Control of nitrogen fixation and ammonia excretion in *Azorhizobium caulinodans*

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Abstract

Due to the costly energy demands of nitrogen (N) fixation, diazotrophic bacteria have evolved complex regulatory networks that permit expression of the catalyst nitrogenase only under conditions of N starvation, whereas the same condition stimulates upregulation of high-affinity ammonia (NH\(_3\)) assimilation by glutamine synthetase (GS), preventing excess release of excess NH\(_3\) for plants. Diazotrophic bacteria can be engineered to excrete NH\(_3\) by interference with GS, however control is required to minimise growth penalties and prevent unintended provision of NH\(_3\) to non-target plants. Here, we tested two strategies to control GS regulation and NH\(_3\) excretion in our model cereal symbiont *Azorhizobium caulinodans* AcLP, a derivative of ORS571. We first attempted to recapitulate previous work where mutation of both P\(_{II}\) homologues *glnB* and *glnK* stimulated GS shutdown but found that one of these genes was essential for growth. Secondly, we expressed unidirectional adenylyl transferases (uATs) in a \(\Delta glnE\) mutant of AcLP which permitted strong GS shutdown and excretion of NH\(_3\) derived from N\(_2\) fixation and completely alleviated negative feedback regulation on nitrogenase expression. We placed a uAT allele under control of the NifA-dependent promoter P\(_{nifH}\), permitting GS shutdown and NH\(_3\) excretion specifically under microaerobic conditions, the same cue that initiates N\(_2\) fixation, then deleted *nifA* and transferred a rhizopine *nifA\(_{L94Q/D95Q}\)-rpoN* controller plasmid into this strain, permitting coupled rhizopine-dependent activation of N\(_2\) fixation and NH\(_3\) excretion. This highly sophisticated and multi-layered control circuitry brings us a step closer to the development of a "synthetic symbioses" where N\(_2\) fixation and NH\(_3\) excretion could be specifically activated in diazotrophic bacteria colonising transgenic rhizopine producing cereals, targeting delivery of fixed N to the crop while preventing interaction with non-target plants.

Author summary

Inoculation of cereal crops with associative diazotrophic bacteria that convert atmospheric nitrogen (N\(_2\)) into ammonia (NH\(_3\)) could be used to sustainably improve delivery of nitrogen to crops. However, due to the costly energy demands of N\(_2\) fixation, bacteria restrict excess production of NH\(_3\) and release to the plants. Diazotrophs can be engineered for excess NH\(_3\) production and release, however genetic control is required to minimise...
growth penalties and prevent unintended provision of NH$_3$ to non-target weed species. Here, we engineer coupled control of N$_2$ fixation and NH$_3$ release in response to the signalling molecule rhizopine supplemented *in vitro*. This control circuitry represents a prototype for the future development of a “synthetic symbiosis” where bacterial N$_2$ fixation and NH$_3$ excretion could be specifically activated following colonisation of transgenic rhizopine producing cereals in the field, minimising bacterial energy requirements and preventing provision of NH$_3$ to non-target plants.

**Introduction**

Nitrogen (N) is an essential constituent of all biological organisms, but metabolically accessible forms are scarce in most environments [1], restricting biomass production. In agriculture, productivity of cereal crops, which are a staple of human dietary requirements, requires large-scale supplementation with synthetic N fertilisers to meet global food security requirements [2]. However, synthesis and excessive application of N fertilisers has a large energy cost, causes CO$_2$ release and results in loss of reduced N to the environment, which has doubled reactive N in the atmosphere and polluted waterways causing eutrophication and oxygen-depleted dead zones [3]. In contrast, N fertilisers are largely unaffordable to small-hold farmers in developing countries such as those in Sub-Saharan Africa [4], restricting yields to a fraction of their maximum potential [5]. Inoculation of cereals with root-associative diazotrophic bacteria that convert atmospheric N$_2$ gas to ammonia (NH$_3$) through the action of oxygen-labile nitrogenase represents an affordable and sustainable alternative to the use of N fertilisers in agriculture [6–8]. Although associative diazotrophs have been estimated to fix up to 25 kg N ha$^{-1}$ year$^{-1}$ in agricultural systems [9], responses to inoculation are typically inconsistent due to sub-optimal competitiveness for root colonisation and persistence in soil [10–13]. Furthermore, due to the costly energy demands of N$_2$ fixation, which consumes at least 16 mol ATP per mol N$_2$ fixed *in vitro*, bacteria have evolved complex regulatory networks that permit expression and activity of the N$_2$-fixing catalyst nitrogenase only under conditions of N starvation, whereas the same condition stimulates upregulation of high-affinity NH$_3$ assimilation by glutamine synthetase (glnA, GS), preventing excess release of excess NH$_3$ for plants [14,15].

Associative diazotrophic bacteria can be engineered for excess production and excretion of NH$_3$ by several strategies [14,16,17]. For example, in *Azotobacter vinelandii*, insertionional inactivation of nifL, which encodes an oxygen as well as N and carbon sensing anti-activator of the nitrogenase master regulator NifA, drives constitutive nitrogenase activity resulting in excretion of NH$_3$ from the cell [18–21]. The same effect was achieved by expressing mutant nifA alleles that are resistant to inhibition by NifL [19,22,23]. While excess NH$_3$ production itself is likely to activate regulatory feedback mechanisms reducing GS biosynthetic activity and NH$_3$ assimilation [16], mutating glnA [24–28] or genes involved in GS regulation may also be required to inhibit NH$_3$ assimilation more strongly and favour optimal NH$_3$ excretion [29,30].

Bacterial GS belongs to the “class I” type enzymes comprised of 12 identical subunits which are each adenylylated or deadenylated by a bidirectional adenylyl transferase (AT, encoded by glnE) at the Tyr$^{397}$ residue, with the fully deadenylated GS form being biosynthetically active and vice versa [31]. Directionality of the A'Tase reaction is regulated by the post-translational modification state of P$_{II}$ signal transduction proteins [32]. The activity of P$_{II}$ proteins is regulated by uridylylation/deuridylylation by the bidirectional uridylyltransferase (UT) GlnD which represents the most basal regulator in the cascade and can directly sense N status of the cell [33]. GlnD uridylylates P$_{II}$ under conditions of N-starvation and the resulting P$_{II}$-UMP
ultimately triggers dephosphorylation of ATase and hence deadenylylation and activation of GS [34]. In Azorhizobium caulindodans (Ac), insertional inactivation of both PII homologues glnB and glnK produced a mutant that was unable to activate GS by deadenylylation, driving NH$_3$-insensitive N$_2$ fixation and excretion of NH$_3$ into the growth media [29]. Critically, this engineering strategy does not appear to be universally applicable as PII is essential for NifA and nitrogenase activity in some bacteria [35,36], whereas it is essential for growth in others [37,38]. In a A$\Delta$hnE ATase mutant of Azospirillum brasilense, complementation with unidirectional adenylyltransferase (uAT) alleles that encoded only the C-terminal adenylylation domain [32] drove strong adenylylation of GS resulting in excretion of NH$_3$ into the growth media [30]. This strategy likely represents a more universally applicable approach for engineering NH$_3$ excretion in diazotrophs because the ATase is highly conserved, has a specific function, and can be readily mutated across diverse diazotrophic bacterial taxa [16,39–41], albeit the mutation appears to be lethal in the heterotroph Mycobacterium tuberculosis [42,43].

From an agricultural perspective, there are three major caveats of engineering diazotrophic bacteria for excessive production and excretion of NH$_3$: i) uncontrolled nifA and (or) nitrogenase expression has a severe energy burden on the cell that could abolish competitiveness for root colonisation; ii) interference with GS activity typically renders strains auxotrophic for the essential amino acid glutamine, which could further reduce competitiveness; and iii) NH$_3$-excreting bacteria have potential to supply NH$_3$ to non-target weed species following promiscuous colonisation in the field. Therefore, establishing control of N$_2$ fixation and NH$_3$ excretion will be crucial for the optimisation of strains as agricultural inoculants. Control of NH$_3$ excretion has already been achieved in A. vinelandii by establishing IPTG-dependent expression of glnA [28], and in A. brasilense by establishing anhydro-tetracycline inducible expression of uATs [30,44]. However, use of plant-derived signals to control N$_2$-fixation and NH$_3$ excretion would be far more applicable in the environment and could impart partner-specificity to target delivery of fixed N to crops and prevent interactions with non-target host plants following promiscuous colonisation [45,46].

We previously developed synthetic rhizopine signalling between barley and the model endophyte Azorhizobium caulindodans AcLP that stimulates transcriptional activation of the mutant nitrogenase master regulator nifA$_{L94Q/D95Q}$, which partially escapes nitrogen regulation, and when paired with the sigma factor RpoN drives N$_2$ fixation in bacteria colonising rhizopine producing (RhiP) barley roots [45,47,48]. Here, we demonstrate that wild-type and engineered Ac strains do not release fixed N as NH$_3$ into the growth media when cultured under N$_2$-fixing conditions and therefore sought to engineer this trait by interfering with high-affinity NH$_3$ assimilation catalysed by GS. In our attempts to recapitulate NH$_3$ excreting glnB glnK double mutants of AcLP [29], we found that deletion of both PII homologues was only possible when second copy of glnB was first integrated into the chromosome suggesting one of the PII homologues were essential for growth. GS and nitrogenase activity in the resulting strain exhibited minimal variation from that of the wild-type, but nevertheless the strain excreted low levels of NH$_3$ into the growth media. To optimise rates of NH$_3$ excretion, we utilised a second engineering strategy where an AcLP glnE mutant was complemented with uATs. In congruency with similar experiments performed in A. brasilense [30], uAT expression drove strong shutdown of GS, but also completely alleviated negative feedback inhibition of nitrogenase by NH$_3$ and stimulated NH$_3$ excretion. By placing uAT expression under control of NifA, we established control of these traits in response to microaerobic conditions, the same cue that initiates N$_2$ fixation, then transferred rhizopine control of nifA$_{L94Q/D95Q}$RpoN into this strain linking activation of N$_2$-fixation and NH$_3$ excretion (Fig 1). This highly sophisticated control circuitry represents a significant milestone in the development of a “synthetic symbiosis” where N$_2$ fixation and NH$_3$ excretion could be activated in bacteria specifically colonising...
Results

Deletion or strong repression of the P_{II} genes is lethal

It was previously demonstrated that insertional inactivation of the Ac P_{II} genes glnB and glnK stimulates shutdown of GS by adenylylation and alleviates negative feedback inhibition of target rhizopine producing cereals, targeting delivery of N to the crops while avoiding potential interactions with non-target plants.
nitrogenase by the product NH$_3$, preventing NH$_3$ assimilation and favouring excretion into the growth media [29]. We attempted to recapitulate these experiments in AcLP, a derivative of Ac harbouring a mini-Tn7 attB integration site stably recombined into its chromosome, by constructing a markerless deletion of glnB and replacing glnK with an omega (O)-spectinomycin resistance cassette. Although the single ΔglnB and ΔglnK::O$^-$Sp mutations were readily acquired, we were unable to acquire the double mutant by introduction of the ΔglnK::O$^-$Sp mutation into AcLPΔglnB when selection was performed on rich or minimal media supplemented with glutamine as a sole N source, suggesting the resulting phenotype was lethal. To explore this notion further, we integrated into the chromosome of AcLPΔglnB a construct encoding glnB with the strong ribosome binding site (RBS) RStd expressed from the IPTG derepressible promoter P$^{lac}$ (Fig 2A) and were subsequently able to acquire the ΔglnB ΔglnK::O$^-$Sp double mutation when selection was performed on rich media in the absence of IPTG, confirming that one of the P$^{II}$ proteins was essential for growth.

We next sought to test whether reduced translation of the introduced glnB gene would stimulate GS shutdown and NH$_3$ excretion by tuning the ribosome binding site (RBS). Seven synthetic RBS’ were experimentally demonstrated to produce translation rates spanning two to three orders of magnitude (S1 Fig), but only when glnB was fused to the strongest RBS RStd and integrated into the AcLPΔglnB chromosome were we able to subsequently isolate the ΔglnK::O$^-$Sp mutation (hereby termed strain AcRGl), suggesting that glnB had been repressed as much as was tolerable. We assessed total GS specific activity and that of the unadenylylated

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Fig 2. Strong repression of glnB in a glnK mutant has minimal effect on glutamine synthetase and nitrogenase activity but drives low-level ammonia excretion. (a) Strategy for generating strain AcRGl with the double ΔglnB and ΔglnK::O$^-$Sp mutation following integration of an IPTG-derepressible glnB gene into the chromosome of AcLP. (b) Activity of the unadenylylated (active) form of GS in n = 5 wild-type (WT) or AcRGl cultures incubated for 24-h as determined by γ-glutamyl transferase assays in the presence or absence of 60 mM MgCl$_2$ (see S2 Fig for total activity). (c) Nitrogenase activity measured by acetylene reduction in n = 6 cultures between 3-h- 21-h. Cultures for all assays were grown in N$_2$-fixing conditions (N-free UMS media with 3% O$_2$ in the headspace). Error bars represent one SEM. Independent two-tailed students t-tests were used to compare means. Exact P-values are provided where P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001. The wild-type AcLP was used as a reference group for comparison of means in panel (d).

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active enzyme in AcRGI by performing γ-glutamyl transferase assays on whole cells in the presence or absence of 60 mM MgCl₂ which specifically inhibits the adenylylated enzyme [49], and found that mutant exhibited higher total GS activity compared to the wild-type (S2A Fig), presumably due to elevated glnA expression (S2B Fig) as is typical of glnB mutants [26,29], whereas the adenylylation state of GS (depicted here as percentage of unadenyllylated GS activity) was unchanged (Fig 2B). We also found that the specific nitrogenase activity of strain AcRGI was no different from that of the wild-type, being repressed by supplementation of 10 mM NH₄Cl into the growth media (Fig 2C). Spectrophotometric quantification of NH₃ was next performed using the indophenol method [50] on the strains grown for 24-h under N₂-fixing conditions (here defined as N-free UMS with O₂ in the headspace adjusted to 3%). No NH₃ was detected in the wild-type or ΔglnB and ΔglnK::ΩSp single mutants, whereas we detected trace amounts of NH₃ in the growth media of strain AcRGI (Fig 2D). Given that construction of the AcLPΔglnB ΔglnK::ΩSp double mutant was lethal, and strong repression of glnB had minimal effect on GS and nitrogenase regulation permitting only low-level NH₃ excretion, we concluded that these strategies were inadequate to establish control of NH₃ excretion in AcLP and opted to pursue an alternative strategy.

**uAT expression drives GS inactivation and ammonia excretion**

In a ΔglnE mutant of *A. brasilense*, controlled expression of a N-terminal truncated uAT consisting of only the AT adenylylation domain resulted in unidirectional activity driving strong inactivation of GS by adenylylation and excretion of NH₃ into the growth media [30]. We recapitulated these experiments in a ΔglnE mutant of AcLP by using the *Sinorhizobium melloti* derived Pnoda promoter (S3 Fig), to drive expression of a series of truncated uATs derived from *Ac* or those previously described for *E. coli* (Fig 3A and 3B) [30]. We assessed GS specific activity and that of the unadenyllylated enzyme using γ-glutamyl transferase assays on cells grown in N₂-fixing conditions for 3-h and confirmed that leaky non-induced uAT expression stimulated GS adenylylation (Fig 3C), while having minimal effect on total GS specific activity relative to wild-type bacteria (S4 Fig). The strains also excreted between 0.1–1.5 mM of NH₃ after 24-h incubation in N₂-fixing conditions, whereas the wild-type and ΔglnE mutant did not excrete detectable levels of NH₃ (Fig 3D). Interestingly, we found that NH₃ excretion was sub-optimal when the Pnoda promoter controlling uAT expression was induced with 5 uM narinogenin (S5 Fig), suggesting that strong uAT overexpression is metabolically detrimental, as was observed in *A. brasilense* [44]. This indicated that more finely tuned uAT expression would be critical to achieve stringent control of GS adenylylation in AcLP.

**Shutdown of glutamine biosynthesis alleviates negative feedback on nitrogenase**

Expression of uAT restricts glutamine production via the high affinity GS-dependent NH₃ assimilation pathway, providing us with a unique opportunity to tease apart the effects of NH₃ and glutamine on the nitrogenase (*nif*) gene expression. We postulated that NH₃ must first be converted into glutamine to mediate repression of *nif* genes and tested this hypothesis first by examining expression of PnifiH fused to GFP on plasmid pOPS1213 in wild-type bacteria and in AcLPΔglnE expressing the uAT-Ac2 allele from the non-induced Pnoda promoter on a second plasmid. As expected, PnifiH::GFP activity in both strains grown under microaerobic conditions (3% O₂ in the headspace) was strongly repressed by supplementation with 10 mM glutamine however, while PnifiH::GFP was repressed in the wild-type by supplementation with 10 mM NH₄Cl, PnifiH::GFP expression was not repressed by NH₄Cl in AcLPΔglnE expressing uAT-Ac2 (Fig 4A). We observed a similar pattern when nitrogenase activity was assessed by ARAs (Fig 4B and 4C), indicating that
NH₃ itself has no effect on negative feedback regulation of nif genes but must be converted into glutamine or potentially other amino acids to facilitate repression. Engineering NH₃ excreting bacteria by targeted GS shutdown therefore has two advantages; i) alleviating negative feedback regulation of nif genes and ii) preventing NH₃ assimilation to favour release.

NifA control of uAT expression

As a direct consequence of engineering NH₃ excretion through GS interference, bacteria typically become auxotrophic for glutamine. While this may not be non-problematic for cultures grown in vitro under gnotobiotic conditions, glutamine auxotrophs in the field would be unable to compete or persist in the soil and rhizosphere. In rhizobia-legume symbioses, rhizobia only restrict NH₃ assimilation after infecting the low-oxygen environment of the nodule and differentiating into an N₂ fixing bacteroid [51,52], allowing them to maintain competitiveness during their free-living state in the soil. To mimic this oxygen-dependent regulation, we fused the uAT-Ac2 allele to native or synthetic RBSs and placed these under control of the NifA-inducible PnifH promoter on mini-Tn7 delivery plasmids, then integrated these into the chromosome of AcLPΔglnE, creating strains AcPU-RStd, AcPU-R1, AcPU-R22, AcPU-R31, AcPU-Rnat and AcPU-R28 (Fig 5A). When grown under aerobic (21% O₂) conditions in the presence of 10 mM NH₄Cl, growth of AcLPΔglnE expressing the uAT-Ac2 allele from the non-induced PnodA promoter was almost entirely abolished compared to where glutamine was
provided as a source of N (Figs 5B and S6). In contrast, the growth characteristics of strains expressing \textit{uAT-Ac2} from the \textit{PnifH} promoter were reminiscent of the wild-type \textit{Ac}, except for strains where \textit{uAT-Ac2} was fused to the strongest RBS' RStd or R1, which increased mean generation times (MGT) but did not affect the total biomass at stationary phase (Figs 5B and S6).

We next assessed GS regulation by \(\gamma\)-glutamyltransferase assays and confirmed that under aerobic conditions in the presence of 10 mM NH\(_4\)Cl, the percentage of active deadenylylated GS activity in strains \textit{Ac-PU-R1}, \textit{Ac-PU-R22}, and \textit{Ac-PU-R3} closely resembled that of the wild-type, suggesting that NH\(_3\) assimilation was functional. When grown under microaerobic conditions (3% O\(_2\)) in the presence or absence of 10 mM NH\(_4\)Cl, GS in wild-type \textit{Ac-LP} was activated by deadenylylation, whereas GS in all \textit{AcLP\(\Delta\)glnE} strains expressing \textit{uAT-Ac2} from the \textit{PnifH} promoter became more heavily inactivated by adenylylation under the same conditions (Fig 5C), with the percentage unadenylated GS activity correlating negatively with the strength of RBS fused to \textit{uAT-Ac2}. We finally performed NH\(_3\) excretion assays on the engineered strains and found that each excreted NH\(_3\) into the growth media after 24-h, except for where \textit{uAT-Ac2} was fused to the weakest RBS [R28] (Fig 5D). Overall, the data suggested that by expressing \textit{uATs} from the \textit{PnifH} promoter, GS shutdown could be controlled in response to atmospheric oxygen tension in a similar manner to the activation of N\(_2\)-fixation.

**Rhizopine-dependent control of nitrogen fixation, GS adenylylation and ammonia excretion**

While NifA-dependent expression of nitrogenase and \textit{uAT-Ac2} in \textit{Ac\(\Delta\)glnE} drives N\(_2\) fixation and GS inactivation leading to NH\(_3\) excretion, the lack of plant host-specific signalling to drive these processes could permit bacteria to supply NH\(_3\) to target crops and non-target weed...
species alike. We previously used synthetic rhizopine signalling to establish control of a mutant nifA allele (encoding NifA<sub>L94Q/D95Q</sub>) and rpoN in AcLPΔnifA carrying plasmid pSIN02, which drove partially NH<sub>3</sub>-resistant activation of nitrogenase activity specifically by bacteria occupying the roots of transgenic RhiP barley [47]. We performed NH<sub>3</sub> excretion assays on AcLPΔnifA carrying pSIN02 and found that this strain did not secrete NH<sub>3</sub> into the growth media (S7 Fig). Thus, we opted to establish rhizopine control of the nifA<sub>L94Q/D95Q</sub>-rpoN operon in our strain AcPU-R22 where uAT-Ac2 expression placed under control by NifA. We first tested in AcLP, induction of a new rhizopine receiver plasmid pSIR03 which was derived from the high-copy rhizopine receiver pSIR03 but carried an RK2 replicon for more stable low-copy maintenance. Using GFP induction assays, we demonstrated that pSIR03 (Fig 6A) has a dynamic range of 162-fold in response to the rhizopine scylo-inosamine (SI) and was induced
in 93.08 ± [SEM] 0.32% of cells in populations when 10 µM SI was supplemented in vitro (Fig 6B and 6C and S1 Table). We deleted the native nifA gene from strain AcPU-R22 and introduced a rhizopine nifA L94Q/D95Q -rpoN controller plasmid pSIN04 which was derived from pSIN03 (Fig 6D). Expression of nifA L94Q/D95Q -rpoN under microaerobic conditions by addition of 10 µM SI into the media resulted in tightly controlled activation of nitrogenase that was unimpeded by addition of 10 mM of NH₃ (Fig 6E). Moreover, GS was strongly adenylylated by addition of 10 SI to the media in both aerobic and microaerobic conditions (Fig 6F).

Because NifA in many diazotrophs is inactivated when cells are grown at 21% O₂ [53], we subsequently tested O₂ tolerance of our NifA L94Q/D95Q mutant protein by inducing expression of nifA L94Q/D95Q -rpoN in AcLPΔnifA carrying pSIN03 with rhizopine and monitoring activation of the PnifH::GFP promoter fusion (S8A Fig). Interestingly, the NifA L94Q/D95Q protein activated PnifH::GFP 13-fold ± [SEM] 1.5 and 98-fold ± [SEM] 2.8 under aerobic and microaerobic conditions, respectively (S8B Fig), suggesting that the protein is tolerant to oxygen.
We next assessed growth of our engineered strain AcPU-R22ΔnifA carrying pSIN04 where NH₄Cl was provided as a sole source of N (S9 Fig). As expected, growth was strongly inhibited in the presence of 10 μM SI, indicating that the strain was unable to assimilate NH₃ in this state. When grown in the absence of nitrogen under N₂-fixing conditions, the strain excreted 812.58 ± [SEM] 5.59 μM OD₆₀₀nm⁻¹ NH₃ into the media after 24-h incubation at an optimal rate of 65.13 ± 7.35 μM OD₆₀₀nm⁻¹ h⁻¹ (Fig 6G). These experiments confirmed that we had established tight rhizopine control of N₂-fixation, GS adenylylation and NH₃ excretion in our engineered AcLP strain.

Discussion

In this study, we employed two strategies to interfere with GS and stimulate NH₃ excretion in AcLP. For our first strategy, we attempted to recapitulate previous experiments where insertional inactivation of the Pₙ genes glnB and glnK stimulated shutdown of GS by adenylylation and alleviated negative feedback inhibition of nitrogenase by the product NH₃, preventing NH₃ assimilation and favouring excretion into the growth media [29]. Although we could delete either of the glnB or glnK genes from AcLP, we were unable to delete both genes in the same strain unless a second copy of the omega interposon into the internal coding sequence and was therefore more likely to have abolished the function of the protein [54]. Interestingly, similar glnB and glnK antibiotic cassette insertions have been made in the phototrophic diazotroph Rhodobacter capsulatus, resulting in NH₃-insensitive NifA and nitrogenase expression and activity [55]. However, attempts to delete both genes were also unsuccessful in this bacterium [56]. Regardless of why deleting glnB and glnK is lethal, reproducing exact copies of the original glnB and glnK mutants [29] would likely be required to establish control of NH₃ excretion in AcLP, as we have shown here that deletion of glnK paired with strong repression of glnB had minimal effect on nitrogenase or GS regulation and permitted only low-level NH₃ excretion.

As was previously demonstrated in A. brasilense [30], expression of E. coli or Ac-derived uATs in our AcLPglnE mutant resulted in strong GS shutdown and high rates of NH₃ excretion when grown in N₂-fixing conditions. We also found that while nitrogenase expression and activity is repressed in microaerobic NH₃ or glutamine-fed cultures of AcLP, shutdown of glutamine biosynthesis by uAT expression resulted in nitrogenase expression that was unpinned by NH₃ but still repressed by glutamine, suggesting that NH₃ must first be converted to glutamine or potentially other amino acids such as asparagine [57] to facilitate repression. This same effect was previously reported in phototrophic Anabaena spp [58,59] and Rhodobacter sphaeroides [60] where GS activity was shutdown using the chemical inhibitor L-Methionine sulfoximine, and in Klebsiella pneumoniae mutants unable to grow on NH₃ as a sole source of N [61]. Moreover, In R. capsulatus, where N₂-fixation is repressed in response to added NH₃ at three levels; a) NtrC-dependent transcription of nifA; b) NifA-dependent transcription of nitrogenase; and c) DraT-DraG-dependent ADP ribosylation of nitrogenase [62,63]; all three levels of regulation were non-responsive to NH₃ following shutdown of GS by insertional inactivation of both Pₙ genes [55]. Thus, it seems plausible that shutdown of glutamine biosynthesis from NH₃ and glutamate abolishes NH₃-dependent regulation of N₂-fixation in genetically diverse bacteria. Targeted GS shutdown therefore affects NH₃ excretion on two fronts,
allowing sustained nitrogenase expression and activity in the presence of fixed N₂ and preventing assimilation of NH₃, favouring excretion into the environment.

Without establishing control of GS shutdown, engineered NH₃ excreting diazotrophs are typically auxotrophic for glutamine, which would render them non-competitive in the environment [14,17]. Here, we placed expression of the uAT-Ac2 allele under control of the NifA-inducible nitrogenase promoter PnifH which, when tuned correctly, triggered GS shutdown and NH₃ excretion specifically under N₂-fixing conditions. In the field, this could allow bacteria to retain competitiveness prior to forming oxygen-deplete biofilms on the surface of roots [64], however lack of host-specific control could permit provision of NH₃ to non-target plant species. Thus, we further modified the engineered strain AcPU-R22 by deleting nifA and bringing the mutant nifA_L94Q/D95Q and rpoN alleles under rhizopine-inducible control, permitting in vitro rhizopine-dependent activation of nitrogenase activity, GS shutdown and NH₃ excretion. In future, we aim to further demonstrate activation of these processes by the bacteria colonising the roots or rhizosphere of transgenic rhizopine producing (RhiP) barley [48], though we acknowledge that this will first require optimisation of rhizopine perception by AcLP carrying a rhizopine biosensor plasmid. At present, current rhizopine biosensors only permit perception of rhizopine by 10–25% of cells colonising RhiP barley roots, and in congruency, activation of in situ nitrogenase activity in these populations amounts to approximately 15% of that observed in wild-type AcLP cells colonising wild-type barley [47]. In addition to improving rhizopine perception, developing strategies to stabilise function of the engineered genes in situ will be crucial to generate a practical interaction between the bacteria and plants.

Although we have demonstrated controlled activation of N₂ fixation and NH₃ excretion in response to rhizopine, it remains likely that increased energy demand in this state might be detrimental to viability and competitiveness on the root surface [15]. Rhizobia overcome this problem by engaging in stringent signalling with the legume that permits partner-specific infection of nodules [65]. Inside the nodule, the bacteria are provided with low-oxygen conditions conducive to nitrogenase stability, they can escape the fierce competition of the rhizosphere, and are fed carbon in the form of dicarboxylates [51,52]. Engineering a nodule-like niche with stringent entry requirements into cereals will likely be important to maximise the effectiveness of inoculation with engineered NH₃ excreting inoculants. The strains developed here could be adapted for entry of such an environment and therefore, this work represents significant advancement towards the development of both associative and more intimate “synthetic N₂-fixing symbiosis” with cereals.

**Materials and methods**

**Bacterial strains and plasmids**

Bacteria used in this study (S1 File) were cultured in TY [66] or UMS [67,68] media supplemented with 300 μM nicotinic acid and 20 mM succinate as previously described [47]. Plasmids (S2 Table) were constructed using HiFi assembly (New England Biolabs) or BEVA modular golden-gate assembly [69,70] as outlined in the S1 File and were mobilised into Azorhizobium by diparental mating with *E. coli* ST18 [71]. For mini-Tn7 integration into the chromosome, tri-parental matings were used to additionally mobilise the transposase helper plasmid pTNS3, which carries an R6K origin of replication that is not maintained in *Azorhizobium* [72].

Gene deletion and replacement mutant strains were constructed by mobilising the relevant suicide plasmid, derived from pK19mobSacB (S2 Table and S1 File), into the target strain and selecting for single-crossover integration into the chromosomal region of interest by plating cells on selective UMS or TY agar media supplemented with 100 μg mL⁻¹ kanamycin. Single-crossover mutants were subsequently grown in non-selective media until stationary phase and
plated in serial dilutions onto UMS or TY agar supplemented with 10% (v/v) sucrose to select for double crossover deletion or replacement of the target gene. For the ΔglnK::ΩSp replacement plasmid pOPS1564 only, 100 μg mL⁻¹ spectinomycin and 1 mM IPTG was added to the media unless otherwise stated. Single colonies were patched onto the same media used for double-crossover selection plus and minus 100 μg mL⁻¹ kanamycin and kanamycin sensitive colonies were screened by PCR and sanger sequencing for deletion or replacement of the target gene.

All AcLP ΔglnB ΔglnK::ΩSp mutant strains were constructed by first deleting glnB from AcLP using plasmid pOPS1691, then subsequently integrating the ΔglnK::ΩSp replacement plasmid pOPS1564 into the target chromosomal region by single-crossover. Because replacement of ΔglnK::ΩSp was not possible on three separate occasions, mini-Tn7 delivery plasmids carrying an IPTG-derepressible copy of glnB (S1 File) were integrated into the engineered attB site prior to selecting for selecting for double-crossover replacement of glnK with the ΩSp interposon as described above.

Growth curves

Growth curves were performed in triplicate by streaking single colonies onto 10 mL TY agar slopes and incubating for 3-days prior to three washes in PBS and inoculation at OD600λ⁢nm 0.01 into 500 μL UMS media in 24-well plates. The OD600λ⁢nm was monitored at 20 min intervals in an Omega FLUOstar plate reader set to shake cultures at 700 rpm at 37˚C until stationary phase. Growth statistics were calculated using the R package GrowthCurver [73].

GS transferase assays

Six-millilitre UMS cultures were initially grown in 30 mL glass universal vials sealed with silicone rubber septa as described for RT-qPCR experiments. After 3-h or 24-h incubation, 1 mL of culture was sampled for protein determination using a Millipore BCA protein assay kit. Five hundred microlitres of CTAB (1 mg mL⁻¹) was added to the remaining cultures which were incubated at room temperature for a further 3 mins prior to harvesting by centrifugation at 4˚C. Cells were washed once with 5 mL 1% (w/v) KCL and finally resuspended in 500 μL of the same buffer and stored on ice. GS transferase assays were performed on 50 μL aliquots the permeabilized cells as previously described [16]. The assays were performed in 500 μL total volumes with 30 min incubation in the presence or absence of 60 μM added MgCl₂ to determine the total GS transferase activity and the activity of the “active” unadenylated enzyme, respectively [49]. The GS transferase buffer was adjusted to pH 7.0, as this was previously estimated as the iso-activity point for Ac [74]. Following addition of the FeCl₃ stop reagent, reaction tubes were centrifuged for 5 min at 13,000 g and 200 μL was transferred to clear, flat bottomed 96-well plates for spectrophotometric quantification of the product L-Glutamyl-γ-Hydroxamate (LGH) at 562λ⁢nm in a Promega GloMax multi-detection system.

Acetylene reduction assays

Cultures for ARAs were prepared and analysed as previously described [47, 75] and 1 mL samples of the headspace atmosphere were analysed using a PerkinElmer Clarus 480 gas chromatograph equipped with a HayeSep N (80–100 MESH) 584 column at 3-h, 5-h, 21-h, 23-h and 25-h incubation, unless otherwise stated.

Ammonia excretion assays

Three-millilitre UMS cultures were initially grown in 30 mL glass universal vials sealed with silicone rubber septa as described for RT-qPCR experiments. OD600λ⁢nm was recorded and
NH$_3$ was quantified in spent supernatants using the spectrophotometric indophenol assay as previously described [16]. A calibration curve was performed for each experiment using freshly made dilutions of NH$_4$Cl in UMS ranging from 5 μM–1 mM. Absorbance of indophenol blue was quantified in a Genesys 150 UV visible spectrophotometer (Thermo Scientific) at 652 nm after 4-h incubation at room temperature.

**RT-qPCR**

For RT-qPCR experiments, $n = 5$ single colonies were streaked onto 10 mL UMS agar slopes supplemented with 20 mM succinate, 10 mM NH$_4$Cl and 300 μM nicotinate and grown for 2-days at 37°C. Cells were washed three times from the slopes with PBS, resuspended in UMS supplemented with the relevant carbon and N sources at OD600=0.3 in 30 mL glass universal vials and transferred with the lid off into a sealed atmosphere cabinet adjusted to 3% O$_2$ by flushing with N$_2$ gas. After 30 min, cultures were sealed with silicone rubber septa and incubated at 37°C with rigorous shaking for 3-h. Cells were next harvested by centrifugation at 4°C, lysed using a FastPrep-24 5G instrument and cellular debris was removed by a second round of centrifugation. RNA was extracted from the resulting lysate using a Qiagen RNeasy extraction kit and tested for quality and purity using an Agilent Experion Bioanalyzer with RNA Stdsens chips. gDNA was depleted from RNA by treatment with Invitrogen Turbo DNase as per the manufacturer’s recommendations and 5 μg was used to generate cDNA using an Invitrogen SuperScript IV reverse transcriptase kit as per the manufacturer’s recommendations. The final cDNA template was diluted 1:20 with water and 1 μL was added to each 20 μL RT-qPCR reaction prepared in 96-well plates with Applied Biosystems PowerUp SYBR green master mix. Reactions were run using an Applied Biosystems ViiA 7 Real-Time PCR system. RT-qPCR primers were initially tested for amplification efficiency and target specificity by generating a standard curve of amplification with 5-fold dilutions of AcLP gDNA. The housekeeping gene primer targeted recA and was validated previously [76], whereas the glnA primers designed here had the following sequence glnA F 5’- CCGCTGACCAA CTCCTACA glnA R 5’- CCATGAACAGGGCCGAGA.

**GFP reporter assays and flow-cytometry**

GFP reporter assays and flow-cytometry experiments were performed on 24-h incubated cultures as previously described [47]. Inducers were added directly to the growth media at the time of inoculation where relevant.

**Supporting information**

**S1 Fig. Characterisation of synthetic ribosome binding sites in AcLP.** Each RBS was fused to GFP under expression by the strong synthetic promoter J23104 on plasmid pOGG024 and GFP was measured after 24-h incubation in UMS media ($n = 3$). Relative luminescence units are defined here as GFP fluorescence/OD600 nm. The RBS nucleotide sequences are provided in S1 File.

(TIF)

**S2 Fig. Expression and total activity of GS is elevated in AcRGl.** (a) Total specific activity of both adenylated (inactive) and unadenylated (active) forms of GS was measured in whole cells grown for 24-h as determined by γ-glutamyl transferase assays ($n = 5$). (b) glnA expression was quantified relative to the housekeeping gene recA by RT-qPCR in cells growth for 3-h. All cultures for assays were grown in N$_2$-fixing conditions (N-free UMS media with 3% O$_2$ in the headspace). Error bars represent one SEM. Independent two-tailed students t-tests were used.
to compare means. ***P < 0.001.

(S3 Fig) Induction of the *Sinorhizobium meliloti* 1021 naringenin-inducible P*nodA* promoter in AcLP (a) Genetic schematic (not to scale) of the low-copy (RK2 replicon) naringenin-inducible GFP reporter plasmid pOPS1536. (b) GFP induction in AcLP (n = 3) harbouring pOPS1536 in response to naringenin supplemented *in vitro*. Relative luminescence units are defined here as GFP fluorescence/OD600nm.

(S4 Fig) Total activity of GS in Δ*glnE* mutants expressing uATs from the non-induced P*nodA* promoter (a) Total specific activity of both adenylated (inactive) and unadenylated (active) forms of GS was measured in whole cells grown in N2-fixing conditions (N-free UMS media with 3% O2 in the headspace) for 3-h as determined by γ-glutamyl transferase assays (n = 5 for wild-type AcLP or n = 3 for other strains). Error bars represent one SEM. Independent two-tailed students t-tests were used to compare means against the wild-type (WT) AcLP as a reference. Not significant (ns) indicates P > 0.05, *P < 0.05.

(S5 Fig) Ammonia excretion is suboptimal in Δ*glnE* mutants expressing uATs from the P*nodA* promoter induced with naringenin. Spectrophotometric determination of NH3 in media of cultures induced with 5 μM naringenin grown for 24-h in N2-fixing conditions (N-free UMS media with 3% O2 in the headspace). Error bars represent one SEM. n = 3 for wild-type AcLP ΔglnE or n = 6 for other strains.

(S6 Fig) Growth statistics for control strains and Δ*glnE* mutants expressing uATs. Mean generation times and the max OD600nm (i.e. the carrying capacity, k) were calculated from standard curves of cultures grown in UMS media at 21% O2. Strains highlighted in white are wild-type (WT) AcLP and AcLP ΔglnE controls, strains highlighted in pink are AcLP ΔglnE carrying P*nodA* [RBS] uAT-DT16 modules on parent plasmid pOGG093 and strains highlighted in blue are AcLP ΔglnE carrying mini-Tn7 integrated P*nifH* [RBS] uAT-Ac2-DT16 modules.

(S7 Fig) Rhizopine control of nitrogen fixation alone does not permit ammonia excretion. Spectrophotometric determination of NH3 in media of n = 3 cultures grown for 24-h in N2-fixing conditions. Error bars represent one SEM. Strain *Azospirillum brasilense* HM053 was used here as a positive control.

(S8 Fig) NifA*L94Q/D95Q* activity is tolerant to ambient environmental oxygen tensions. (a) Genetic schematic (not to scale) of the rhizopine nifA*L94Q/D95Q*-rpoN controller plasmid with P*nifH*::GFP reporter fusion pSIN03. (b) P*nifH* promoter activity was measured in n = 4 cultures grown for 24-h under the conditions indicated. Relative fluorescence units (RFU) are defined here as GFP fluorescence/OD600nm. Error bars represent one SEM. Independent two-tailed students t-tests with Bonferroni-holm adjustment were used to compare means. P > 0.05. **P < 0.01, ***P < 0.001.

(S9 Fig) Growth statistics for AcPU-R22Δ*nifA* carrying pSIN04. Growth of treatment and control strains was assessed in UMS media supplemented with 10 mM NH4Cl as a sole source of N and in the presence of absence of 10 μM of the rhizopine *scilho*-inosamine (SI) (a) Growth
curves are representative of $n = 3$ replicates per treatment condition. (b) Mean generation times (MGTs) were calculated from the growth curves using Growthcurver [73]. Error bars represent one SEM. Independent two-tailed students t-tests were used to compare means. Exact P values are provided where P > 0.05. *P < 0.05.

(TIF)

S1 Table. Flow-cytometry statistics for rhizopine-inducible GFP expression in AcLP carrying pOPS1052.

(DOCX)

S2 Table. Plasmids used in this study.

(DOCX)

S1 File. Bacterial strains, golden-gate pieces, plasmid construction and oligonucleotides.

(XLSX)

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