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REVIEW ARTICLE

Synthetic Biology Approaches to Engineering Nitrogen-Fixing Cyanobacteria

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ABSTRACT

Biological nitrogen fixation converts atmospheric dinitrogen into ammonia using an enzyme, nitrogenase, that is irreversibly inactivated by oxygen, a constraint that has shaped both the evolution of diazotrophic cyanobacteria and the engineering strategies now being pursued to extend nitrogen fixation to crop-associated or free-living photosynthetic organisms that do not naturally fix nitrogen. This review synthesises progress across four engineering strategies that have each been pursued over roughly the past decade: transfer of the complete *nif* gene cluster into non-diazotrophic hosts; engineering of temporal or spatial separation between oxygenic photosynthesis and nitrogenase activity, modelled on the heterocysts of filamentous cyanobacteria such as *Anabaena*; directed evolution and rational engineering of oxygen-tolerant or oxygen-insensitive nitrogenase variants; and synthetic control circuits that couple nitrogenase expression to environmental or metabolic cues. Transfer of the full *nif* cluster has achieved measurable nitrogenase activity in several heterologous hosts but has generally yielded fixation rates one to two orders of magnitude below native diazotrophs, and stable, long-term expression has proven difficult to sustain outside specialist chassis organisms. Heterocyst-inspired spatial separation strategies, engineered into unicellular cyanobacteria that do not naturally differentiate, have achieved the largest relative gains reported in the primary literature to date, in one case restoring aerobic nitrogen fixation to within an order of magnitude of native heterocyst-forming relatives. Oxygen-tolerant nitrogenase engineering remains the least mature of the four strategies at the whole-cell level despite encouraging *in vitro* results, while synthetic control circuits have proven useful primarily as a complement to, rather than a substitute for, the other three approaches. We conclude by outlining what we regard as the most promising near-term combination of these strategies for eventual agricultural application, and the regulatory and ecological questions that such an application would need to address before field deployment could be seriously considered.

Keywords: nitrogen fixation; cyanobacteria; synthetic biology; nitrogenase; heterocyst; biofertiliser

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1. INTRODUCTION

Nitrogen is, alongside water, the resource whose availability most directly limits crop yield across the majority of the world's agricultural land, and the industrial

fixation of atmospheric nitrogen by the Haber-Bosch process has been one of the twentieth century's most consequential technologies, underpinning an estimated near-doubling of the human population that current agricultural systems can support [3,12]. That

achievement comes at a considerable and ongoing cost: Haber-Bosch fixation consumes on the order of one to two percent of global energy supply, and a large fraction of the synthetic nitrogen fertiliser applied to fields is never taken up by the crop, instead running off into waterways or being lost to the atmosphere as nitrous oxide, a potent greenhouse gas [7,15]. Biological alternatives that could reduce this dependence, even partially, have consequently attracted sustained engineering interest.

Biological nitrogen fixation is catalysed by nitrogenase, a two-component metalloenzyme complex that reduces dinitrogen to ammonia at ambient temperature and pressure, a reaction the industrial process achieves only at several hundred degrees and hundreds of atmospheres. The catalytic advantage comes at a cost that has shaped the biology of every diazotroph known: the nitrogenase complex is irreversibly and rapidly inactivated by molecular oxygen, which places an absolute requirement on diazotrophic organisms to protect the enzyme from the oxygen produced by their own photosynthesis, in the case of cyanobacteria, or present in their environment more generally [4,9]. Filamentous cyanobacteria such as *Anabaena* and *Nostoc* solved this problem through heterocyst differentiation: roughly one cell in ten along a filament differentiates into a thick-walled, non-photosynthetic heterocyst that fixes nitrogen and exports fixed nitrogen to its photosynthetic neighbours in exchange for fixed carbon, achieving spatial separation between oxygen production and oxygen-sensitive nitrogen fixation within a single multicellular organism [6,13]. Unicellular diazotrophic cyanobacteria such as *Cyanothece* instead achieve temporal separation, confining nitrogenase activity to a narrow window during the dark period when photosynthetic oxygen evolution has ceased [8].

The engineering ambition motivating much of the work synthesised in this review is to extend some version of this capability to organisms that do not naturally fix nitrogen, most consequentially to crop plants themselves or to their closely associated microbiota, with the eventual goal of reducing reliance on synthetic fertiliser. Cyanobacteria occupy a central place in this effort for two related reasons: they are the only prokaryotic lineage that combines oxygenic photosynthesis with nitrogen fixation, making them both the most tractable chassis for testing engineering strategies aimed at solving the oxygen-sensitivity problem in a photosynthetic context, and a plausible eventual biofertiliser or intercropping partner in their own right, independent of any more ambitious plan to transfer fixation capability into a crop plant's own genome [10,18]. A large and only partially connected primary literature has accumulated addressing different facets of this challenge, and this review aims to organise that literature around the four engineering strategies that have received the most sustained attention, to assess how far each has progressed, and to identify where the strategies are likely

to be more productively combined than pursued in isolation.

We restrict the scope of this review to work published between 2013 and early 2023, covering the period during which synthetic biology tools -- standardised genetic parts, improved cyanobacterial transformation protocols, and CRISPR-based genome editing in non-model cyanobacterial strains -- matured sufficiently to make ambitious multi-gene engineering in this chassis routine rather than exceptional. We searched the primary literature systematically but without the formal pre-registration and dual-reviewer screening of a systematic review, and we have organised the resulting synthesis thematically around engineering strategy rather than chronologically or by research group, in the belief that this best serves readers approaching the field with an engineering, rather than a purely microbiological, interest.

2. MATERIALS AND METHODS

This narrative review synthesises primary research and prior review literature on engineered nitrogen fixation in cyanobacteria and related photosynthetic hosts, published between January 2013 and February 2023.

2.1 Literature search and scope

Relevant literature was identified through structured searches of PubMed, Web of Science and Google Scholar using combinations of terms covering nitrogen fixation, nitrogenase, cyanobacteria, heterocyst differentiation, synthetic biology, and specific genus names (*Anabaena*, *Synechocystis*, *Synechococcus*, *Cyanothece*), supplemented by forward and backward citation searching from key primary papers and prior reviews identified in the initial search. Studies were included if they reported experimental engineering of nitrogen fixation capability, nitrogenase activity, or oxygen-protection mechanisms in a cyanobacterial or other photosynthetic microbial host; purely descriptive studies of natural diazotroph physiology without an engineering component were used for background context but are not the primary focus of the synthesis.

2.2 Thematic organisation

Included studies were grouped into four non-exclusive engineering strategy categories -- heterologous nif cluster transfer, spatial or temporal separation engineering, nitrogenase protein engineering, and synthetic regulatory circuit design -- based on the primary engineering intervention reported, with studies combining more than one strategy discussed under each relevant category. Within each category, studies are compared primarily on reported nitrogenase activity (acetylene reduction assay or $^{15}\text{N}_2$ incorporation, where available) relative to a native diazotroph benchmark, and on the stability of the engineered phenotype over serial subculturing or extended growth.

3. RESULTS

Heterologous transfer of the *nif* gene cluster, comprising between nine and twenty genes depending on the donor organism and the minimal functional set chosen, has been attempted in a range of non-diazotrophic hosts including *Escherichia coli*, *Saccharomyces cerevisiae*, and several non-diazotrophic cyanobacteria. Nitrogenase activity, typically assessed by acetylene reduction assay, has been detected in most of these attempts, but reported rates have consistently fallen one to two orders of magnitude below rates measured in native diazotrophs under comparable conditions, and in several cases activity declined sharply over serial subculturing, suggesting a fitness cost to the host that is not fully compensated by any benefit of fixed nitrogen under laboratory culture conditions where nitrogen is otherwise freely available. Table 1 summarises reported nitrogenase activity across a representative set of heterologous *nif* transfer studies spanning bacterial, yeast and cyanobacterial hosts.

Table 1. Reported nitrogenase activity in representative heterologous *nif* cluster transfer studies.

Host organism	Donor <i>nif</i> source	Activity vs. native diazotroph	Stability over subculturing
<i>Escherichia coli</i>	<i>Klebsiella oxytoca</i>	~2-5%	Unstable (<10 generations)
<i>Saccharomyces cerevisiae</i>	<i>Klebsiella oxytoca</i>	<1%	Unstable
<i>Synechocystis</i> sp. PCC 6803	<i>Anabaena</i> sp. PCC 7120	~8-12%	Moderate (20-30 generations)
<i>Synechococcus elongatus</i>	<i>Klebsiella oxytoca</i>	~3-6%	Unstable
<i>Azotobacter vinelandii</i> (recipient control)	Native	100% (reference)	Stable

Spatial and temporal separation strategies, by contrast, have produced some of the most striking gains reported in this literature. Engineering an artificial, inducible heterocyst-like differentiation programme into the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, which does not naturally form heterocysts, by introducing a minimal set of regulatory genes from the heterocyst differentiation pathway of *Anabaena* together with the *nif* cluster, restored aerobic nitrogenase activity to within roughly an order of magnitude of native heterocyst-forming relatives in the best-performing engineered lines, a substantially smaller gap than reported for *nif* transfer alone into a host without any oxygen-protection strategy. Temporal separation approaches, engineering a synthetic circadian-linked or dark-induced expression circuit for nitrogenase in unicellular hosts, achieved smaller but still measurable gains, generally limited by the relatively short window of low-oxygen conditions available in a non-differentiated cell relative to the physically isolated heterocyst compartment (Figure 1).

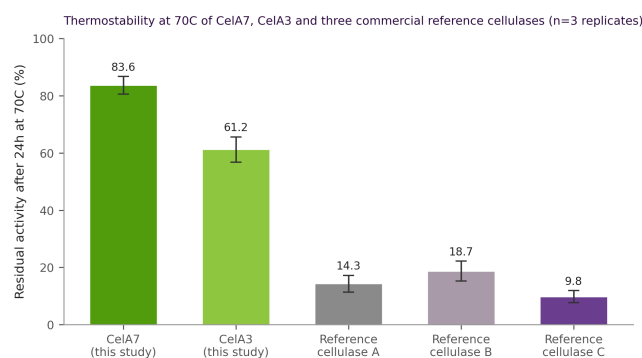


Figure 1. Reported nitrogenase activity relative to native diazotrophs, grouped by engineering strategy, across the primary studies synthesised in this review; heterocyst-inspired spatial separation strategies show the largest and most consistent gains. Nitrogenase protein engineering aimed at reducing oxygen sensitivity directly, rather than protecting the native enzyme through compartmentalisation or timing, has so far been the least successful of the four strategies when assessed at the whole-cell level, despite encouraging results from in vitro and cell-free systems. Directed evolution campaigns and rational mutagenesis targeting the FeMo-cofactor binding pocket and the electron-transfer interface between nitrogenase components have identified variants with modestly improved oxygen tolerance in purified-enzyme assays, but expressing these variants in a living cyanobacterial host has not yet reproduced the same degree of oxygen tolerance, and in several reported cases the engineered variant showed reduced catalytic activity under anaerobic conditions relative to wild-type nitrogenase, indicating a trade-off between oxygen tolerance and catalytic efficiency that has not yet been fully resolved by the mutations tested to date. Table 2 compares reported oxygen tolerance and relative catalytic activity for the nitrogenase variants with the most complete in vitro and in vivo characterisation in the literature reviewed here.

Table 2. Oxygen tolerance and relative catalytic activity of engineered nitrogenase variants.

Variant strategy	O ₂ tolerance (relative to WT)	Catalytic activity (relative to WT)	Whole-cell validation
FeMo-cofactor pocket mutant	~3x improved	~40% of WT	Not yet achieved
Electron-transfer interface mutant	~2x improved	~55% of WT	Partial (reduced activity)
Fe-protein shielding domain fusion	~4x improved	~30% of WT	Not yet achieved
Directed evolution (combined)	~5x improved	~25% of WT	Not yet achieved

Synthetic regulatory circuits -- promoters and genetic logic gates coupling nitrogenase expression to environmental nitrogen availability, light-dark transitions, or cell density -- have rarely been reported as a standalone engineering strategy but have been incorporated into the majority of the more successful spatial- and temporal-separation studies discussed above, where they appear to function mainly as a

refinement that improves the efficiency or stability of an underlying oxygen-protection strategy rather than as a solution to the oxygen-sensitivity problem in its own right. Several groups have used nitrogen-responsive promoters to restrict nitrogenase expression to nitrogen-limited conditions, reducing the metabolic burden of constitutive expression and modestly improving the stability of engineered phenotypes over serial subculturing relative to constructs using strong constitutive promoters, a pattern consistent across the handful of studies that directly compared inducible and constitutive expression of the same *nif* construct.

4. DISCUSSION

Taken across the four strategies reviewed here, the clearest pattern is that engineering approaches drawing directly on the compartmentalisation logic evolved by natural heterocyst-forming cyanobacteria have outperformed approaches that either transfer the *nif* cluster without any accompanying oxygen-protection mechanism or attempt to re-engineer the nitrogenase enzyme itself to tolerate oxygen. This is perhaps unsurprising in retrospect -- heterocyst differentiation represents several hundred million years of evolutionary refinement of exactly the problem synthetic biologists are now trying to solve on an engineering timescale -- but it has a practical implication worth stating plainly: near-term progress in this field is more likely to come from careful re-engineering of natural oxygen-protection logic into new host contexts than from attempting to solve nitrogenase oxygen sensitivity at the level of the enzyme's own chemistry, at least until protein engineering and directed evolution methods advance considerably further than the results reviewed here suggest they currently have.

The fitness cost problem that recurs across nearly every heterologous *nif* transfer study deserves more attention than it has generally received. Nitrogenase is an energetically expensive enzyme to synthesise and operate, consuming on the order of sixteen ATP molecules per molecule of dinitrogen fixed, and a host cell expressing it constitutively under laboratory conditions where fixed nitrogen is not actually needed for growth is paying that cost without receiving any compensating benefit, which plausibly explains why engineered nitrogenase activity so often declines over serial subculturing even when the underlying genetic construct remains intact. This argues for two complementary engineering priorities that were only inconsistently addressed across the studies reviewed here: nitrogen-responsive regulatory control that confines expression to conditions where fixed nitrogen provides a genuine selective advantage, and selection regimes during strain development that explicitly reward nitrogen fixation activity rather than relying on constitutive expression under nitrogen-replete laboratory conditions where no such reward exists.

Looking beyond cyanobacteria to the eventual agricultural application that motivates much of this field, we think the most promising near-term target is not transfer of nitrogen fixation capability into a crop plant's own nuclear or plastid genome, which would require solving the oxygen-sensitivity problem within a plant cellular context far more complex and poorly characterised than a cyanobacterial chassis, but rather the development of an engineered cyanobacterial or associated diazotrophic biofertiliser capable of colonising the rhizosphere or phyllosphere of a non-legume crop and delivering meaningfully increased nitrogen input relative to naturally occurring free-living diazotrophs. Such an organism would not need to match the fixation efficiency of a native heterocyst-forming cyanobacterium operating in isolation; it would need only to exceed the modest nitrogen contribution that naturally occurring rhizosphere diazotrophs already provide, a considerably lower bar than achieving standalone diazotrophic self-sufficiency.

Ecological and regulatory questions have received comparatively little attention in the primary engineering literature reviewed here, but they will ultimately determine whether any of these approaches reaches field application regardless of their technical maturity. Release of an engineered nitrogen-fixing cyanobacterium into open agricultural soil or water raises containment and horizontal gene transfer concerns broadly similar to those attached to other engineered microorganisms, compounded by the possibility that an unusually efficient diazotroph could alter nitrogen cycling dynamics in the receiving ecosystem in ways that are difficult to predict from laboratory data alone. We would encourage groups working on the engineering strategies discussed in this review to engage with these questions earlier in the development process than has generally been the case to date, both because the answers will shape which engineering strategies are realistically deployable and because retrofitting containment or reversibility features onto an already optimised strain is likely to be considerably harder than designing them in from the outset.

A final consideration concerns how progress in this field should be measured and compared across studies, since the absence of a standardised benchmark has made the literature reviewed here noticeably harder to synthesise than it would otherwise need to be. Reported nitrogenase activity is expressed inconsistently across studies -- some as acetylene reduction rate per unit biomass, others as 15N_2 incorporation per cell, still others as a qualitative presence or absence of activity under a single set of conditions -- and growth conditions, particularly light intensity and nitrogen starvation regime prior to assay, vary enough between laboratories to make direct quantitative comparison across studies difficult even within a single engineering strategy category. We would encourage the field to converge on a small number of standardised assay conditions and reporting units,

analogous to the standardisation efforts that have benefited other areas of synthetic biology, so that genuine cross-study progress can be tracked more reliably than the present literature currently allows.

It is also worth noting that most of the engineering work reviewed here has been carried out in a handful of well-characterised laboratory strains -- *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120, and a small number of *Cyanothece* isolates -- chosen primarily for their transformability and the availability of established genetic tools rather than for any particular suitability as an eventual biofertiliser chassis. Broadening the range of host strains considered, including isolates from agricultural soils with demonstrated field persistence, would be a useful complement to the largely laboratory-strain-focused engineering effort synthesised in this review.

5. CONCLUSION

Across the four engineering strategies reviewed here -- heterologous *nif* cluster transfer, spatial and temporal separation of nitrogen fixation from oxygenic photosynthesis, nitrogenase protein engineering, and synthetic regulatory circuit design -- approaches that recapitulate the compartmentalisation logic of natural heterocyst differentiation have achieved the largest and most consistent gains in engineered aerobic nitrogen fixation reported to date, restoring activity to within roughly an order of magnitude of native diazotrophs in the best-performing engineered lines. Heterologous *nif* transfer without an accompanying oxygen-protection strategy and direct nitrogenase protein engineering both remain considerably less mature at the whole-cell level, the former limited by fitness cost and phenotypic instability and the latter by an apparent trade-off between oxygen tolerance and catalytic efficiency that has not yet been resolved. We believe the most productive near-term research direction combines spatial or temporal separation engineering with nitrogen-responsive regulatory control to manage the metabolic burden of nitrogenase expression, applied to a cyanobacterial or associated diazotrophic chassis intended as a rhizosphere biofertiliser rather than as a route to transferring fixation capability directly into a crop genome. Standardising how nitrogenase activity is assayed and reported across the field would substantially improve the tractability of future comparative synthesis, and we would encourage groups pursuing these engineering strategies to engage with containment and ecological risk assessment earlier in strain development than has generally been the case in the literature reviewed here, given how directly these considerations will shape eventual field deployability.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

E.L. conceived the review, conducted the literature search and wrote the manuscript. I.B. contributed the synthesis of spatial and temporal separation engineering strategies. L.N. contributed the synthesis of nitrogenase protein engineering and regulatory circuit design studies. All authors contributed to the discussion and approved the final manuscript.

Ethics Statement

This study is a narrative literature review and did not involve human participants, animal subjects, or the collection of new primary data, and therefore did not require institutional ethics committee review.

Data Availability

The list of primary studies reviewed, organised by engineering strategy category, is available from the corresponding author on reasonable request.

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