Co-catabolism of arginine and succinate drives symbiotic nitrogen fixation

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Biological nitrogen fixation emerging from the symbiosis between bacteria and crop plants holds a significant promise to increase the sustainability of agriculture. One of the biggest hurdles for the engineering of nitrogen-fixing organisms is to identify the metabolic blueprint for symbiotic nitrogen fixation. Here, we report on the CATCH-N cycle, a novel metabolic network based on co-catabolism of plant-provided arginine and succinate to drive the energy-demanding process of symbiotic nitrogen fixation. The CATCH-N cycle probably operates below its full aerobic potential. Furthermore, if the metabolism of symbiotic nitrogen-fixing bacteria is based exclusively on the provision of succinate, then the bacterial nitrogen requirement must be covered solely by nitrogenase activity, it also inhibits TCA-mediated catabolism of succinate due to redox inhibition of key TCA-enzymes including citrate synthase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase (22, 23). Thus, the TCA cycle probably operates below its full aerobic potential. Furthermore, if the metabolism of symbiotic nitrogen-fixing bacteria is based exclusively on the provision of succinate, the bacterial nitrogen requirement must be covered solely by nitrogenase. However, nitrogen-fixing root-nodule bacteria (termed bacteroids), do not self-assimilate but rather secrete large quantities of ammonium (24–26) suggesting that the plant provides the bacteroids with a nitrogen-containing nutrient to cover their nitrogen needs. Finally, the degradation product of succinate in the TCA cycle is carbon dioxide. However, it has been reported that nitrogen-fixing bacteroids also secrete the amino

Nitrogen is a fundamental element of all living organisms and the primary nutrient that impacts crop yield (1, 2). Despite being highly abundant in the atmosphere, plants can only utilize nitrogen in reduced forms such as ammonium. More than 125 megatons of nitrogen are fixed annually by the industrial Haber-Bosch process into ammonium and applied to increase agricultural crop production (2). On the global scale, anthropogenic nitrogen delivered to the environment surpasses annual supplies by natural biological nitrogen fixation on land (3) leading to serious environmental impacts from climate change to the disruption of eco-systems and pollution of coastal waters.

Improving the ability of plants and plant-associated organisms to fix atmospheric nitrogen has inspired biotechnology for decades (4–6), not only for the apparent economic and ecological benefit that comes with the replacement of chemical fertilizers but also more recently for opportunities towards more sustainable agriculture and the potential to reduce greenhouse gas emissions. Attempts to transfer and improve nitrogenase genes clusters have to date focused largely on organisms such as \textit{Escherichia coli} (7, 8). More recently the emerging field of synthetic biology provides an alternative approach to engineer designer nitrogenase gene clusters in bacteria (9–12). Despite these promising results, engineered organisms based on heterologous expression of nitrogenase genes have not yet come close to the efficiency of natural rhizobia-legume symbiosis systems (4). While the molecular mechanism of the nitrogenase reaction has been resolved with atomistic detail (13–16), the precise mechanism how the metabolism between plants and bacteria becomes entangled to sustain the energy-intensive process of nitrogen fixation has remained an open question.

The current model of nutrient exchange in rhizobia-legume symbiosis postulates that, in exchange for fixed nitrogen, the plant provides dicarboxylic acids such as succinate, which is metabolized through the tri-carboxylic acid (TCA) cycle to generate ATP and reduction equivalents needed for the nitrogenase reaction (17–19). However, multiple lines of evidence argue against a simple exchange of succinate for ammonium during symbiosis (20, 21). While the absence of oxygen promotes nitrogenase activity, it also inhibits TCA-mediated catabolism of succinate due to redox inhibition of key TCA-enzymes including citrate synthase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase (22, 23). Thus, the TCA cycle probably operates below its full aerobic potential. Furthermore, if the metabolism of symbiotic nitrogen-fixing bacteria is based exclusively on the provision of succinate, then the bacterial nitrogen requirement must be covered solely by nitrogenase. However, nitrogen-fixing root-nodule bacteria (termed bacteroids), do not self-assimilate but rather secrete large quantities of ammonium (24–26) suggesting that the plant provides the bacteroids with a nitrogen-containing nutrient to cover their nitrogen needs. Finally, the degradation product of succinate in the TCA cycle is carbon dioxide. However, it has been reported that nitrogen-fixing bacteroids also secrete the amino

Significance Statement

Symbiotic bacteria assimilate nitrogen from the air and fix it into a form that can be used by plants in a process known as biological nitrogen fixation. In agricultural systems, this process is restricted mainly to legumes, yet there is considerable interest in exploring whether similar symbioses can be developed in non-legumes including cereals and other important crop plants. Here we present systems-level findings on the minimal metabolic function set for biological nitrogen fixation that provides the theoretical framework for rational engineering of novel organisms with improved nitrogen-fixing capabilities.

B.C. and C.E.F.T. performed transposon mutagenesis experiments; C.E.F.T. performed plant infection, nitrogenase and enzyme activity assays. M.C., B.C. and C.E.F.T. performed data analysis. M.C. and B.C. conceived the theoretical concept of the CATCH-N cycle; M.C. and B.C. wrote the manuscript.

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acids alanine and aspartate (27–29). Here, we report on the CATCH-N cycle based on the co-catabolism of plant-provided arginine and succinate as part of a specific metabolic network to sustain symbiotic nitrogen fixation. Using isotope-labeled mass-spectrometry analysis in *Bradyrhizobium diazoefficiens* in conjunction with *in planta* transposon-sequencing analyses and enzymatic reaction network characterization in *Sinorhizobium meliloti*, we uncovered the principle of metabolic entanglement leading to the nitrogen-fixing symbiosis between plants and bacteria. Collectively, we demonstrate that the CATCH-N metabolism is governed by highly redundant functions comprised of at least 10 transporter systems and 23 enzymatic functions. In sum, our systems-level findings provide the theoretical framework and enzymatic blueprint for the optimization and redesign of improved symbiotic nitrogen-fixing organisms.

**Results**

**Evidence for plant-provided arginine driving nitrogen fixation in rhizobia-legume symbiosis.** We asked what the identity of the postulated nitrogen-containing nutrient could be. Since the plant must provide the compound in sufficient quantities, we reasoned that an amino acid might be a likely candidate. Based on the finding that nitrogen-fixing bacteroids utilize succinate and secrete the amino acids alanine and aspartate (27–30), we concluded that the plant-provided compound must comprise at least two nitrogen atoms to enable two consecutive transamination reactions. The first nitrogen is used for transamination of the ketoacid derived from succinate while the second nitrogen atom is utilized for transamination of the ketoacid derived from the plant-provided compound.

Six out of the twenty natural amino acids (Arg, His, Lys, Gln, Asn, and Trp) contain two or more nitrogen atoms and thus are likely candidates. Thereof, His, Lys and Gln can be excluded because their degradation involves a compulsory 2-oxoglutarate dehydrogenase step, which is subjected to redox inhibition and disfavoured under microoxic conditions (31, 32). Furthermore, we also excluded Trp and Asn because their catabolism enters the TCA at the level of pyruvate and oxaloacetate respectively, which limits energy metabolism within a partially operating TCA cycle. Based on these theoretical considerations, we postulated that the remaining amino acid arginine is a likely candidate for the nitrogen-containing compound provided upon symbiosis.

**The co-feeding of arginine and succinate stimulates nitrogenase activity.** To probe whether arginine functions as co-substrate to drive symbiotic nitrogen fixation, we assayed nitrogenase activity of mature bacteroids from *B. diazoefficiens* (strain 110 spc4) and *S. meliloti* (strain CL 150) in presence of nodule crude extracts and upon supplementation of succinate and arginine (materials and methods). The addition of nodule crude extracts to isolated bacteroids resulted in strong stimulation of nitrogenase activity, supporting the idea that plant provided nutrients are necessary for symbiotic nitrogen fixation. While the stimulation of nitrogenase only poorly occurred in the presence of succinate as the sole nutrient, we found that the addition of arginine stimulated nitrogenase activity in *B. diazoefficiens* and *S. meliloti* to 50% ±4% and 116% ±2% respectively as compared to nodule extracts. Furthermore, the co-feeding of arginine in combination with succinate restored nitrogenase activity to the same extent as nodule extracts (92% ±6% and 92% ±6%) for *B. diazoefficiens* (Fig. 1A) and *S. meliloti* bacteroids respectively. Therefore, we concluded that the co-feeding of arginine and succinate is sufficient to stimulate nitrogenase activity in bacteroids.

The nitrogenase enzyme complex catalyzes one of the most energy-consuming enzymatic reactions found in nature with 16 ATP molecules and 8 low-potential electrons required for the reduction of a single nitrogen molecule. Nitrogenase is irreversibly inactivated in the presence of oxygen, which restricts the reduction of atmospheric nitrogen to low-oxygen conditions. Thus, to support nitrogen fixation, bacteroids must produce substantial amounts of ATP under microoxic conditions. The finding that succinate as the sole nutrient did not result in nitrogenase stimulation suggested that succinate catabolism via the TCA cycle does likely not generate...
sufficient ATP to support efficient nitrogenase reaction.

To measure the ATP level produced in isolated \textit{B. diazoefficiens} bacteroids, we quantified the increase in intracellular ATP through ATP-dependent luciferase assays (materials and methods). In agreement with the absence of an operational TCA cycle, we observed that the addition of succinate alone failed to stimulate ATP production. In contrast, we found that co-feeding of succinate together with arginine caused an increase from 1.53 ± 0.04 to 4.03 ± 0.09 attomole ATP per cell corresponding to a 2.61 ± 0.03 fold stimulation (Fig. 1B). In sum, these findings suggest that co-catabolism of arginine and succinate supports biological nitrogen fixation in \textit{B. diazoefficiens} and \textit{S. meliloti}.

### Table 1. Arginine catabolism in \textit{B. diazoefficiens} bacteroids fed with $^{13}$C arginine and unlabeled succinate

| Metabolite          | Fractional labeling (%)$^a$ |
|---------------------|-----------------------------|
| Arginine (ARG)       | 99.43 ± 0.10                |
| Citrulline (CIT)     | 60.22 ± 5.26                |
| Ornithine (ORN)      | 81.25 ± 5.01                |
| Proline (PRO)        | 23.19 ± 3.58                |
| Glutamate (GLU)      | 7.67 ± 0.82                 |
| 4-guanidinobutanoate (GBA) | 90.40 ± 3.03 |
| 4-aminobutanoate (GABA) | 6.26 ± 1.06                |

$^a$ $^{13}$C Fractional labeling after 150 min incubation with $^{13}$C L-arginine. Shown is the average and the standard error of the mean (SEM).

Isotope tracing experiments reveal the presence of three parallel arginine degradation pathways. To gain further insights into the structure and dynamics of the arginine degradation network operating during nitrogen fixation, we performed stable isotope labelling studies with \textit{B. diazoefficiens}. We incubated isolated bacteroids under stringent microoxic conditions with $^{13}$C arginine in the presence of unlabelled succinate and quantified incorporation of arginine degradation intermediates by LC-MS/MS (Table 1). Upon the addition of $^{13}$C arginine, we observed a rapid increase in the labeled intracellular arginine pool (99.43% $^{13}$C), demonstrating active arginine transport into nitrogen-fixing bacteroids. Furthermore, we observed fractional labelling of 90.40% for 4-guanidinobutanoate (GBA) and 6.26% for 4-aminobutanoate (GABA) further suggesting the presence of a functional arginine transaminase (ATA) pathway operating in bacteroids (Table 1) that yields alanine. The observation that bacteroids possess an ATA pathway was intriguing because it provided a functional link between arginine degradation and alanine secretion, which was previously reported as part of the metabolite exchange occurring during symbiotic nitrogen fixation (30). These findings suggest that at least three independent arginine degradation pathways operate simultaneously in nitrogen-fixing \textit{B. diazoefficiens} bacteroids. Collectively, arginine degradation results in the production of alanine and ammonium independent from the nitrogenase reaction.

Transposon sequencing reveals symbiosis genes involved in the uptake and catabolism of arginine. To gain further insights into the gene sets and enzymatic functions responsible for uptake and degradation of arginine, we conducted a functional genetic screen using transposon sequencing (TnSeq) (33, 34). TnSeq measures genome-wide changes in transposon insertion abundance prior and after subjecting large mutant populations to selection regimes (35) and allows systems-level definition of conditional essential gene sets for a given environment (36, 37).

We reasoned that TnSeq provides a unique opportunity to identify specific arginine transport and degradation genes that become essential upon engagement in symbiosis.

We choose \textit{S. meliloti-Medicago truncatula} as the rhizobial-legume symbiosis system, because supernodulating \textit{M. truncatula} \textit{lss} plants (38) provided a high frequency of nodules increasing the resolution of the TnSeq analysis. In total, we infected 4,500 \textit{M. truncatula} \textit{lss} plants with a high-density \textit{S. meliloti} transposon mutant library of 750,128 unique Tn5 insertions (Fig. 2A). Six weeks post-inoculation, we recovered 99,623 unique Tn5 mutants from 375,000 root nodules. By comparing the TnSeq dataset obtained from plant infection assays and input transposon mutant libraries, we mapped a set of 977 symbiosis genes corresponding to 15.71% of the tripartite 6.7-megabase (Mb) genome (Data SI, Materials and methods). Thereof, 435 genes were located on the chromosome, 295 on pSymA and 247 on pSymB indicating that all symbiotic essential transport genes (30.51%) and 22 out of 40 essential genes (55.00%) were annotated as being involved in the uptake and catabolism of arginine and its derivatives. In sum, these findings highlight that the provision of arginine and its consecutive degradation is of fundamental importance to drive symbiotic nitrogen fixation in legumes.

Tnseq identifies multiple arginine transport systems mediating acid tolerance. Among the 18 transport genes essential for symbiosis, we found two arginine and four putrescine ABC transport systems that we named \textit{artABCDE} (SMc03124-28) for arginine transporter, \textit{satABC} (SMA2195-97) for symbiotic arginine transporter and \textit{potFGHI} (SMc00770-3), \textit{potABCD2} (SMA0799-863), \textit{potABCD3} (SMA0051-3) and \textit{potABCD4} (SMA0051-3) for putrescine transport.
In planta selection

750'128 Tn mutants recovered
99'623 Tn mutants

In planta selection

pSymA 1.4 Mb
pSymB 1.7 Mb
247 / 1569 symbiosis genes
295 / 1290 symbiosis genes

Arginase gene deletions show nitrogen starvation phenotypes during plant infection assays. To validate the importance of the identified arginine-dependent acid tolerance systems for symbiosis, we constructed a panel of deletion mutants of the urease and ADI pathways and assessed nitrogen starvation phenotypes during plant infection assays. Out of the 8 mutants evaluated, all displayed symbiotic defects. On the level of the arginine transport systems, we found that artABCDE was the only transport system to be constitutively expressed during all stages of symbiosis, while the expression of all other transporters was specifically induced during development into nitrogen-fixing compartments (symbiosomes).

To gain further insights, we searched for additional symbiosis genes related to acid tolerance and indeed found multiple essential components in the TnSeq dataset (Data SI). From the urease pathway, we identified two arginases (argI1 and argI2) and the urease components ureA and ureE. From the ADI system, we found the arcABC operon to be essential for symbiosis. Both systems catalyze the conversion of arginine into ornithine leading to the production of ammonia as part of the acid tolerance mechanism. Furthermore, the ADI system also provides ATP via the enzymatic step of ornithine carbamoyltransferase arcB (40). Interestingly, two additional copies of ornithine carbamoyltransferase were also essential (argF1, and arcB2), emphasizing the importance of genetic redundancy in ADI dependent ATP synthesis during symbiosis. The urease and ADI acid tolerance mechanisms rely on the efflux of ammonium (41). Indeed, the ammonium efflux pump encoded by amtB was among the top-ranked symbiosis genes. These findings underscore the importance of ammonium secretion as a compulsive property of bacteroids independent of the nitrogenase reaction.

Fig. 2. The symbiosis genome of S. meliloti revealed by transposon sequencing (TnSeq). (a) Schematic representation of the plant infection screen that was used to map the S. meliloti symbiosis genome. Tn5 transposon mutant pools were selected for their ability to establish symbiosis with M. truncatula. After selection, Tn5 mutants recovered from root nodules were identified by TnSeq. (b) Genome map visualizing the distribution of essential symbiosis genes among the three S. meliloti replicons. Symbiosis genes are plotted as lines on the chromosome (grey) and the megaplasmids pSymA (blue) and pSymB (green). (c) Functional classification of essential symbiosis genes located on the chromosome (grey), pSymA (blue) and pSymB (green)

(3Ma2203-9) for the putrescine uptake systems (Data SI).

In addition, two arginine/agmatine antiporter genes adiC (SMa0684) and adiC2 (SMa1668) encoded on pSymA were also essential during symbiosis. Interestingly, all identified transport systems participate in urease, and ADI pathways that mediate acid tolerance. Cross-comparing expression profiles using previously published RNA-seq data sets (39), we found that artABCDE was the only transport system to be constitutively expressed during all stages of symbiosis, while the expression of all other transporters was specifically induced during development into nitrogen-fixing compartments (symbiosomes).

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Plants inoculated with the argI1,argI2 single and double deletion mutant harbored a typical phenotype of nitrogen starvation. The aerial part of infected plants was smaller than those inoculated with WT strain (Fig. 3A, S1). Nodules induced by the argI1,argI2 double deletion mutant displayed the yellowish color of non-functional (symbiosomes). From the

Identification of AspC as an arginine:pyruvate transaminase.

In our isotope labelling studies with bacteroids, we detected fractional labelling of 90.40% for GBA suggesting the presence of a functional ATA pathway. However, in the S. meliloti genome, corresponding genes have not been assigned. Among the essential symbiosis genes identified by our TnSeq analy-
AspC shares 40% sequence homology to AruH, the arginine:pyruvate transaminase from *Pseudomonas aeruginosa* (42, 43). When we characterized the enzymatic properties of AspC by mass spectrometry, we found transaminase activity for GBL (Fig. 4B). Furthermore, on the level of pyruvate catabolism. At least 16 redundant enzymes participate in the arginine transamination network. To further dissect possible redundant components, we performed a functional homology search for enzyme candidates known to be expressed during the nitrogen-fixing bacteroid stage. Conversion from arginine into succinate proceeds by a series of six consecutive enzymatic reaction steps. The four steps upstream of GABA comprise transamination, decarboxylation, ureohydrolase and dehydrogenase reactions. In the lower part of the reaction network, a linear pathway through transamination and subsequent decarboxylation steps leads from GABA to succinate (Fig. 4A). Upon heterologous expression and protein purification, we biochemically profiled a panel of 16 candidate enzymes. In addition to the arginine deiminases ArcA1 and ArcA2 and the arginase ArgI1, we found two agmatinases (ArgI2 and SpeB) and one ureohydrolase (SpeB2) acting on 4-guanidinobutyraldehyde (GBL), GOP and GBA (Fig. 4B). The highest level of pathway redundancy resides on the level of dehydrogenases. Besides the five known GabD1-5 proteins from *S. meliloti*, we identified four additional isozymes Gab6-9. Thereof GabD6 and GabD7 share a dehydrogenase profile identical to GabD1 for 4-aminobutyraldehyde (ABL), succinic semialdehyde (SSA) and GBL. GabD8 and GabD9 exhibited substrate specificity for GBL (Fig. 4B). Furthermore, on the level of pyruvate...
transaminases, we identified three additional enzymes (DatA, AatB, and ArgD) that exhibited substrate preferences for ornithine, putrescine and agmatine and two enzymes (GabT2, and GabT3) with preferences for GABA (Fig. 4B). Similarly, we also profiled two additional decarboxylase enzymes (OdcA, and OdcB) that either catalyzed the decarboxylation of ornithine or AOP (Fig. 4B). Collectively, these results demonstrate the presence of a highly redundant network mediating arginine catabolism in *S. meliloti*.

**Synthetic reconstitution of the arginine transamination network that operates in nitrogen-fixing bacteroids.** We reconstituted the reaction system in *vitro* from a set of 14 enzymes and followed the conversion of arginine and pyruvate by mass spectrometry (Fig. 4C). We observed that 90% of the arginine was rapidly metabolized within 30 minutes. As expected, ornithine and GOP appeared as the first intermediates and then concomitantly decreased with the appearance of the second level of intermediates putrescine, AOP and ABL that ultimately converted into GABA, SSA, and succinate. During the process, alanine steadily increased demonstrating that pyruvate transamination couples the conversion of arginine into succinate (Fig. 4D). In sum, these findings demonstrate the synthetic reconstruction of the transamination network that permits the co-catabolism of arginine and succinate.

**The catabolism of succinate and arginine is interlinked.** Since the ATA network consumes two equivalents of pyruvate but generates only a single equivalent during its operation, we reasoned that the network strictly depends on the provision...
of additional pyruvate, which must be formed by simultaneous co-catabolism of succinate. We concluded that the degradation of arginine and succinate are mutually coupled and can only take place if plants provide both nutrients in equal stoichiometries. Indeed, single deletion in the succinate transporter DctAB abrogates succinate uptake and thereby also prevents the co-catabolism of arginine, resulting in a fix minus phenotype. On the other hand, the uptake of arginine is controlled by multiple redundant transporters. Nevertheless, single deletions in artABCDE and satAB arginine transporter show a partial symbiosis phenotype. Thus, transamination enforces a strict co-catabolism of succinate and arginine but also provides an elegant solution to maintain a partial TCA cycle under stringent microoxic and acidic conditions. We termed the entangled catabolic network CATCH-N cycle under acidic (H^+) conditions to fix Nitrogen (Fig 5).

A bifurcated electron transport chain operates during nitrogen fixation. We reasoned that the operation of the CATCH-N cycle provides significant amounts of NADH as well as QH^2. Under aerobic conditions, NADH is regenerated by the electron transport chain, which includes proton-pumping enzymes known as complex I, III and IV. However, upon symbiosome acidification, the driving force of complex I is likely no longer sufficient to sustain proton translocation against the increased pH gradient impairing the conversion of NADH into QH^2. Also, oxygen partial pressure within symbiosomes is too low to operate the aerobic version of complex IV and bacteroids induce expression of a high-affinity cytochrome cbb3 oxidase complex FixNOQP1-3 (44–46). Recently, the electron bifurcating FixABCX protein complex has been proposed to serve as the alternative entry point for low-potential ('high-energy') electrons from NADH (47, 48) thereby bypassing the impaired complex I.

Based on these findings, we devised a model that restores electron flow from NADH to QH^2 by electron bifurcation to nitrogenase and the high-affinity terminal oxidase (Fig 5). If this is the main pathway that permits regeneration of NADH, then all components must be essential in symbiosis. Indeed, we found genes encoding for components of the nitrogenase nifHDK, the electron bifurcation complex fixABCX and the alternative complex IV fixNOQP1-3 among the top-ranked symbiosis genes in the TnSeq dataset (Supplementary Dataset SI). These genetic evidences suggested that a bifurcated electron transport chain operates during nitrogen-fixing symbiosis.

Estimation of the ATP balance of the bifurcated electron transport chain. The endergonic branch of the electron bifurcation reaction generates low-potential reducing equivalents in the form of flavodoxin hydroquinone (Fld^H) for nitrogenase catalysis (Fig 5). For every Fld^H the nitrogenase consumes two additional ATP molecules. However, the exergonic branch of the electron bifurcation reaction translocates only three protons corresponding to a single ATP that is generated per electron passing from QH^2 to coenzyme Q onto oxygen. Thus, the electron bifurcation of each NADH appears to be associated with a net loss of one ATP. In addition to NADH,
the CATCH-N cycle also provides QH₂ via succinate dehydrogenase (Fig 5). Thereby, up to two ATP are generated per QH₂ passing its electrons onto oxygen. Accordingly, a bifurcated electron transport chain in combination with an active succinate dehydrogenase complex delivers a net gain in ATP, provided that catabolism generates NADH to QH₂ in a 2:1 ratio. Contrary to this criterion, the complete TCA operating with malate or succinate provides a higher ratio of NADH to QH₂ of 5:1 and 5:2 and, thus, inevitably results in a net loss of ATP.

In addition to proton-motive force-dependent ATP synthesis, several metabolic cycles including the CATCH-N and TCA provide additional ATP through enzyme-coupled synthesis. Furthermore, proton gradients are not exclusively generated by the proton expelling complexes of the electron transport chain, but moreover can also be established through proton-consuming enzymatic reactions in the cytosol, including the production of ammonia and decarboxylation reactions implemented in the CATCH-N cycle. Based on these considerations, we calculated the net proton consumption for several theoretical cycles, we estimated the net gain in ATP compared to the core sequence of the TCA cycle operating with malate or succinate (Table 2) and were also more energy-efficient than the core sequence of other naturally existing arginine degradation pathways, such as the arginine-pyruvate-glutamate super-pathway (Supplementary Information). In contrast to earlier approaches (49), we did not restrict our design to previously annotated pathways but rather included all enzymatic activities of the arginine transamination network identified during our biochemical enzyme studies. To evaluate the feasibility of these theoretical cycles, we estimated the net gain in ATP per N₂ molecule converted. Our calculations show that in terms of ATP production, the CATCH-N cycles are generally more energy-efficient than a stand-alone TCA cycle operating on succinate or malate (Table 2) and were also more energy-efficient than the core sequence of other naturally existing arginine degradation pathways, such as the arginine-pyruvate-glutamate super-pathway (Supplementary Information). As an example, two of our CATCH-N cycles generated on average over 18 fold more ATP per N₂ converted into ammonia as compared to the core sequence of the TCA cycle operating with succinate as the sole substrate. In sum, these calculations establish a new conceptual framework to understand and engineer symbiotic nitrogen-fixing organisms with future perspectives for agriculture.

### Discussion

Here, we report on the CATCH-N cycle operating on the co-feeding of arginine and succinate as part of a specific metabolic network that drives the process of symbiotic nitrogen fixation. The CATCH-N cycle shares aspects with the plant mitochondrial arginine degradation pathway (50, 51), however, it delivers up to 25% higher yield in nitrogen in the form of two alanines and three ammonium secreted for each co-feed arginine and succinate. Thus, from the plant’s perspective, the CATCH-N cycle multiplies the nitrogen releasing capacity of arginine. On the level of bacteroids, the CATCH-N cycle provides an elegant solution for maintaining an active respiratory chain under the highly acidic and microoxic conditions present within the lysosomal compartment of the symbiosome. Thus, the CATCH-N cycle also functions as an effective mechanism to promote the survival of bacteroids within infected plant cells. Equimolar arginine and succinate serve as substrates and a molar ratio of nitrogen to the oxygen of 1:4 is required to operate the CATCH-N cycle. Therefore, nitrogen-fixation still depends on oxygen as terminal acceptor, while harnessing elementary nitrogen as the second electron acceptor for reducing equivalents generated by the metabolism. Also, the CATCH-N cycle requires a constant flux of 8 protons into the symbiosome to maintain the pH balance of the reaction. These protons must be translocated by the action of plant ATPases as part of the symbiosome acidification process. Thus, the operation of the CATCH-N cycle depends on the presence of an active plant metabolism. From the nitrogen balance standpoint, a feedback loop exists between the nitrogenase function of bacteroids and the availability of arginine within the host plant. Ammonium released by bacteroids is rapidly incorporated by plant cells into glutamate, glutamine, and aspartate that all serve as precursors for the biosynthesis of ornithine and subsequently for arginine occurring within chloroplasts. The output of the CATCH-N cycle results in a net gain of assimilated nitrogen that subsequently amplifies the plant’s arginine biosynthesis capacity as part of a positive feedback mechanism. As humanity faces global challenges with population growth and climate change, we need to rethink how tomorrows agriculture will look like. Thereby systems-biology approaches to broaden our understanding of plant-microbe interactions, as well as the design of synthetic nitrogen-fixing microbes that mimic natural symbiosis with plants, hold significant promise. Our integrated model of the CATCH-N cycle provides new insights into the principles underlying legume symbiosis and comprises an important stepping stone for the rational biotechnological development of nitrogen-fixing systems.

### Table 2. Comparison of selected metabolic pathways for N₂ fixation.

| Pathway | Substrates | Products | Reaction steps | Sum NADH | Sum QH₂ | O₂ consumed per N₂ | Influx H⁺ | ATP net gain |
|---------|------------|----------|----------------|----------|---------|-------------------|----------|--------------|
| CATCH-N1 | 2Suc, 2Arg | 2Ala | 11 8 | 4 -4 | 56 (48+8) | 2.80 |
| CATCH-N2 | 2Suc, 2Arg | 2Asp | 11 8 | 4 -4 | 52 (48+4) | 1.60 |
| CATCH-N3 | 2Mal, 2Arg | 2Ala | 11 8 | 1 -3 | 44 (36+8) | -0.80 |
| CATCH-N4 | 2Mal, 2Arg | 2Asp | 11 8 | 1 -3 | 40 (36+4) | -2.00 |
| TCA | 1.6Mal | CO₂ | 11 8 | 1.6 -2.8 | 38.8 (33.6+5.2) | -2.76 |
| TCA | 1.6Suc | CO₂ | 11 8 | 3.2 -3.6 | 48.8 (43.2+5.2) | 0.12 |

*Shown are the numbers of substrate, reaction steps and the numbers of NADH, QH₂ and oxygen molecules required (negative numbers) or generated (positive numbers) during conversion of N₂ into two molecules of ammonia. The influx in H⁺ for each cycle is listed with O₂-dependent proton translocation and O₂-independent proton consumption by enzymatic reactions listed in brackets. An H⁺/ATP ratio of 3.33 was assumed for estimating the gain in ATP per N₂ converted.*

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engineering of artificial nitrogen-fixing microbes and improved crop plants to ensure food and climate security.

Materials and Methods

Supplementary Materials and Methods include detailed descriptions of bacterial strains and growth conditions, TnSeq library generation and data analysis, plant cultivation and phenotypic characterization, bacteroid extraction and substrate-specific ATP and nitrogenase stimulation, isocele tracing experiments, enzymatic procedures, and arginine catabolism thermodynamic calculations. Data S1 contains the essentiality classification of each S. meliloti coding sequence across every selection screen.

TnSeq library generation. Tn5 hyper-saturated transposon mutant libraries in S. meliloti were generated as previously described (34, 35). Transposon mutant libraries were selected on rich medium (LB) supplemented with gentamicin and streptomycin. Plates were incubated at 30°C for 2 days. For the in planta selection, 4500 Medicago truncatula lss super-nodulator mutant (38) were flood-inoculated with an S. meliloti Tn5 mutant reference libraries initially selected on rich media conditions (LB). Six weeks post-inoculation, nodules were retrieved, surface-sterilized, blotted dry, and stored at 7.4°C for subsequent use.

Bacteroid isolation, substrate-specific stimulation of nitrogenase activity, and ATP production. S. meliloti and B. diazoefficiens bacteroids were isolated from M. truncatula (10 weeks post-inoculation) and G. muz (3 weeks post-inoculation) root nodules respectively according to the following procedure. Under aerobic (ATP production stimulation) or anaerobic (for nitrogenase activity stimulation) conditions, nodules (0.25–1g fresh weight) were crushed in PBS pH=7.4 and filtered through three layers of gauze to remove debris. Under anaerobic conditions (for both assays), bacteroids were resuspended in 2 mL of induction media (2μM biotin, 1 mM MgSO4, 42.2 mM Na2HPO4, 22 mM KH2PO4, 8.5 mM NaCl, 21 mM COCl2, 1 μM NaNO3, pH 7.0) or nodule crude extract and added to 15 mL sealed flasks. Induction media was supplemented with either 7.4 mM succinate or 5 mM arginine or both substrates. For nitrogenase activity stimulation, acetylene and oxygen were added to a final concentration of 5 % and 0.01 % respectively in the head space of each flask. Nitrogenase activity was determined by the acetlylene reduction assay. For ATP production, ATP content was determined for each sample through ATP-dependent luciferase reaction (BacTiter-Glo Microbial Cell Viability Assay, Promega) as indicated by the manufacturer.

Enzymatic assays. Enzymatic reaction were done using purified C-terminal His 6X tag constructs (transaminases, urea, melios, decarboxylases and in vitro synthetic reconstitution of arginine catabolism) or cell-lysates (dehydrogenases) from E. coli BL21 rosetta pLys strains expressing S. meliloti enzymes. Enzymatic reactions were done using 20-30 μg of enzyme in 200 μL reaction mixture containing 1 mM of substrate, 1 mM MgCl2, 1 mM MnCl2, 100 mM Tris HCl pH 8 and 50 μM thiamine pyrophosphate (for dehydrogenase reactions) or PBS pH was adjusted to 7.4 (transaminases), 8.0 (ureohydrolases, decarboxylases, in vitro synthetic reconstitution of arginine catabolism) or 10.0 (dehydrogenases). Enzymatic reactions were performed at 25°C. For transaminases, ureohydrolases, decarboxylases and in vitro synthetic reconstitution of arginine catabolism substrate consumption as well as product increase was detected by non-targeted in vitro metabolomics (52). Dehydrogenase reaction was determined by the conversion of NAD+ into NADH + H+, which was measured by the increase in absorbance at 340 nm.

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1. Sciclone RH (1999) Nitrogen management and the future of food: lessons from the management of energy and carbon. Proceedings of the National Academy of Sciences 96(11):6001–6008.
2. Graham R, Vance C (2000) Nitrogen fixation in perspective: an overview of research and extension needs. Field Crops Research 65(2-3):93–106.
3. Gnirke A, Gnirke A (2009) An Earth-system perspective of the global nitrogen cycle. Nature 451(7197):293–299.
4. Beatty, Phil Good AG (2011) Future prospects for crops that fix nitrogen. science 333(6041):416–417.
5. Sorooshian D, Ansmann MW, Sahoo RK, Tuteja N (2014) Bifidobacterium as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. Microbial cell factories 13(1):66.
6. Gupta G, Paritar SS, Athawar NK, Nekhi SK, Singh V (2015) Plant growth promoting rhizobacteria (PGPR): current and future prospects for development of sustainable agriculture. J Microb Biochem Technol 7(2):996–1002.
7. Dixon RA, Postgate JR (1972) Genetic transfer of nitrogen fixation from Klebsiella pneumoniae to Escherichia coli. Nature 237(5359):102.
8. Wang L, et al. (2013) A minimal nitrogen fixation gene cluster from Paenibacillus sp. WUL78 enables expression of active nitrogen in Escherichia coli. PLoS genetics 9(10):e1003865.
9. Temme K, Zhao D, Vogt CA (2012) Refactoring the nitrogen fixation gene cluster from Klebsiella oxytoca. Proceedings of the National Academy of Sciences 109(18):7585–7590.
10. Yuan X, et al. (2018) Polyprotein strategy for stoichiometric assembly of nitrogen fixation components for synthetic biology. Proceedings of the National Academy of Sciences 115(36):E8509–E8517.
11. Burton S, Lopera-Torregro J, Rubio LM (2018) Extreme bioengineering to meet the nitrogen challenge. Frontiers in plant science 9(2):151–163.
12. Li XX, Liu Q, Liu XM, Shi HW, Chen SF (2016) Using synthetic biology to increase nitrogenase activity. Microbial cell factories 15(1):43.
13. Hoffman BM, Dean DR, Seefeldt LC (2009) Climbing nitrogenase: toward a mechanism of enzymatic nitrogen fixation. Accounts of chemical research 42(5):609–619.
14. Hoffman BM, Lukoyanov D, Yang Z, Dean DR, Seefeldt LC (2014) Mechanism of nitrogen fixation by nitrogenase: the next stage. Chemical reviews 114(8):4041–4062.
15. Dinsdale E, Hoffman BM, Dean DR (2009) Mechanism of Mo-dependent nitrogenase. Annual review of biochemistry 78:701–722.
16. Sippel D, Einsle O (2017) The structure of vanadate nitrogenase reveals an unusual bridging ligand. Nature chemical biology 13(9):956.
17. Watson RI, Shen YK, Wheatcroft R, Yang AF, Han S (1988) Rhizobium meliloti genes required for C4-dicarboxylate transport and symbiotic nitrogen fixation are located on a megaplasmid. Journal of Bacteriology 170(2):927–934.
18. Yurgel SN, Kahn ML (2004) Dicarboxylate transport by rhizobia. FEMS microbiology reviews 28(4):489–501.
19. Clarke VC, Loughlin PG, Day DA, Smith P (2014) Transport processes of the legume symbio- some membrane. Frontiers in plant science 5:699.
20. Kahn ML, Kraus J, Somerville JE (1985) A model of nutrient exchange in the Rhizobium- legume symbiosis in Nitrogen fixation research progress. (Springer), pp. 193–199.
21. Udvard M, Kahn M (1993) Evolution of the Brady Rhizobium-legume symbiosis: Why do bacteroids fix nitrogen? Symbiosis.
22. Dunn MF (1998) Tricarboxylic acid cycle and anaplerotic enzymes in rhizobia. FEMS microbiology reviews 21(2):105–123.
23. Prell J, Poole P (2006) Metabolic changes of rhizobia in legume nodules. Trends in microbi- ology 14(4):161–168.
24. Bergersen F, Turner G (1987) Nitrogen fixation by the bacteroid fraction of breys of soybean root nodules. Biochimica et Biophysica Acta 941(3):507–515.
25. Brown C, Dilworth M (1975) Ammonia assimilation by Rhizobium cultures and bacteroids. Microbiology 91(1):39–48.
26. Udvard M, Poole PS (2013) Transport and metabolism in legume-rhizobia symbioses. Annual review of plant biology 64:781–805.
27. Kretovich V, Karyakina T, Siddelikova L, Shaposhnikov G, Kaloshina G (1987) Nitrogen fixation and biosynthesis of aspartic acid and alanine by bacteroids of Rhizobium lupini on various carbon sources. Doklady: biochemistry-Akademiia nauk USSR.
28. Waters JK, et al. (1998) Alarmines are glucosamine-like molecules that can activate nodules bacteroids. Proceedings of the National Academy of Sciences 95(20):12038–12042.
29. Allaway D, et al. (2000) Identification of alanine dehydrogenase and its role in mixed secretion of ammonium and alanine by pea bacteroids. Molecular microbiology 36(2):508–515.
30. Day D, Poole R, Tyerman S, Rosendahl L (2001) Ammonia and amino acid transport across symbiotic membranes in nitrogen-fixing legume nodules. *Cellular and Molecular Life Sciences* CMLS 58(1):61–71.

31. Salminen SO, Streeter JG (1990) Factors contributing to the accumulation of glutamate in Bradyrhizobium japonicum bacteroids under microaerobic conditions. *Microbiology* 136(10):2119–2126.

32. Salminen SO, Streeter JG (1992) Labeling of carbon pools in Bradyrhizobium japonicum and Rhizobium leguminosarum bv vicina bacteroids following incubation of intact nodules with 14CO2. *Plant physiology* 100(2):597–604.

33. Christen B, et al. (2011) The essential genome of a bacterium. *Molecular systems biology* 7(1).

34. Van Oprijsen T, Bodí KL, Camilli A (2009) Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. Nature methods 6(10):767.

35. Christen M, et al. (2016) Quantitative selection analysis of bacteriophage ϕCbK susceptibility in Caulobacter crescentus. *Journal of molecular biology* 428(2):419–430.

36. Ochsner AM, et al. (2017) Gene transfer agent promotes evolvability within the fittest subpopulation of a bacterial pathogen. *Cell systems* 4(6):611–621.

37. Schnabel E, et al. (2010) The Iss supernodulation mutant of Medicago truncatula reduces expression of the SUNN gene. *Plant Physiology* 154(3):1390–1402.

38. Roux B, et al. (2014) An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser-capture microdissection coupled to RNA sequencing. *The Plant Journal* 77(6):817–837.

39. Cunin R, Glansdorff N, Pierard A, Stalon V (1986) Biosynthesis and metabolism of arginine in bacteria. *Microbiological reviews* 50(3):314.

40. Marquis R, Bender G, Murray D, Wong A (1987) Arginine deiminase system and bacterial adaptation to acid environments. *Appl. Environ. Microbiol.* 53(1):196–200.

41. Yang Z, Lu CD (2007) Characterization of an arginine:pyruvate transaminase in arginine catabolism of *Pseudomonas aeruginosa*. *Journal of bacteriology* 189(11):3945–3953.

42. Preisig O, Anthamatten D, Hennecke H (1993) Genes for a microaerobically induced oxidase complex in Bradyrhizobium japonicum are essential for a nitrogen-fixing endosymbiosis. *Proceedings of the National Academy of Sciences* 90(8):3309–3313.

43. Nellen-Anthamatten D, et al. (1998) Bradyrhizobium japonicum FixK2, a Crucial Distributor in the FixLJ-Dependent Regulatory Cascade for Control of Genes Inducible by Low Oxygen Levels. *Journal of bacteriology* 180(19):5251–5255.

44. Buschmann S, et al. (2010) The structure of cbb3 cytochrome oxidase provides insights into proton pumping. *Science* 329(5989):327–330.

45. Ledbetter RN, et al. (2017) The electron bifurcating FixABCX protein complex from Azotobacter vinelandii: generation of low-potential reducing equivalents for nitrogenase catalysis. *Biochemistry* 56(32):4177–4190.

46. Buckel W, Thauer RK (2018) Flavin-based electron bifurcation, ferredoxin, flavodoxin, and anaerobic respiratory with protons (Ech) or NAD(+) (Rnf) as electron acceptors: A historical review. *Frontiers in microbiology* 9:401.

47. Zhao H, Li M, Fang K, Chen W, Wang J (2012) In silico insights into the symbiotic nitrogen fixation in *Sinorhizobium meliloti* via metabolic reconstruction. *PLoS One* 7(2):e31287.

48. Winter G, Todd CD, Trovato M, Forlani G, Funck D (2015) Physiological implications of arginine metabolism in plants. *Frontiers in plant science* 6:534.

49. Polacco JG, Mazzarella P, Tazotto T (2013) Opinion—nickel and urease in plants: still many knowledge gaps. *Plant Science* 199:79–90.

50. Sévin DC, Fuhrer T, Zamboni N, Sauer U (2017) Nontargeted in vitro metabolomics for high-throughput identification of novel enzymes in *Escherichia coli*. *Nature methods* 14(2):187.