Such "negative results" are of course never definitive, and one must allow for the possibility that the necessary growth conditions have not been provided. Nevertheless, these observations suggested that growth and N_2 fixation do not occur simultaneously and that simply recovering the ability to assimilate ammonium, as in the case for RC3205, is still insufficient to allow N_2-dependent growth.

Experiments presented here show (*i*) that a *Rhizobium* cell cannot simultaneously grow and fix N_2; (*ii*) that under nitrogen-limited, low-O_2 conditions, some *Rhizobium* cells in the population switch to a nongrowing cell state by a stochastic process; and (*iii*) that N_2 fixation occurs only therein.

MATERIALS AND METHODS

Bacterial Strains. *Rhizobium* sp. "cowpea" strains have been described (7–9) and are summarized in Table 1. Minimal growth medium, MS, contains 0.2% succinic acid titrated to pH 6.3 with NaOH, 7.5 mM potassium phosphate, 0.2 mM $MgSO_4$, 0.05 mM $CaCl_2$, 1 μM $FeCl_3$, and 1 μM Na_2MoO_4 plus indicated amounts of L-glutamate as the source of nitrogen. Viable cell counts were obtained by plating on a GYPC medium (0.4% D-glucose/0.2% yeast extract/7.5 mM potassium phosphate, pH 6.3/0.2% salt-free Casamino acids/1.5% agar) at 30°C.

Growth Experiments. *Rhizobium* liquid suspension cultures were maintained in constant-temperature baths at 28°C with continuous sparging by indicated gas mixtures. Triton

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Abbreviation: GlnSase, glutamine synthetase.

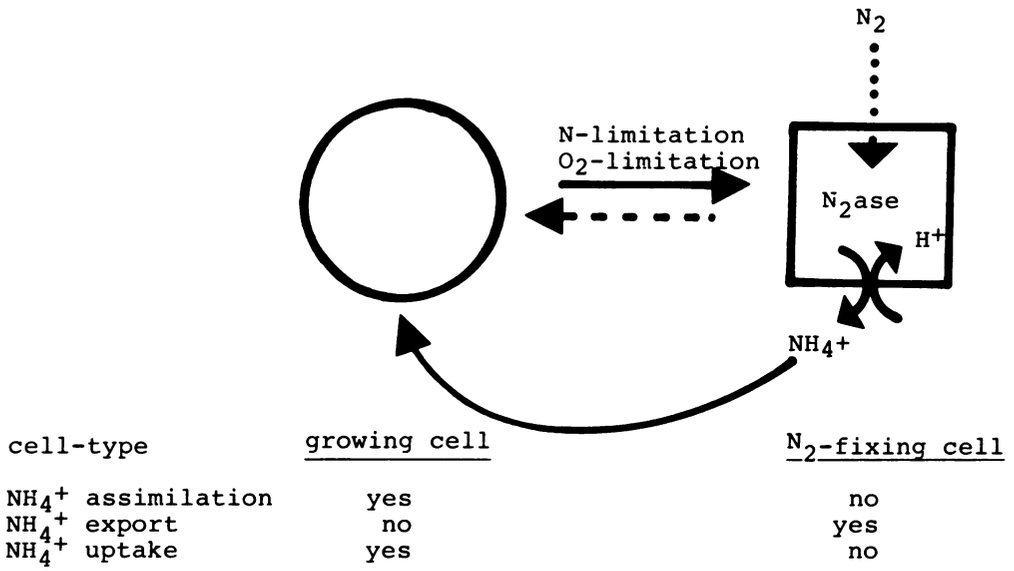

FIG. 1. Model for *Rhizobium* N_2-fixing syntrophism.

X-100 at 0.1% was included in media to suppress cell aggregation, which decreased cell counts and increased fluctuations in multiple trials but was otherwise without effect when compared to control cultures. Gases were passed through a high-pressure nitrocellulose filter (0.45 μm) and then through 20% H_2SO_4 to strip gases of contaminating NH_3. Dissolved O_2 concentrations were monitored by an O_2 electrode (Clark type), corrected for ionic strength of the medium and barometric pressure, connected to an operational amplifier circuit (10). Limitation of nutrient levels in cultures (i.e., low bacterial cell densities) allowed achievement of steady-state O_2 because rates of O_2 introduction were much greater than rates of utilization.

RESULTS

Low O_2 and Limiting Nitrogen Induce Loss of *Rhizobium* Colony-Forming Ability. *Rhizobium* sp. RC3200 cultured at 3 μM O_2 in limiting nitrogen lost the ability to form colonies. A limiting-nitrogen condition was achieved, by adding only marginal amounts of a utilizable nitrogen source (L-glutamate) such that utilizable nitrogen (exclusive of N_2) was exhausted during the course of the experiment. Regardless of initial L-glutamate levels supplied, RC3200 cultures at 3 μM O_2 lost the ability to form colonies when L-glutamate was exhausted (Fig. 2*a*). The optimal viable cell titers reached by such cultures were directly proportional to amounts of L-glutamate initially supplied and were similar whether growth was aerobic or microaerobic (data not presented). Viable cell titers were independent of plating media [i.e., whether rich (GYPC) or chemically defined minimal (MS + glutamate) media were used] and were independent of plating conditions (i.e., aerobic or microaerobic).

To test whether loss of growth or ability to form colonies or both, specifically involved a block in RC3200 ammonium assimilation, these experiments were repeated with ammonium assimilation-constitutive strain RC3205 (8). RC3205 nitrogen-limited microaerobic cultures at 3 μM O_2 also lost ability to form colonies; however, the behavior of this strain was more complex (Fig. 2*b*). Viable rhizobia could be subdivided into two classes according to time-dependent colony-forming abilities: those that yielded colonies at normal times (5 days at 30°C) and those that required 2 additional days to do so (delayed colony-forming phenotype, designated Dcf; Fig. 2*b*). These assignments were unambiguous and reproducible. Dcf rhizobia first appeared at the time of nitrogen-limitation onset (L-glutamate exhaustion): at their zenith

Table 1. Bacterial strains

Strain	Phenotype	GlnSase I	GlnSase II	Nif
RC3200	wt	+	+	+
RC3201	Gln^-	ad	−	−
RC3205	Gln^+ rev of RC3201	un[c]	−	++
RC3312	Ery^R, Nif^-	+	+	−
RC3505	Cam^R	+	+	+

ad, Adenylylated; un, constitutively unadenylylated; Nif, phenotype (both in culture and in planta) of N_2 fixation; Cam^R, chloramphenicol-resistant; Ery^R, erythromycin-resistant; wt, wild type.

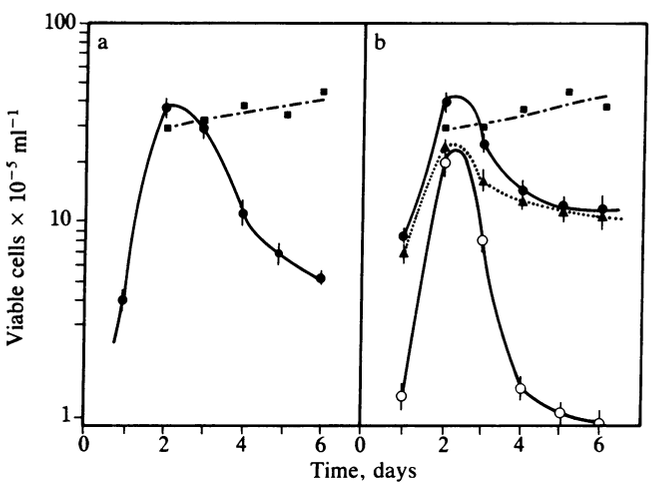

FIG. 2. Viable cell count determinations of RC3200 and RC3205 nitrogen-limited liquid cultures under low O_2. *Rhizobium* strains RC3200 and RC3205 were cultured in MS medium with 6.6 μM L-glutamate as the utilizable nitrogen source and were plated on GYPC medium. Cultures were continuously sparged with 0.2% O_2/1.0% CO_2/>98% N_2. Dissolved O_2 was monitored as described. Multiple, independent trials showed reproducibilities in viable cell count determinations within 5% of the mean of three experiments. (*a*) RC3200. (*b*) RC3205. Viable cell counts were subdivided into those that yielded colonies at normal times (▲), delayed types (Dcf phenotype) (○), and total viable cells (●). For both cultures, total cell counts in nitrogen-limited phase were determined by light microscopy with a Petroff–Hauser counting chamber (■). Data are the means of five trials.

they comprised 50% of total viable cells but were always transient and thereafter declined. It was concluded that RC3200 and RC3205 behaved similarly in nitrogen-limited culture at 3 μM O_2, but RC3205 exhibited in addition a transient population of Dcf cells, which may represent "partially switched" cells still able to revert to the vegetative state. Furthermore, the ability to assimilate ammonium does not allow RC3205 to grow on N_2.

A series of such RC3200 growth experiments, where viable cell counts were determined in limiting nitrogen as a function of steady-state O_2 tension, was conducted (Fig. 3). In such conditions, loss of colony-forming ability under N_2 was evident only within a small window of steady-state O_2, being optimal at 3 μM O_2. Here some 90% of total cells could be considered unable to form colonies. Total cell counts obtained by light microscopy in Petroff–Hauser counting chambers during the limiting-nitrogen phases of cultures correlated well with maximum viable cell titers observed at the ends of exponential growth phases (Fig. 2 *a* and *b*). Therefore, no diminution of total cells in limiting-nitrogen culture phases occurred. Total cell protein as measured by a modified Coomassie blue procedure (11) remained constant or increased slightly during limiting-nitrogen phase (data not presented).

When argon replaced N_2, loss of colony-forming ability was observed at steady-state O_2 levels below 3 μM (Fig. 3). Therefore, the distinctly different behavior of cultures under N_2 as opposed to argon in limiting O_2 must result from N_2 fixation. It was concluded that nitrogen limitation triggered loss of colony-forming ability in liquid cultures at 3 μM O_2 and that, at steady-state O_2 levels below 1 μM O_2, loss of colony-forming ability was obscured by N_2 fixation.

Loss of RC3200 colony-forming ability is greatest at 3 μM O_2 in nitrogen limitation (Fig. 3), whereas *Rhizobium* N_2 fixation is optimum below 1 μM O_2 (9, 12–14). Under such O_2-limited conditions, rhizobia synthesize new set(s) of cytochromes and cytochrome oxidases ($K_m[O_2]$ = 1–10 × 10^{-9} M) allowing active oxidative phosphorylation at nanomolar O_2 levels (12). Correspondingly, RC3200 fails to exhibit productive N_2 fixation at 3 μM steady-state O_2 (9). Taken together with the results of physiology experiments presented above, these observations suggested the following hypothesis: loss of colony-forming ability at 3 μM O_2 in nitrogen limitation represents a switch to a nonviable cell state prerequired for N_2 fixation. If true, this would explain why loss of colony-forming ability is obscured below 1 μM O_2 under N_2: here productive N_2 fixation would contribute ammonium to the nitrogen pool of the medium and alleviate nitrogen limitation, which in turn would inhibit loss of colony-forming ability among remaining vegetative cells.

***Rhizobium* N_2 Fixation Allows Syntrophism.** To address this hypothesis, further experiments were conducted with Nif^- strains of RC3200 isolated after hydroxylamine mutagenesis. One such Nif^- strain, RC3312 (ref. 9; Table 1), proved to be pleiotropic. Although nodulation-proficient (Nod^+), it synthesized neither nitrogenase nor cytochrome P-450 nor 3-hydroxybutyrate dehydrogenase either in O_2-limited culture or in bacteroids obtained after nodulation of *Vigna sinesis* (data not presented). Nevertheless, Nif^+ spontaneous revertants of RC3312 could be isolated at a frequency of $\approx 1 \times 10^{-8}$ that recovered a wild-type phenotype. When RC3312 was cultured at 3 μM O_2 in nitrogen-limited conditions, no loss of colony-forming ability was observed (Fig. 4*a*). RC3312 cultures simply appeared to enter stationary phase upon nitrogen limitation in all O_2 environments tested. It was concluded that RC3312 is blocked from switching to the nongrowing cell state.

RC3312 (Nif^-) and RC3505 (Nif^+, Table 1) were then cocultured under nitrogen-limited, O_2-limited (<1 μM) conditions, allowing productive N_2 fixation. When the limited amount of L-glutamate added was exhausted, this mixed culture was primed for N_2 fixation-dependent growth. RC3312 continued to grow, while RC3505 exhibited loss of colony-forming ability (Fig. 4*b*)! This cooperative growth did not occur when argon was substituted for N_2 nor at steady-state 3 μM O_2 (data not presented) and, therefore, was the result of N_2 fixation. Because RC3200 exports ammonium produced by N_2 fixation (6, 15), RC3505 was presumably cross-feeding RC3312 ammonium under these conditions. RC3312 preferentially utilized this ammonium. This led to continued

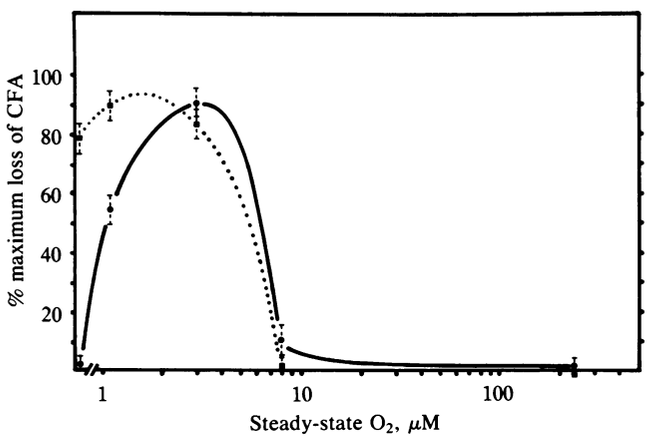

FIG. 3. Loss of RC3200 colony-forming ability (CFA) as a function of steady-state dissolved O_2. Growth experiments were conducted as described in Fig. 2 except that L-glutamate was present at 6.6 μM. Viable cell counts obtained at the end of the exponential growth phase were compared with those obtained from the horizontal asymptote of growth curves in nitrogen-limited phase (Fig. 2) to yield a measure of maximum loss of CFA of the total cell population. This determination was made for cultures in the range of steady-state O_2 concentrations indicated, where N_2 (●) or argon (■) was the predominant sparge gas. The data points corresponding to lowest steady-state O_2 were obtained by continuous sparging with 20 ppm of O_2, at which level (<1 μM) dissolved O_2 could not be monitored accurately.

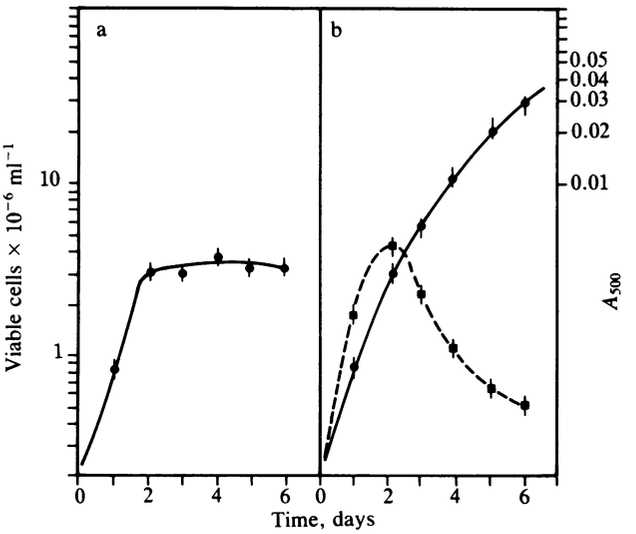

FIG. 4. Syntrophism. (*a*) RC3312 (Table 1) was cultured in MS medium with 6.6 μM glutamate as described in Fig. 2. Viable cell counts versus time were determined as described in Fig. 2. (*b*) RC3312 (●) and RC3505 (Table 1) (■) were cultured together under nitrogen- and O_2-limited conditions as described in Fig. 2, except that O_2 in the sparge gas mixture was lowered to 20 ppm. Viable cell counts versus time were determined by double plating on GYPC medium with Ery (200 $\mu g \cdot ml^{-1}$) and GYPC medium with Cam (200 $\mu g \cdot ml^{-1}$).

nitrogen limitation of the mixed culture amid N_2 fixation and further induced RC3505 cells to switch to N_2 fixation, resulting in their loss of colony-forming ability.

DISCUSSION

Rhizobium sp. cells lose colony-forming ability when cultured at low O_2 in limiting nitrogen. Because this phenomenon is observed only under a narrow range of conditions, it appears to represent a major shift in the metabolic activity of the culture. Because colony-forming ability is irrevocably lost, the switch in cell state seems irreversible. Specific conditions allowing reversal to the vegetative cell state may exist but remain undiscovered. Given these observations on the metabolic shift, the nongrowing cell state appears predisposed for N_2 fixation. Loss of colony-forming ability is obscured in limiting O_2, where N_2 fixation occurs. However, substitution of argon for N_2 yields continued loss of colony-forming ability in limiting O_2.

Despite alleviation of nitrogen limitation by N_2 fixation (i.e., ammonium production under N_2), RC3200 and RC3205 cultures nevertheless fail to grow (8, 9), whereas inclusion of L-glutamine (as the nitrogen source) allows growth of liquid cultures under limiting O_2 and represses N_2 fixation. Failure to grow on ammonium under such conditions cannot result solely from inactivation of ammonium assimilation via the GlnSase enzymes because this phenomenon extends to RC3205, which possesses constitutively unadenylylated GlnSase I. As alluded to above, rhizobia biosynthesize new sets of cytochrome oxidases with extremely high affinities for O_2; thus, growth of cultures can occur readily at nanomolar levels of steady-state dissolved O_2 (12, 13). Taken together, these observations suggest that ammonium produced by N_2 fixation counteracts loss of colony-forming ability in limiting O_2.

Such ambiguities raise the question of the metabolic role(s) of ammonium in RC3200. All available evidence suggests that RC3200 is unresponsive to ammonium, which is always assimilated poorly and is in fact actively exported during N_2 fixation (6, 7, 15). Ammonium uptake in "slow-growing" rhizobia results from transmembrane diffusion of free ammonia, a function of intra- and extracellular pH, and from low-level fortuitous transport by amino acid porters. Because ammonium export is not constitutive but is induced prior to onset of N_2 fixation (7, 15), this process, as well, may be conducted by cells that have switched to the nongrowing cell state. This would prevent intracellular ammonium from reaching concentrations sufficient to allow net assimilation. Indeed, evidence from adenylylation studies of GlnSase I indicates that intracellular ammonium is certainly less than 10 μM and probably much less under these conditions (6, 7). Therefore, failure to observe cooperative growth of RC3200 O_2-limited cultures without first adding RC3312 suggests that RC3312 is also defective in ammonium export and, therefore, accumulates ammonium.

Alleviation of nitrogen limitation in mixed cultures by RC3505-mediated N_2 fixation facilitated selective growth of RC3312 Nif^-. This selective growth may relate to (*i*) no loss of RC3312 colony-forming ability under 3 μM O_2 (Fig. 4*a*)—i.e., a presumed inability of RC3312 to switch the cell state to one allowing N_2 fixation—or to (*ii*) the ability of RC3312 to accumulate sufficient intracellular ammonium to allow assimilation, or to both.

The observations described above suggest that *Rhizobium* grows and fixes N_2 in different cell states and that these processes are mutually exclusive. During N_2 fixation, electron flux must be partitioned to directly reduce N_2 and to drive oxidative phosphorylation. Given the high ATP requirement and electron flux of N_2 fixation, segregating growth and N_2 fixation into different cell states may have been advantageous. N_2-fixing filamentous cyanobacteria, which conduct N_2 fixation in specialized, nongrowing heterocysts, also have successfully used this metabolic strategy. Furthermore, free ammonia, a powerful nucleophile, interferes with biosynthetic processes. Therefore, ammonia cytotoxicity also may be mitigated by segregating growth and N_2 fixation.

Free-living, N_2-fixing RC3200 cells are, therefore, formally similar to symbiotic "bacteroids" (16).

Liquid suspension cultures contrive to allow all cells to experience the same milieu. In soil environments, rhizobia could assume specialized cell states within a colony that would be determined by differences in nutrient and O_2 accessibility—i.e., colony geometry. Superficial cells would experience greatest availabilities, and their growth would be enhanced; some interior stratum of cells under nitrogen and O_2 limitation would be optimally adapted for N_2 fixation and could export fixed nitrogen to superficial cells to allow their continued proliferation. This "altruism" would confer on syntrophism an appropriate selective advantage, provided rhizobia selectively cross-feed their own siblings.

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