Recipient-Biased Competition for an Intracellularly Generated Cross-Fed Nutrient Is Required for Coexistence of Microbial Mutualists

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ABSTRACT Many mutualistic microbial relationships are based on nutrient cross-feeding. Traditionally, cross-feeding is viewed as being unidirectional, from the producer to the recipient. This is likely true when a producer’s waste, such as a fermentation product, has value only for a recipient. However, in some cases the cross-fed nutrient holds value for both the producer and the recipient. In such cases, there is potential for nutrient re-acquisition by producer cells in a population, leading to competition against recipients. Here, we investigated the consequences of interpartner competition for cross-fed nutrients on mutualism dynamics by using an anaerobic coculture pairing fermentative Escherichia coli and phototrophic Rhodopseudomonas palustris. In this coculture, E. coli excretes waste organic acids that provide a carbon source for R. palustris. In return, R. palustris cross-feeds E. coli ammonium (NH4+), a compound that both species value. To explore the potential for interpartner competition, we first used a kinetic model to simulate cocultures with varied affinities for NH4+ in each species. The model predicted that interpartner competition for NH4+ could profoundly impact population dynamics. We then experimentally tested the predictions by culturing mutants lacking NH4+ transporters in both NH4+ competition assays and mutualistic cocultures. Both theoretical and experimental results indicated that the recipient must have a competitive advantage in acquiring cross-fed NH4+ to sustain the mutualism. This recipient-biased competitive advantage is predicted to be crucial, particularly when the communally valuable nutrient is generated intracellularly. Thus, the very metabolites that form the basis for mutualistic cross-feeding can also be subject to competition between mutualistic partners.

IMPORTANCE Mutualistic relationships, particularly those based on nutrient cross-feeding, promote stability of diverse ecosystems and drive global biogeochemical cycles. Cross-fed nutrients within these systems can be either waste products valued by only one partner or nutrients valued by both partners. Here, we explored how interpartner competition for a communally valuable cross-fed nutrient impacts mutualism dynamics. We discovered that mutualism stability necessitates that the recipient have a competitive advantage against the producer in obtaining the cross-fed nutrient, provided that the nutrient is generated intracellularly. We propose that the requirement for recipient-biased competition is a general rule for mutualistic coexistence based on the transfer of intracellularly generated, communally valuable resources.

KEYWORDS cross-feeding, coculture, fermentation, hydrogen, microbial communities, mutualism, nitrogen fixation, purple bacteria, synthetic ecology

Mutualisms, or mutually beneficial relationships between organisms, are ubiquitous and play important roles in diverse ecosystems (1). Mutualistic cross-feeding of resources between microbes can have broad impacts, ranging from influencing host health (2, 3) to driving global biogeochemical cycles (4–7). Cross-fed metabolites are
often regarded as nutrients due to the value they provide to a dependent partner, the recipient. However, for the partner producing the nutrient, the producer, a cross-fed nutrient’s value can vary. On one extreme, the cross-fed metabolite is valued by the recipient but not the producer, as is the case for fermentative waste products (8–11). In other cases, a cross-fed metabolite holds value for both the recipient and the producer, as is the case for vitamin B12 (7, 12, 13) and ammonium (NH₄⁺) (14, 15). Such communally valuable cross-fed nutrients are subject to partial privatization (16), wherein the producer has mechanisms to retain a portion of the nutrient pool for itself. While most mutualism cross-feeding studies only consider unidirectional metabolite transfer from producer to recipient, we hypothesized that partially privatized cross-fed resources could be subject to competition between partner populations. Such competition from partial privatization mechanisms seems likely, considering that competition for exogenous limiting resources is known to affect mutualism stability (9, 17–20). Similarly, others have shown that adding an exogenous source of a cross-fed nutrient can shift relationships between microbial partners from being mutualistic to competitive (21).

One example of cross-feeding that could involve competition between mutualistic partners is NH₄⁺ excretion by N₂-fixing bacteria (Fig. 1A), hereon called N₂ fixers (14, 15). During N₂ fixation, the enzyme nitrogenase converts N₂ gas into two NH₃ molecules (22). In an aqueous environment, NH₃ is in equilibrium with NH₄⁺. At neutral pH, NH₄⁺ is the predominant form, but small amounts of NH₃ can potentially leave the cell by passive diffusion across the membrane; this passive diffusion is referred to here as NH₃ excretion (23) (Fig. 1B). This inherent “leakiness” for NH₃ likely fosters NH₄⁺ cross-feeding, as extracellular NH₃ is available to neighboring microbes. Importantly, these neighbors can include clonal N₂ fixers, as NH₃/NH₄⁺ is a preferred nitrogen source for most microbes. At concentrations above 20 μM, extracellular NH₃ can be acquired by passive diffusion; below 20 μM, NH₄⁺ is specifically bound and transported as NH₃ by AmtB transporters (Fig. 1B) (24). AmtB-like transporters are conserved throughout all domains of life (25). There is growing evidence that AmtB is used by N₂ fixers to recapture excreted NH₃ lost by passive diffusion, as ΔAmtB mutants accumulate NH₄⁺ in culture supernatants, whereas wild-type strains do not (26–28). Thus, during NH₄⁺ cross-feeding, AmtB likely facilitates both NH₄⁺ acquisition by a recipient partner and recapture of NH₄⁺ by the N₂ fixer.

Assessment of the effects of interpartner competition for a cross-fed nutrient would require a level of experimental control not possible in most natural settings. However, synthetic microbial communities, or cocultures, are well-suited to address such questions (29–31). We previously developed a bacterial coculture that features cross-feeding of waste products (organic acids) from Escherichia coli and a communally valuable nutrient (NH₄⁺) from Rhodopseudomonas palustris Nx (Fig. 1A) (28). We demonstrated

![FIG 1 An obligate bacterial mutualism based on cross-feeding of essential nutrients. (A) Escherichia coli (Ec) anaerobically ferments glucose into excreted organic acids that Rhodopseudomonas palustris Nx (Rp Nx) can consume (acetate, lactate, and succinate) and other products that R. palustris cannot consume (formate [For] and ethanol [EtOH]). R. palustris Nx grows photoheterotrophically, wherein organic compounds are used for carbon and electrons and light is used for energy. In return, R. palustris Nx constitutively fixes N₂ gas and excretes NH₄⁺, supplying E. coli with essential nitrogen. (B) NH₄⁺ can be passively lost from cells as NH₃. Both species have high-affinity NH₄⁺ transporters (AmtB) that facilitate NH₄⁺ uptake. NH₃ is the predominant form at neutral pH, as indicated by the enlarged arrowheads of the double-sided arrows.](mbio.asm.org/figure?id=1)
that this coculture supports stable coexistence and reproducible growth and metabolic trends when started from a wide range of starting species ratios, including single colonies (28). Here, using both a kinetic model and genetic manipulation to alter the affinity of each species in the coculture for NH$_4^+$, we demonstrate that interpartner competition for excreted NH$_4^+$ plays a direct role in maintaining coexistence. Specifically, insufficient competition by E. coli for NH$_4^+$ resulted in a collapse of the mutualism. Mutualism collapse could be delayed or potentially avoided through higher NH$_4^+$ excretion by R. palustris or increased E. coli population size. Our results suggest that for obligate mutualisms based on an intracellularly generated cross-fed nutrient, competition for that nutrient must be biased in favor of the recipient to avoid mutualism collapse and the potential extinction of both species.

RESULTS

Competition for cross-fed NH$_4^+$ is predicted to shape mutualism population dynamics. Within our coculture (Fig. 1A), E. coli ferments sugars into waste organic acids, providing essential carbon and electrons to R. palustris Nx. R. palustris Nx converts N$_2$ into NH$_4^+$ and is genetically engineered to excrete low micromolar amounts of NH$_4^+$, providing essential nitrogen for E. coli (28). The R. palustris parent strain does not support coculture growth with E. coli due to insufficient NH$_4^+$ excretion (28). NH$_4^+$ excretion by R. palustris Nx is due to a 48-nucleotide internal deletion in the gene for the master transcriptional regulator of nitrogenase, nifA, which results in constitutive nitrogenase activity even in the presence of normally inhibitory NH$_4^+$ (32). In contrast to organic acids, which are only useful to R. palustris, NH$_4^+$ produced by R. palustris Nx is essential for the growth of both species; R. palustris uses some NH$_4^+$ that it converted from N$_2$ for its own biosynthesis and excretes the rest, which serves as the nitrogen source for E. coli. However, R. palustris Nx can also take up extracellular NH$_4^+$ (32). Thus, we hypothesized that competition for excreted NH$_4^+$ between the R. palustris Nx producer population and the E. coli recipient population could influence mutualism dynamics.

We first explored whether competition for cross-fed NH$_4^+$ could affect the mutualism by using SyFFoN, a mathematical model describing our coculture (28, 33). SyFFoN simulates population and metabolic dynamics in batch cocultures based on Monod equations with experimentally determined parameter values. Graphical details for individual functions and parameter value choices have been described elsewhere (33). As previous versions described NH$_4^+$ uptake kinetics only for E. coli (28, 33), we amended SyFFoN to include both an R. palustris NH$_4^+$ uptake affinity constant ($K_m$) and a higher R. palustris maximum growth rate ($\mu_{\text{max}}$) when NH$_4^+$ is used (Fig. 2A; see also Table S1 and Text S1). Simulations from the amended model, SyFFoN v3, and the previous version, SyFFoN v2 (33), were comparable (Fig. S1). We then simulated batch cocultures, wherein the relative affinity for NH$_4^+$ varied between the two species by increasing the $K_m$ value for NH$_4^+$ from the default value of 0.01 mM in either species (Fig. 2B). We did not decrease $K_m$ values, because NH$_4^+$ transporters are regarded as high-affinity transporters (34), and therefore we assumed that a higher affinity was less likely physiologically. The model predicted that net growth of both species is achieved only when the R. palustris affinity for NH$_4^+$ is low relative to that of E. coli (R. palustris: E. coli affinity ratio, <1; herein affinity values are the inverse of $K_m$ values), as E. coli can acquire enough excreted NH$_4^+$ to be able to grow. In contrast, when the R. palustris affinity for NH$_4^+$ is high relative to that of E. coli (R. palustris:E. coli affinity ratio, >1), E. coli growth is no longer supported, because E. coli cannot compete for excreted NH$_4^+$. These trends are minimally impacted by the increase in the R. palustris growth rate when reacquiring NH$_4^+$ (Fig. S2). Changing the default $K_m$ value (e.g., to 1 \(\mu\text{M}\)) affected the simulated cell density values but not the overall trends. Despite the lack of E. coli growth, high R. palustris cell densities were still predicted (Fig. 2B), due to persistent, low-level organic acid cross-feeding stemming from E. coli maintenance metabolism, which can support R. palustris growth even when E. coli is not growing (33). In contrast, NH$_4^+$ cross-feeding from R. palustris to E. coli functions solely in a
growth-dependent manner, as the organic acids from *E. coli* serve both as the electron source for nitrogenase and the carbon source for *R. palustris* growth. The SyFFoN prediction that mutualism stability requires that *E. coli* have a higher affinity for NH$_4^+$ than does *R. palustris* might seem at odds with other models of resource competition, wherein an increased cost of cooperation and/or decreased resource capture by the cooperator (as should be the case when *E. coli* further outcompetes *R. palustris* for NH$_4^+$) can result in extinction of the cooperator (35, 36). We reasoned that the population-level outcome from altering the affinity for a communally valuable nutrient depends on whether the nutrient is generated intra- or extracellularly. Intracellular generation of a communally valuable nutrient would enforce partial privatization, as the producer would have a steep advantage in retaining a sufficient portion of the nutrient pool. No matter what the recipient affinity for the nutrient, it could never overcome the advantage imparted by the physical boundary of the producer's cell envelope. Extracellular generation, on the other hand, such as the enzymatic release of sugar monomers from extracellular polysaccharides, can result in the majority of the nutrient being lost to neighboring cells, making the ability of the producer to capture the nutrient more important (35, 37). The producer advantage of intracellular nutrient generation is built into SyFFoN, as N$_2$ and NH$_4^+$ are treated as two separate nitrogen sources; while both species can acquire extracellular NH$_4^+$, there is also a direct route for N$_2$ into an *R. palustris* biomass, bypassing NH$_4^+$ (Fig. 2A; Text S1). To assess whether the intrinsic partial privatization provided by this direct route was responsible for the SyFFoN prediction, we modified SyFFoN so that all N$_2$ went through NH$_4^+$ before it could be assimilated by either species (Fig. 2C), mimicking extracellular...
generation of NH₄⁺. In this configuration, a disproportionately high affinity for NH₄⁺ by either species prevented the growth of either one or both species (Fig. 2D). In the range where net growth of both species was predicted, coculture growth was dependent on preferential access by R. palustris, the producer rather than the recipient (Fig. 2D), similar to predictions from studies between cooperator and competitor cells (35, 37).

Thus, the requirement that the E. coli recipient be more competitive for NH₄⁺ to maintain coexistence is expected to only be true for intracellularly generated NH₄⁺.

Genetic disruption of AmtB NH₄⁺ transporters affects relative affinities for NH₄⁺. Bacterial cells generally acquire NH₄⁺ through two mechanisms: passive diffusion of NH₃ or uptake by AmtB transporters (Fig. 1B) (24). We hypothesized that deletion of the amtB gene in either species would result in a lower affinity for NH₄⁺ in that species and thus could be used to test how the relative NH₄⁺ affinity impacts coculture dynamics. We generated ΔAmtB mutants of both E. coli and R. palustris and first characterized the effects of the mutations in monocultures. Deletion of amtB in E. coli had no effect on growth or fermentation profiles when 15 mM NH₄Cl was provided (Fig. S3), consistent with previous observations where ΔAmtB growth defects were only apparent at NH₄⁺ concentrations below 20 μM (24). In R. palustris ΔAmtB monocultures with N₂ as the nitrogen source, growth trends were equivalent to those of the parent strain; however, R. palustris ΔAmtB excreted more NH₄⁺ than the parent strain and about a third of that excreted by R. palustris Nx (Fig. S3C and D). In line with our hypothesis, NH₄⁺ excretion by R. palustris ΔAmtB could be due to a decreased ability to reacquire NH₄⁺ lost by diffusion, resulting in increased net NH₄⁺ excretion. Alternatively, we considered that NH₄⁺ excretion by R. palustris ΔAmtB could be due to improper nitrogenase regulation in response to NH₄⁺ (27, 38). However, we found that nitrogenase activity in R. palustris ΔAmtB responded similarly to NH₄⁺-induced inhibition as in the parental strain (Fig. S4). These observations demonstrated that R. palustris ΔAmtB NH₄⁺ excretion is likely due to a poor ability to reacquire NH₄⁺ lost by diffusion.

To test our hypothesis that deletion of amtB would lower cellular affinity for NH₄⁺, we directly tested all possible E. coli and R. palustris strain combinations in competition assays in which ample carbon was available for each species but the NH₄⁺ concentration was kept low. Specifically, a small amount of NH₄⁺ was added every hour to bring the final NH₄⁺ concentration to approximately 5 μM, although it is possible that the NH₄⁺ concentration exceeded 5 μM at early time points when consumption rates could have been slow due to low cell densities (Fig. 3). In this competition assay, the species
that is more competitive for NH₄⁺ should reach a higher cell density than the other species. In all cases, wild-type (WT) *E. coli* was more competitive for NH₄⁺ than any *R. palustris* strain. However, each *R. palustris* strain was able to outcompete *E. coli ΔAmtB* (Fig. 3), even though the *E. coli* maximum growth rate is 4.6 times higher than that of *R. palustris* (Fig. S3). Even *R. palustris* strains lacking AmtB outcompeted *E. coli ΔAmtB* (Fig. 3), indicating that *R. palustris* has a higher affinity for NH₄⁺ than *E. coli*, independent of AmtB. These data confirmed that deletion of *amtB* was an effective means by which to lower the relative affinity for NH₄⁺ in each mutualistic partner.

**Alteration of relative NH₄⁺ affinities affects mutualistic partner frequencies.**

We then examined how relative affinities for excreted NH₄⁺ influenced mutualism dynamics by comparing the growth trends of cocultures containing either WT *E. coli* or *E. coli ΔAmtB*, paired with either *R. palustris ΔAmtB*, *R. palustris Nx*, or *R. palustris NxΔAmtB*, the latter of which we previously determined exhibited 3-fold-higher NH₄⁺ excretion levels than the N× strain in monoculture (28). We did not use the *R. palustris* parent strain, because it was previously determined not to support coculture growth due to insufficient NH₄⁺ excretion (28). For each *R. palustris* partner, cocultures with *E. coli ΔAmtB* grew slower than cocultures with WT *E. coli* (Fig. 4A and B). *E. coli ΔAmtB* also constituted a lower percentage of the population and achieved lower cell densities than did WT *E. coli* when paired with the same *R. palustris* strain (Fig. 4C). These lower frequencies were consistent with the competitive disadvantage of *E. coli ΔAmtB* for excreted NH₄⁺ (Fig. 3). AmtB is only expected to be important for NH₄⁺ acquisition when concentrations are below 20 μM (24). In agreement with this expectation, supplementing cocultures with 15 mM NH₄Cl led to rapid growth and domination by *E. coli ΔAmtB* (Fig. S5), which resembled those characteristics of previous cocultures with WT *E. coli* that were supplemented with 15 mM NH₄Cl (28). The low final cell density in cocultures with 15 mM NH₄Cl (Fig. S5) is due to rapid organic acid excretion associated with the high *E. coli* growth rate, which leads to culture acidification that prevents *R. palustris* growth (28).
For *R. palustris* strains lacking AmtB, the effects on population trends varied. Consistent with our previous work, *R. palustris NxDΔAmtB* supported higher WT *E. coli* percentages and cell densities (Fig. 4C) (28). Similar to adding 15 mM NH$_4^+$, the high NH$_4^+$ excretion level from *R. palustris NxDΔAmtB* (Fig. 5D) resulted in faster *E. coli* growth and accumulation of consumable organic acids (acetate, succinate, and lactate), which acidify the medium and inhibit *R. palustris* growth (Fig. 4D) (28). Surprisingly, although *R. palustris ΔAmtB* excreted the least amount of NH$_4^+$ in monoculture, it supported a higher WT *E. coli* population in coculture, and consumable organic acids accumulated (Fig. 4C and D). These trends resembled those from cocultures with *R. palustris NxDΔAmtB* (Fig. 4C and D). Unlike Nx strains, which have constitutive nitrogenase activity due to a mutation in the transcriptional activator nifA (32), *R. palustris ΔAmtB* has WT nifA. Thus, *R. palustris ΔAmtB* can likely still regulate nitrogenase expression, and thereby its activity, in response to nitrogen starvation. We hypothesized that in coculture with WT *E. coli*, *R. palustris ΔAmtB* might experience heightened nitrogen starvation, as NH$_4^+$ consumption by WT *E. coli* would limit NH$_4^+$ reacquisition by *R. palustris ΔAmtB* (in an *R. palustris ΔAmtB* monoculture, any lost NH$_4^+$ would simply benefit its clones). We therefore tested whether coculture conditions stimulated higher nitrogenase activity by using an acetylene reduction assay. In agreement with our hypothesis, *R. palustris ΔAmtB* had increased nitrogenase activity under coculture conditions compared to monocultures, whereas *R. palustris Nx*, which exhibits constitutive nitrogenase activity, showed similar levels under both conditions (Fig. 5E). Thus, the relatively greater WT *E. coli* population in coculture with *R. palustris ΔAmtB* was likely due to both the competitive advantage for acquiring NH$_4^+$ over *R. palustris ΔAmtB* (Fig. 3) and the higher NH$_4^+$ cross-feeding levels associated with increased nitrogenase activity.

**E. coli must have a competitive advantage for NH$_4^+$ acquisition to avoid mutualism collapse.** Unlike all other pairings, cocultures of *R. palustris ΔAmtB* paired with *E. coli ΔAmtB* showed little growth when started from a single colony of each species (Fig. 4A), a method that we routinely use to initiate cocultures (28, 33). We reasoned that the higher *R. palustris ΔAmtB* affinity for NH$_4^+$ relative to *E. coli ΔAmtB* (Fig. 3) likely led to community collapse, as predicted by SyFFoN (Fig. 2B). Even though SyFFoN predicted *R. palustris* growth when outcompeting *E. coli* for NH$_4^+$ (Fig. 2B), SyFFoN likely underestimated the time required to achieve these densities, if they would be achieved at all, as SyFFoN does not take into account cell death, which is known to occur when *E. coli* growth is prevented (33). Consistent with the hypothesis that poor coculture growth was due to a competitive disadvantage of *E. coli ΔAmtB* for NH$_4^+$, SyFFoN simulations indicated that starting with a more dilute *R. palustris* inoculum would increase the probability that any given *E. coli ΔAmtB* cell would acquire NH$_4^+$ when in competition with *R. palustris* and thereby overcome the competitive disadvantage of *E. coli ΔAmtB* for NH$_4^+$ (Fig. 5F). Indeed, we observed greater growth of both species when cocultures were inoculated at ratios equal or higher relative densities of *E. coli ΔAmtB* versus *R. palustris ΔAmtB* (Fig. 5F).

The explanation that mutualism collapse was due to a competitive advantage of *R. palustris ΔAmtB over E. coli ΔAmtB* for NH$_4^+$ called into question why cocultures pairing *E. coli ΔAmtB* with either *R. palustris Nx* or *R. palustris NxDΔAmtB* did not collapse as well (Fig. 4), given that in all of these pairings *E. coli ΔAmtB* was at a competitive disadvantage (Fig. 3). We hypothesized that a relatively high NH$_4^+$ excretion level by these latter *R. palustris* strains (Fig. 5D) could compensate for a low *E. coli* NH$_4^+$ affinity. To explore this hypothesis, we simulated cocultures with the *R. palustris* affinity for NH$_4^+$ set high relative to that of *E. coli* (R. palustris:E. coli affinity ratio, 1,000) and varied the *R. palustris NH$_4^+$ excretion level (Fig. 5). Indeed, increasing *R. palustris* NH$_4^+$ excretion was predicted to overcome a low *E. coli* affinity for NH$_4^+$ and support growth of both species (Fig. 5). The only exception was at the highest levels of NH$_4^+$ excretion, where *R. palustris* growth was predicted to be inhibited due to rapid *E. coli* growth and subsequent accumulation of organic acids that acidify the environment (Fig. 5), similar to previous observations where we experimentally increased the NH$_4^+$ excretion level.
These simulations suggested that *R. palustris* Nx and NxΔAmtB supported coculture growth with *E. coli* ΔAmtB due to higher NH$_4^+$ excretion levels (Fig. S3D), whereas a combination of low NH$_4^+$ excretion by *R. palustris* ΔAmtB (Fig. S3D) and a low affinity for NH$_4^+$ by *E. coli* ΔAmtB led to collapse of the mutualism in this pairing.

To this point, we had only considered the effect of severe discrepancies in NH$_4^+$ affinities between the two species (e.g., a 1,000-fold difference in $K_m$ values in our simulations) as a mechanism leading to coculture collapse within the time period of a single culturing. However, we wondered if a subtle discrepancy in NH$_4^+$ affinities could lead to coculture collapse if given more time. We therefore simulated serial transfers of cocultures with partners having different relative NH$_4^+$ affinities (Fig. 6A and B). At equivalent NH$_4^+$ affinities (Fig. 6A), both species were predicted to be maintained over serial transfers. However, when the relative affinities approached a threshold (relative *R. palustris*: *E. coli* affinity ratio, 1.5), cell densities of both species were predicted to decrease over serial transfers (Fig. 6B). This decline in coculture growth is due to *E. coli* being slowly but progressively outcompeted for NH$_4^+$ by *R. palustris*. As the difference between the *R. palustris* and *E. coli* populations expands, *R. palustris* cells have a greater chance of acquiring NH$_4^+$ than the smaller *E. coli* population, further starving *E. coli* and simultaneously cutting off *R. palustris* from its supply of organic acids from *E. coli*.

The above prediction prompted us to investigate if cocultures pairing *R. palustris* Nx with *E. coli* ΔAmtB were stable through serial transfers. We focused on cocultures with *R. palustris* Nx rather than *R. palustris* NxΔAmtB, because *R. palustris* Nx has

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FIG 5 Higher *R. palustris* NH$_4^+$ excretion levels are predicted to compensate for a low *E. coli* NH$_4^+$ affinity. Batch cultures (after 300 h) were simulated with a relative NH$_4^+$ affinity of 1,000 (R. palustris: *E. coli* affinity ratio [Rp:Ec]; affinity values are the inverse of $K_m$ values) over different *R. palustris* NH$_4^+$ excretion levels (SyFFoN parameter $R_p$). Final cell densities, solid lines; initial cell densities, dotted lines.

FIG 6 A low *E. coli* affinity for NH$_4^+$ results in coculture collapse over serial transfers when paired with *R. palustris* Nx. (A and B) Simulated batch cultures (300 h) were serially transferred using a 1% inoculum based on the cell density at 300 h for the previous culture. Relative NH$_4^+$ affinity values represent the relative *E. coli* $K_m$ for NH$_4^+$ ($K_A$) divided by that of *R. palustris* ($K_AR$). $K_A$ and $K_AR$ were both 0.01 mM in panel A, $K_A$ was 0.015 mM and $K_AR$ was 0.01 mM in panel B. (C) Change in cell densities of *R. palustris* Nx and *E. coli* ΔAmtB of cocultures grown for 1 week, less than 24 h into stationary phase. A 1% inoculum was used for each subsequent serial transfer. Error bars indicate standard deviations (SD; n = 4). Final *E. coli* cell percentages ± SD for each transfer are shown.
AmtB and would therefore be most likely to outcompete *E. coli* ΔAmtB. Strikingly, over eight serial transfers of cocultures pairing *R. palustris* Nx with *E. coli* ΔAmtB, we observed a significant decrease in cell densities of both partners (Fig. 6C). This decline in coculture growth over serial transfers was in stark contrast to results with cocultures of *R. palustris* Nx paired with WT *E. coli*, which we have serially transferred over 100 times with no extinction events (J. B. McKinlay, unpublished data). These results indicate that the recipient population must have a competitive advantage for a cross-fed nutrient relative to the producer population to avoid mutualism collapse.

**DISCUSSION**

Here, we demonstrated that within a mutualistic relationship, partners can compete for a cross-fed nutrient upon which the mutualistic interaction is based, in this case NH₄⁺. This competition can impact partner frequencies and mutualism stability. We demonstrated that efficient nutrient reacquisition by the producer can render nutrient excretion levels insufficient for mutualistic growth, starving the recipient and leading to tragedy of the commons (Fig. 6) (39). Conversely, recipient-biased competition for a cross-fed nutrient promotes mutualism stability. As noted above, the importance of this recipient-biased competitive advantage likely depends on whether the communally valuable resource is generated intracellularly or extracellularly (compare Fig. 2A and C). Intracellular synthesis ensures that a portion of the nutrient pool can be assimilated by the producing partner regardless of the differential affinity between the partners for that nutrient after excretion (Fig. 2A). Intracellular generation therefore helps stabilize a mutualism against an otherwise-competitive recipient by enforcing partial privatization. The competitive advantage of the recipient is in turn necessary to limit reacquisition of the excreted nutrient by the producer and thereby to drive directionality in nutrient exchange. Although partial privatization has primarily been thought to depend on mechanisms used by the producer to retain a portion of a communally valuable resource (16), our results indicate that the degree of privatization can be influenced by the partner as well; competition for the excreted nutrient pool impacts how much of a cross-fed resource will be shared versus reacquired. In effect, recipient-biased competition for an excreted communally valuable nutrient avoids tragedy of the commons by enforcing partial privatization over complete privatization.

It is expected that for mutualistic relationships based on the extracellular generation of nutrients, such as the release of sugar from a polymer, a high affinity for the nutrient by either partner can collapse the mutualism (Fig. 2D). It has been shown that microbes that excrete sugar polymer-degrading enzymes in the presence of competitors must have an advantage in obtaining the released sugars to proliferate, or even to avoid extinction (35–37). Supplementing a mutualism with an exogenous source of an otherwise-excreted communally valuable nutrient could also be viewed to mimic extracellular production. In these cases, the population outcome is also heavily influenced by the competitive affinities of each partner. For example, progressively adding exogenous nutrients to a yeast coculture stabilized by amino acid cross-feeding was shown to shift a mutualistic relationship to one of competition (21).

The importance of the recipient having the upper hand in interpartner competition likely applies to other synthetic cocultures and natural microbial mutualisms that are based on the cross-feeding of communally valuable nutrients that are generated intracellularly, including amino acids (21, 40, 41) and vitamin B₁₂ (7, 12). The same rule could also apply to interkingdom and nonmicrobial cross-feeding examples, such as those between plants and bacteria, fungi, or pollinators (1). In these cases, any decrease in resource release or emergence of traits allowing for reacquisition of a released resource would be expected to undermine the mutualism. Conversely, some nonmicrobial examples of cooperative feeding would be expected to follow the predictions for microbial mutualisms based on the extracellular generation of a communally valuable resource. For example, cooperative hunting between grouper fish and moray eels (42) or cooperative harvesting of
honey from bee hives between honeyguide birds and humans (43) would be expected to collapse if a single partner were to monopolize the resource (44). Indeed, the cooperative relationship between honeyguide birds and humans has declined in areas that have adopted bee-keeping practices, though in this case such declines are due to a technological advancement rather than evolution (43).

Our study also provided mechanistic insights into acquisition of communally valuable nutrients. AmtB transporters were shown to be crucial determinants of interpartner competition for NH$_4^+$ . We were intrigued to find that when both species lacked AmtB, R. palustris outcompeted E. coli for NH$_4^+$ (Fig. 5), enough to collapse the mutualism within a single culturing (Fig. 3). Whether by maximizing NH$_4^+$ retention or reacquisition, R. palustris, and perhaps other N$_2$ fixers, might have additional mechanisms aside from AmtB to minimize loss of NH$_4^+$ as NH$_3$. These mechanisms could include a relatively low internal pH to favor NH$_4^+$ over NH$_3$, negatively charged surface features, or relatively high affinities by NH$_4^+$-assimilating enzymes, such as glutamine synthetase. There are several reasons why it would be beneficial for N$_2$ fixers to have a superior ability to retain or acquire NH$_4^+$, perhaps by using mechanisms that are independent of AmtB, is not far-fetched. Bacteria are known to exhibit differential mechanisms to compete for nutrients. For example, iron acquisition commonly involves the excretion of iron-binding molecules or proteins called siderophores, which can differ in chemical structure and affinity for iron. These structural differences also influence their potential to be utilized by competitors and therefore their communal value as an extracellularly generated resource (48). Strategies to utilize siderophores as a shared resource are numerous, and they lead to different cooperative or competitive outcomes in microbial communities (48, 49). One must consider that additional mechanisms for acquiring NH$_4^+$ beyond AmtB might likewise exist. Understanding the physiological mechanisms that confer competitive advantages for nutrient acquisition between species will undoubtedly aid in describing the interplay between competition and cooperation within mutualisms.

MATERIALS AND METHODS

Strains and growth conditions. Strains, plasmids, and primers are listed in Table S2. All R. palustris strains contained Δupp and ΔhupS mutations to facilitate accurate CFU measurements by preventing cell aggregation (50) and to prevent H$_2$ uptake, respectively. E. coli was cultivated on Luria-Burtani (LB) agar, and R. palustris was cultivated on defined mineral (PM) agar (51) with 10 mM succinate. (NH$_4$)$_2$SO$_4$ was omitted from PM agar for determining R. palustris CFU. Monocultures and cocultures were grown in 10 ml of defined M9-derived coculture medium (MDC) (28) in 27-ml anaerobic test tubes. To make the medium aerobic, MDC was exposed to N$_2$ via bubbling, and then tubes were sealed with rubber stoppers and aluminum crimps and then autoclaved. After autoclaving, MDC medium was supplemented with cation solution (1% [vol/vol]; 100 mM MgSO$_4$ and 10 mM CaCl$_2$ stock concentration) and glucose (25 mM final concentration), unless indicated otherwise. E. coli monocultures were also supplemented with 15 mM NH$_4$Cl. All cultures were grown at 30°C laying horizontally under a 60-W incandescent bulb with shaking at 150 rpm. Starter cocultures were inoculated with 200 µl MDC containing a suspension of a single colony of each species. Test cocultures were inoculated using a 1% inoculum from starter cocultures. Serial transfers were also inoculated with a 1% inoculum. Kanamycin and gentamicin were added to final concentrations of 100 µg/ml for cultures of R. palustris and 15 µg/ml for E. coli cultures when appropriate.

Generation of R. palustris mutants. R. palustris mutants were derived from wild-type CGA009 (52). Generation of strains CGA4004, CGA4005, and CGA4021 is described elsewhere (28). To generate strain CGA4026 (R. palustris ΔAmtB), the WT nifA gene was amplified using primers JBM1 and JBM2, digested with XbaI and BamHI, and ligated into plasmid pJQ2005K to make pJQnifA16. This suicide vector was then introduced into CGA4021 by conjugation, and sequential selection and screening were performed as described (53) to replace nifA* with WT nifA. Reintroduction of the WT nifA gene was confirmed by PCR and sequencing.

Generation of the E. coli ΔAmtB mutant. P1 transduction (54) was used to introduce ΔamtB-km from the Keio Collection strain JW0441-1 (55) into E. coli MG1655. The ΔamtB-km genotype of kanamycin-resistant colonies was confirmed by PCR and sequencing.
**Analytic procedures.** Cell density was assayed based on the optical density at 660 nm (OD$_{660}$) using a Genesyss 20 visible spectrophotometer (Thermo-Fisher, Waltham, MA). Growth curve readings were obtained in culture tubes without sampling (i.e., tube OD$_{660}$). Specific growth rates were determined using OD$_{660}$ readings between 0.1 and 1.0, a range for which there is a linear correlation between cell density and OD$_{660}$. Final OD$_{660}$ measurements were taken in cuvettes, and samples were diluted into the linear range as necessary. H$_2$ was quantified using a gas chromatograph (Shimazu, Kyoto, Japan) with a thermal conductivity detector as described (56). Glucose, organic acids, formate, and ethanol were quantified using a Shimadzu high-performance liquid chromatograph as described (57). NH$_4^+$ was quantified using an indophenol colorimetric assay as described (28). Acetylene reduction assays (45) were performed by first harvesting cells from 10 ml of medium and resuspending in 10 ml of fresh MDC medium in 27-ml sealed tubes preflushed with argon gas. Suspensions were incubated in light for 1 h at 30°C to recover. Then, 250 µl of 100% acetylene gas was injected into the headspace to initiate the assay, and ethylene production was measured over time by gas chromatography, as described (45). Ethylene levels were normalized to total _R. palustris_ CFU in the 10-ml volume.

**NH$_4^+$ competition assay.** Fed batch cultures were prepared in custom anaerobic 75-ml serum vials with side sampling ports. Each vial contained a stir bar and 30 ml of MDC and was sealed at both ends with rubber stoppers and aluminum crimps. Each vial was supplemented with 25 mM glucose, 1% (vol/vol) cation solution, and 20 mM sodium acetate. Unlike acetic acid, which _E. coli_ excretes, sodium acetate does not change the pH of the medium. Starter monocultures of each species were grown to equivalent CFU (per milliliter) in MDC containing limiting nutrients (3 mM sodium acetate for _R. palustris_ and 1.5 mM NH$_4$Cl for _E. coli_), and 1 ml of each species culture was inoculated into the serum vials. These competition cultures were incubated at 30°C under a 60-W incandescent bulb with stirring at 200 rpm for 96 h. Each serum vial was constantly flushed with Ar to maintain anaerobic conditions. NH$_4$Cl was fed from a 500 µM NH$_4$Cl stock via a peristaltic pump on an automatic timer at a rate of 0.33 ml/min once an hour for a final concentration of ~5 µM upon each addition. The NH$_4^+$ concentration was below the known concentration at which AmtB transporters become important for NH$_4^+$ uptake (24). Samples were taken at 0 and 96 h for quantification of CFU.

**Mathematical modeling.** A Monod model describing bidirectional cross-feeding in batch cultures, called SyFFoN v3 (syntrophy between fermenter and fixer of nitrogen, version 3), was modified from our previous model (33) to allow for competition between _E. coli_ and _R. palustris_ for NH$_4^+$ as follows: (i) an equation for the _R. palustris_ growth rate on NH$_4^+$ was added to boost the _R. palustris_ growth rate when acquiring NH$_4^+$ and (ii) the ability for _R. palustris_ to consume NH$_4^+$ was added along with an _R. palustris_ $K_m$ for NH$_4^+$ ($K_{NH4}^+$). Default NH$_4^+$ $K_m$ values were set to 0.01 mM for both species, to achieve a ratio of 1. To achieve higher _R. palustris_ or _E. coli_ relative NH$_4^+$ affinities, the _E. coli_ or _R. palustris_ $K_m$ value was raised, respectively. Simulated cultures were run for 300 h unless noted otherwise. Normally, full glucose consumption occurs by ~100 h under typical experimental conditions and in corresponding simulations, but 300 h was allowed to capture trends that would take longer to emerge in response to parameter changes while still approximating a reasonable experimental time frame. Equations and default parameter values derived from our experimental data can be found in Text S1 and Table S1. SyFFoN v3 is run in RStudio and is available for download at https://github.com/McKinlab/Coculture-Mutualism.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01620-17.

**TEXT S1**, DOCX file, 0.1 MB.
**FIG S1**, TIF file, 0.4 MB.
**FIG S2**, TIF file, 0.2 MB.
**FIG S3**, TIF file, 0.3 MB.
**FIG S4**, TIF file, 0.3 MB.
**FIG S5**, TIF file, 0.2 MB.
**FIG S6**, TIF file, 0.1 MB.
**FIG S7**, TIF file, 0.2 MB.
**TABLE S1**, DOCX file, 0.02 MB.
**TABLE S2**, DOCX file, 0.02 MB.

**ACKNOWLEDGMENTS**

We thank Richard Phillips (Indiana University) for providing equipment for the NH$_4^+$ competition assay. We also thank Jay Lennon (Indiana University) for helpful discussions on the manuscript.

This work was supported in part by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, under award number DE-SC0008131, by the U.S. Army Research Office, grant W911NF-14-1-0411, and by the Indiana University College of Arts and Sciences.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.
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