Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Use of plant colonizing bacteria as chassis for transfer of N_2-fixation to cereals

Barney A Geddes[1], Min-Hyung Ryu[2], Florence Mus[3], Amaya Garcia Costas[3], John W Peters[3], Christopher A Voigt[2] and Philip Poole[1]

Engineering cereal crops that are self-supported by nitrogen fixation has been a dream since the 1970s when nitrogenase was transferred from *Klebsiella pneumoniae* to *Escherichia coli*. A renewed interest in this area has generated several new approaches with the common aim of transferring nitrogen fixation to cereal crops. Advances in synthetic biology have afforded the tools to rationally engineer microorganisms with traits of interest. Nitrogenase biosynthesis has been a recent target for the application of new synthetic engineering tools. Early successes in this area suggest that the transfer of nitrogenase and other supporting traits to microorganisms that already closely associate with cereal crops is a logical approach to deliver nitrogen to cereal crops.

Addresses
[1] Department of Plant Sciences, Oxford University, Oxford OX1 3RB, United Kingdom
[2] Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[3] Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT, USA

Corresponding author: Poole, Philip (philip.poole@plants.ox.ac.uk)

Current Opinion in Biotechnology 2015, **32**:216–222

This review comes from a themed issue on **Plant biotechnology**

Edited by **Inge Broer** and **George N Skaracis**

http://dx.doi.org/10.1016/j.copbio.2015.01.004

0958-1669/

Introduction

Nitrogen is one of the primary nutrients limiting plant growth in agriculture. Despite its prevalence in the Earth's atmosphere, most nitrogen exists as biologically inaccessible N_2. Legumes have been used for hundreds of years to incorporate nitrogen into cropping systems without fertilization. This is accomplished through symbiotic interactions with diazotrophs called rhizobia, which infect legume root nodules and fix N_2 into biologically accessible ammonia using the enzyme nitrogenase [1]. Nitrogenase only occurs in Prokaryotes [2], with the agriculturally important N_2-fixing symbioses largely restricted to legume [3]. Therefore, biologically fixed nitrogen is not directly available to the most agriculturally important crops including maize, wheat and rice.

In agriculture nitrogen limitation is circumvented by application of fertilizers derived from the Haber–Bosch process of inorganic N_2-fixation. Modern rates of nitrogen fertilizer application have doubled the flux into terrestrial nitrogen cycles [4]. Such a significant perturbation is unsustainable and has resulted in environmental consequences, including the production of potent greenhouse gasses and the eutrophication of water systems [5]. Inorganic fertilization is also economically expensive rendering it inaccessible to some developing nations. Global food demand continues to rise and requires increased crop production [6]. Closing yield gaps in developing nations that are most often caused by lack of nitrogen could contribute significantly towards increasing global food supply [7]. Together these factors have potentiated a renewed focus towards engineering biological nitrogen fixation in cereal crops.

A paradigm of three approaches arose with the common goal of engineering cereal crops that could be self-supported by biological nitrogen fixation. These include engineering perception of rhizobia and subsequent nodule formation by cereals, engineering expression of nitrogenase in organelles of plants, and utilizing endophytic diazotrophs that infect cereals to fix nitrogen for their host plants [8]. These approaches have recently been reviewed both together [9], and independently [10–12]. Here we discuss engineering nitrogen fixation in cereal crops by enhancing pre-existing plant-microbe interactions.

Engineering nitrogen fixation in plant colonizing bacteria

To enhance pre-existing interactions between plants and microbes two distinct, but non-mutually exclusive approaches arise: either engineer increased colonization between plants and highly efficient N_2-fixing microbes or engineer transfer of efficient nitrogen fixation into bacteria that already associate closely with cereals. Since the factors that are required for nitrogen fixation are to date more defined than those that govern colonization, the approach of engineering robust colonizers that fix nitrogen has arguably more practical merit.

Tools from synthetic biology

At the 1975 Asilomar meeting, two challenging problems were identified to exemplify the new recombinant DNA technology: the production of insulin in a recombinant host and the transfer of nitrogen fixation to a cereal crop [13]. The first involved the transfer of a single gene into *E. coli* and was solved in 1978, ultimately leading to Genentech [13]. Nitrogen fixation was similarly transferred from *Klebsiella* to *E. coli* in 1972 [14], but its transfer to a eukaryote (including a plant) or engineering a stable association between a nitrogen-fixing bacterium and a cereal crop has remained elusive. In addition to biochemical challenges, there are several issues that make this a difficult genetic engineering problem. First, the system requires the simultaneous transfer of 9–20 genes, most of which are essential [15•,16•,17••] (Figure 1). Second, the system is very fragile with activity being lost quickly when the expression of any gene is suboptimal [17••,18].

During the last 40 years, there has been a rapid expansion in genetic engineering tools, most recently being pushed by the field of synthetic biology. Advances in four areas may aid the engineering and transfer of nitrogenase activity:

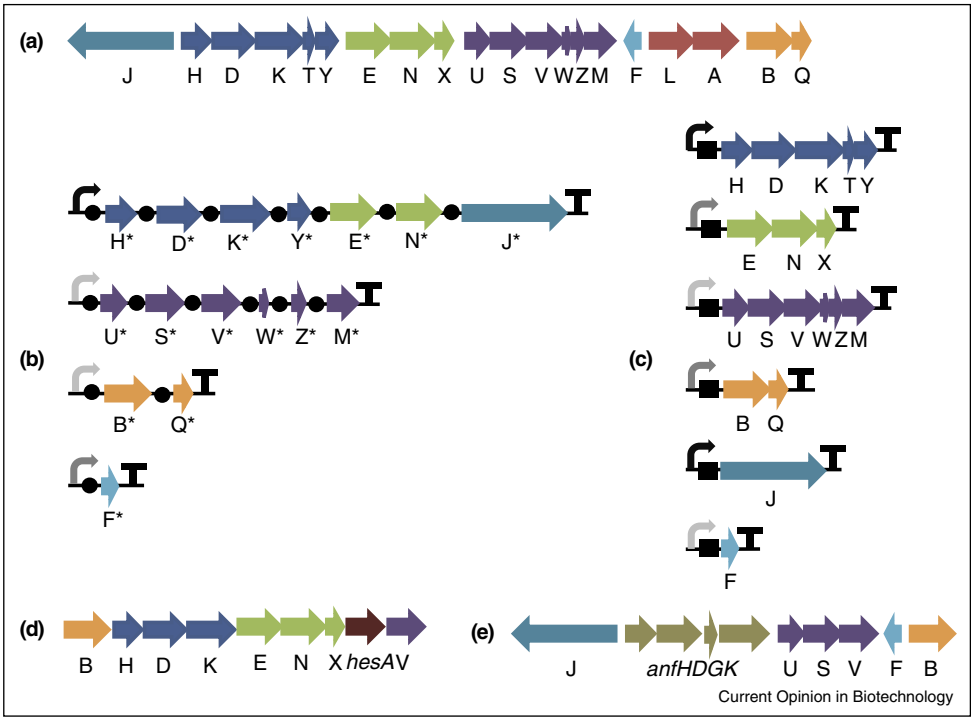
- *Precision expression control*. There are new computational tools and part libraries can be used to fine tune expression over orders of magnitude [19,20]. In addition, there has been work to redefine the 'expression cassette' to include insulator parts to reduce the context dependence of gene expression [21–26]. Increasingly, these tools are being extended from model organisms, such as *E. coli*, to more challenging hosts, including eukaryotes [27–30].
- *Multi-gene DNA synthesis and assembly*. DNA synthesis has become a routine means to obtain genes and to realize designs that require many parts and genetic changes [31–33]. This makes it possible to work with larger *nif* clusters, including those from the sequence database for which no DNA or organism is available. It also enables many designs to be tried at a reasonable cost [18].
- *Synthetic regulation*. Genetic sensors and circuits can be constructed that give cells the ability to receive and process environmental information [34,35]. This allows nitrogenase activity to be turned on under desired conditions, including in coupling activity with sensing association with a plant. Such sensors and circuits have begun to be transferred to plants themselves, enabling the control of gene expression in response to non-native environmental signals [36].
- *Simplifying design*. A goal has been to simplify the process of genetic engineering [37], in essence taking the form of principles of industrial design applied to biology. In part, this involves the deconstruction of genetics into a series of DNA parts, each of which has a modular and defined function. A challenge has been that functions already controlled by the cell – including nitrogen fixation – already have complex and non-modular regulation. A variety of approaches have been taken to simplify native genetics in order to build more modular and engineerable genetic systems [38,39,40••] (Figure 1).

Supporting traits for nitrogen fixation

Nitrogen fixation cassettes that have been refactored using these synthetic biology approaches have proven successful in transferring nitrogen fixation to the facultative anaerobe *E. coli* [17,40••]. However, most microbes that efficiently colonize plants as associative bacteria or endophytes are aerobic organisms. The challenge of transferring nitrogen fixation to aerobic microorganisms is formidable because nitrogenase is oxygen labile. Oxygen toxicity to nitrogenase is circumvented in rhizobial symbioses by specialized root nodules that provide a low-oxygen environment for rhizobia [41]. Some aerobes such as *Azotobacter vinelandii* and *Azorhizobium caulinodans* are capable of free-living nitrogen fixation, and have sophisticated mechanisms of oxygen protection. This is thought to be through maintaining high rates of oxygen consumption at the cell membrane via respiration [42] but it may also involve an alginate oxygen diffusion barrier [43] and conformational protection of nitrogenase by interaction with a specific iron-sulfur protein termed the Shetna protein [44]. Rapid reduction of oxygen is accomplished by remodelling of the electron transport chain to contain alternate terminal oxidases that reduce the efficiency of ATP generation per oxygen reduced. Both *A. vinelandii* and *A. caulinodans* contain alternate terminal oxidases that are expressed under free-living nitrogen fixing conditions [45,46]. One of these, cytochrome *bd*, is highly upregulated under nitrogen fixing conditions and shown to be essential for diazotrophic growth in *A. vinelandii* [45,47] (Figure 2).

Remarkably, the large nitrogen fixation island from *Pseudomonas stutzeri* was transferred to the aerobic associative bacterium *Pseudomonas protegens* Pf-5, conferring the ability to grow micro-aerobically using N_2 as a sole nitrogen source. Moreover, inoculation of *Arabidopsis*, alfalfa, tall fescue and wheat with transgenic *P. protegens* resulted in significant growth promotion effects compared to the near-isogenic wild-type under nitrogen-limited conditions. The transfer of the *P. stutzeri* island resulted in constitutive *nif* expression, probably because *P. protegens* lacks the regulation present in *P. stutzeri* [48••]. The nitrogen fixing island of *P. stutzeri* contains a number of uncharacterized genes that have previously been analysed by mutagenesis and shown to contribute to the efficiency of nitrogen fixation in *P. stutzeri* [49]. Characterizing these components in *P. stutzeri*, and defining a minimal unit for transfer of nitrogen fixation from *P. stutzeri* to *P. protegens* are important targets for *nif* transfer to aerobes.

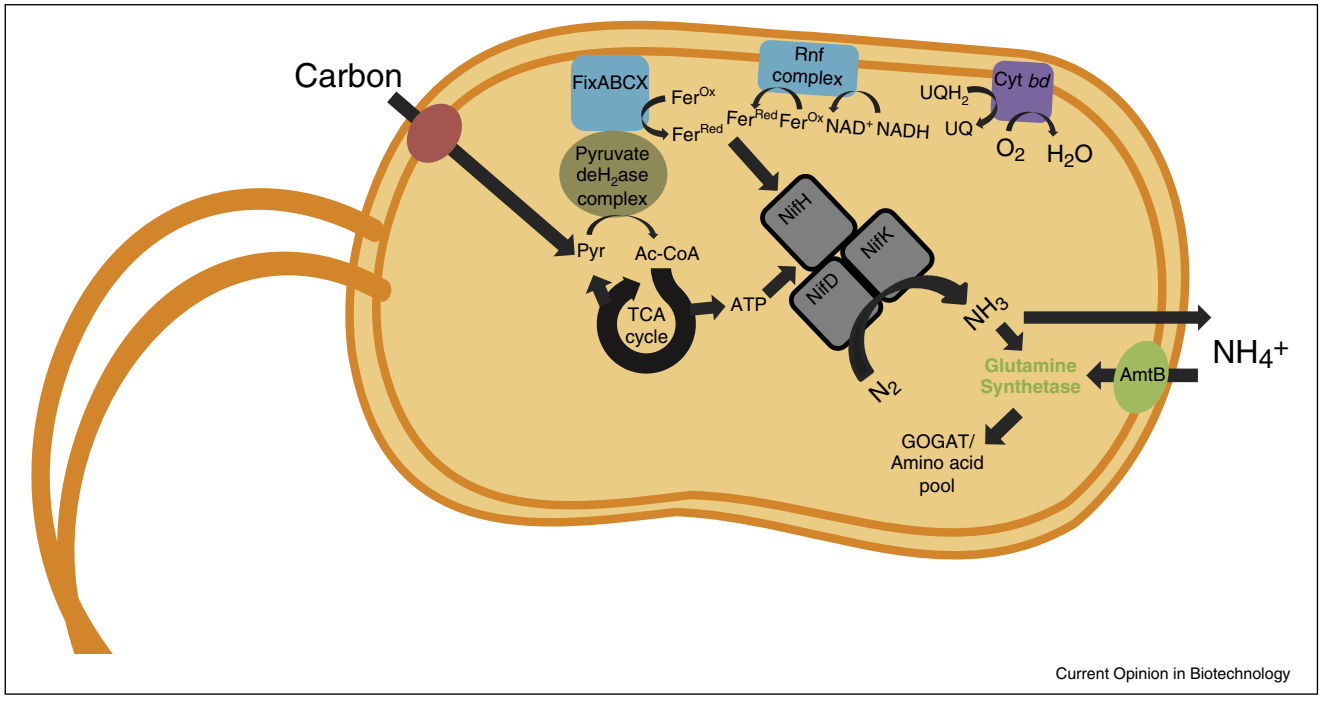
Figure 1



Advances in genetic transfer of nitrogen fixation to *E. coli*. Colored arrows represent *nif* genes annotated by letters below. **(A)** N_2-fixation cluster from *Klebsiella*. Arrow colors correspond to operons. **(B)** and **(C)** Synthetic biology approaches for refactoring *Klebsiella nif* genes. Grey-scale symbols represent synthetic parts. Curved arrows represent T7 promoters with strengths loosely corresponding to shade (dark = strong, light = week). Circles represent synthetic ribosome binding sites. Squares represent *lac* operator sites. T shapes represent terminators. Refactored coding sequences are denoted by *. (C) The approach by Temme *et al.* [17••] eliminated unnecessary genes and used refactored coding sequences and synthetic ribosome binding sites. Inducible expression of T7 RNA polymerase from various controller plasmids was used to control transcription from T7 promoters of varying strengths. **(D)** Wang *et al.* [40••] used native operons, coding sequences and ribosome binding sites transcribed from T7 promoters of varying strengths. A *lac* operator downstream of promoters allowed inducible expression of genes from constitutively transcribed T7 RNA polymerase. (D) N_2-fixation gene cluster from *Paenibacillus* sp. WLY78. Notably genes encoding electron transfer to nitrogenase (*nifF, nifJ*) are absent [15•]. **(E)** Minimal cluster for FeFe nitrogenase N_2-fixation in *E. coli*. Brown arrows represent Fe-nitrogenase biosynthesis genes transferred from *A. vinelandii*. Remaining genes represent a minimal set of *K. pneumoniae* genes required to support nitrogenase biosynthesis. In this system the FeFe nitrogenase circumvents the requirement of the NifEN scaffold for nitrogenase maturation [16•].

Another important consideration for the transfer of nitrogen fixation to aerobes is the mechanism of electron transfer to nitrogenase. While this has been well established for anaerobes, a mechanism of electron transfer to nitrogenase has not been demonstrated for aerobic nitrogen fixation. In the facultative anaerobe *Klebsiella pneumoniae* electrons are transferred to nitrogenase by the flavodoxin NifF which is reduced by the pyruvate:flavodoxin oxidoreductase NifJ [50]. However in aerobic bacteria the oxidative decarboxylation of pyruvate is carried out by the pyruvate dehydrogenase complex rather than pyruvate:flavodoxin oxidoreductase yielding NADH which is a less powerful reductant than flavodoxin or ferredoxin. Nitrogen fixation requires reducing equivalents at lower oxidation-reduction potentials than NADH, which are generated readily by anaerobes but to a lesser extent by aerobes. The Rnf complex and FixABCX are membrane-associated complexes that have been proposed to transfer electrons to nitrogenase during aerobic nitrogen fixation [51,52] (Figure 2). The Rnf complex is thought to reduce nitrogenase with ferredoxin generated by using the proton motive force to drive reverse electron flow through the complex from NADH [52]. Strains carrying mutations in loci that encode Rnf complexes in *A. vinelandii* had significantly reduced nitrogen fixation [53]. Notably, the nitrogen fixation island of *P. stutzeri* also encodes an Rnf complex that is important for nitrogen fixation [54]. FixABCX are widely distributed among aerobic nitrogen fixing bacteria including rhizobia, and essential for symbiotic nitrogen fixation. FixAB belongs to the Electron Transfer Flavoprotein (ETF) family and FixCX is related to Etf-quinone reductase. We propose that FixABCX functions in aerobic nitrogen fixing bacteria to bifurcate electrons between ferredoxin and NADH or a quinone. This is by analogy to electron bifurcation reactions in anaerobic bacteria that have been

Figure 2



Model of traits to support aerobic nitrogen fixation and transfer to cereals. Carbon to energize nitrogen fixation could be supplied from a biased rhizosphere. Electron transfer to nitrogenase could be carried out by FixABCX or the Rnf complex (blue). Molecular details have not clearly been elucidated, but this may be mediated by the reduction of ferredoxin driven by coupled exergonic reactions. Electrons may be transferred from NADH or directly from pyruvate by the pyruvate dehydrogenase complex. Oxygen protection could be carried out by high respiration rates conferred by alternate terminal oxidases (purple). Along with altering the regulation of nitrogenase biosynthesis, enzymes involved in ammonium uptake and assimilation (green) represent targets for genetic modification to engineer enhanced ammonium release to the plant.

shown to be catalysed by ETF complexes [55]. In *A. caulinodans* FixAB are essential for diazotrophic growth and genetic evidence suggests they interact with the pyruvate dehydrogenase complex [56].

Ammonium release by bacteria is a critical attribute for translating engineered nitrogen fixation by bacteria into nitrogen assimilation by plants [57]. The misregulation of the *P. stutzeri nif* cluster in *P. protogens* Pf-5 resulted in significant ammonium release by the bacteria [48••]. Misregulation of nitrogen fixation leading to ammonium excretion has also been observed in *A. vinelandii*, where mutation of the key regulators *nifA* or *nifL*, have resulted in ammonium excretion [58,59]. Ammonium release by an *Azospirillum brasilense* glutamine synthetase (*glnA*) mutant may have increased growth of wheat under nitrogen-limited conditions [60,61] (Figure 2). The deletion of two ammonium transporters encoded by *amtB1* and *amtB2* enhanced ammonium secretion in *P. stutzeri* [62] (Figure 2), which was increased when combined with expression of *nifA* from a constitutive promoter [62]. *A. vinelandii* with combined *nifL* and glutamine synthetase active site mutations also showed high ammonium release [63].

N_2-fixation is energetically demanding, requiring at least 16 ATP and 8 electrons per N_2 fixed [41] and energy supply is likely to limit endophytic N_2-fixation. Although plant roots are generally rich sources of carbon, and as much as 20 percent of photosynthate can be exuded from roots, in a non-sterile setting newly introduced microorganisms will be forced to compete for carbon with the native microbiota. One approach to enhance carbon supply to N_2-fixing microbes would be to provide them with a specialized carbon source that the general microbiota cannot catabolize. This biased rhizosphere hypothesis, has been proposed to enhance the competitiveness of newly introduced microbes in the plant environment [64]. During *Agrobacterium* infection, opine synthesis genes are transferred to the plant resulting in the condensation of carbohydrates with amino acids to form opine compounds that the invading *Agrobacterium* use as a carbon source [65]. Transfer of opine synthesis genes to tobacco plants, and opine catabolism genes to *Pseudomonas* resulted in competitive advantage for colonization to an opine catabolizing strain against the wild-type during colonization of opine-synthesising plant roots [66,67]. Transgenic opine-producing *Arabadopsis thaliana* plants were also shown to reshape populations of opine-catabolizing bacteria [68].

Conclusions

The traits required for colonization of plants are poorly understand and are likely to involve hundreds of genes [69,70]. The daunting task of engineering the ability to colonize and associate with plants can be avoided by using pre-existing endophytes or associative bacteria as chassis for either enhancing, or transferring N_2-fixation using synthetic biology. The utilization of bacteria that already inhabit an ecological niche with cereal crops to engineer nitrogen fixation in non-legume plants has been less favoured than the plant-engineering approaches. However, given the new tools afforded by synthetic biology, and the opportunities they generate for enhancing pre-existing associations, a renewed focus should be placed on this area.

Ultimately it is important to integrate the approaches to transfer nitrogen fixation to cereals. If the ability to perceive rhizobial signalling molecules can be transferred to non-legume plants and result in the formation of an oxygen-limited, nodule-like root organ, then these nodules must become infected with N_2-fixing bacteria. Endophytic or associative organisms that have already been engineered for high nitrogen fixation levels and nitrogen transfer to the plant would be ideal symbionts for this new niche. Thus together these approaches could bring the dream of self-supported nitrogen fixing cereal crops closer to reality.

Acknowledgements

The authors acknowledge funding from the Biotechnology and Biological Sciences Research Council grant BB/L011484/1 to PP and National Science Foundation grant NSF-1331098 to JWP and CV.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Oldroyd GE, Murray JD, Poole PS, Downie JA: **The rules of engagement in the legume-rhizobial symbiosis**. *Annu Rev Genet* 2011, **45**:119-144.
2. Dos Santos PC, Fang Z, Mason SW, Setubal JC, Dixon R: **Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes**. *BMC Genomics* 2012, **13**:162.
3. Werner GD, Cornwell WK, Sprent JI, Kattge J, Kiers ET: **A single evolutionary innovation drives the deep evolution of symbiotic N_2-fixation in angiosperms**. *Nat Commun* 2014, **5**:4087.
4. Galloway JN, Townsend AR, Erisman JW, Bekunda M, Cai Z, Freney JR, Martinelli LA, Seitzinger SP, Sutton MA: **Transformation of the nitrogen cycle: recent trends, questions, and potential solutions**. *Science* 2008, **320**:889-892.
5. Rockström J, Steffen W, Noone K, Persson Å, Chapin FS, Lambin EF, Lenton TM, Scheffer M, Folke C, Schellnhuber HJ *et al.*: **A safe operating space for humanity**. *Nature* 2009, **461**:472-475.
6. Tilman D, Balzer C, Hill J, Befort BL: **Global food demand and the sustainable intensification of agriculture**. *Proc Natl Acad Sci U S A* 2011, **108**:20260-20264.
7. Mueller ND, Gerber JS, Johnston M, Ray DK, Ramankutty N, Foley JA: **Closing yield gaps through nutrient and water management**. *Nature* 2012, **490**:254-257.
8. Beatty PH, Good AG: **Future prospects for cereals that fix nitrogen**. *Science* 2011, **333**:416-417.
9. Oldroyd GE, Dixon R: **Biotechnological solutions to the nitrogen problem**. *Curr Opin Biotechnol* 2014, **26**:19-24.
10. Santi C, Bogusz D, Franche C: **Biological nitrogen fixation in non-legume plants**. *Ann Bot* 2013, **111**:743-767.
11. Curatti L, Rubio LM: **Challenges to develop nitrogen-fixing cereals by direct *nif* gene transfer**. *Plant Sci* 2014, **225**:130-137.
12. Rogers C, Oldroyd GE: **Synthetic biology approaches to engineering the nitrogen symbiosis in cereals**. *J Exp Bot* 2014, **65**:1 [eru098].
13. Simmonds J: *Community matters a history of biological nitrogen fixation and nodulation research, 1965 to 1995*. . [Ph.D. thesis] Troy, NY: Rensselaer Polytechnic Institute, ProQuest; 2007, :. (Publication No. 3299478).
14. Dixon R, Postgate J: **Genetic transfer of nitrogen fixation from *Klebsiella pneumoniae* to *Escherichia coli***. *Nature* 1972, **237**:102-103.
15. • Wang L, Zhang L, Liu Z, Zhao D, Liu X, Zhang B, Xie J, Hong Y, Li P, Chen S: **A minimal nitrogen fixation gene cluster from *Paenibacillus* sp. WLY78 enables expression of active nitrogenase in *Escherichia coli***. *PLOS Genet* 2013, **9**:e1003865.
This paper demonstrates that significantly fewer genes than previously thought are required to transfer nitrogenase to *E. coli*.
16. • Yang J, Xie X, Wang X, Dixon R, Wang Y-P: **Reconstruction and minimal gene requirements for the alternative iron-only nitrogenase in *Escherichia coli***. *Proc Natl Acad Sci U S A* 2014, **111**:E3718-E3725.
This work shows that a reduced set of genes is required for transfer of the Fe nitrogenase relative to the Mo form.
17. •• Temme K, Zhao D, Voigt CA: **Refactoring the nitrogen fixation gene cluster from *Klebsiella oxytoca***. *Proc Natl Acad Sci U S A* 2012, **109**:7085-7090.
This work highlights the potential of synthetic biology for transferring nitrogen fixation by completely refactoring nitrogenase biosynthesis genes removing all native regulation.
18. Smanski MJ, Bhatia S, Zhao D, Park Y, Woodruff LBA, Giannoukos G, Ciulla D, Busby M, Calderon J, Nicol R *et al.*: **Functional optimization of gene clusters by combinatorial design and assembly**. *Nat Biotechnol* 2014, **32**:1241-1249.
19. Salis HM, Mirsky EA, Voigt CA: **Automated design of synthetic ribosome binding sites to control protein expression**. *Nat Biotechnol* 2009, **27**:946-950.
20. Kosuri S, Goodman DB, Cambray G, Mutalik VK, Gao Y, Arkin AP, Endy D, Church GM: **Composability of regulatory sequences controlling transcription and translation in *Escherichia coli***. *Proc Natl Acad Sci U S A* 2013, **110**:14024-14029.
21. Mutalik VK, Guimaraes JC, Cambray G, Mai Q-A, Christoffersen MJ, Martin L, Yu A, Lam C, Rodriguez C, Bennett G *et al.*: **Quantitative estimation of activity and quality for collections of functional genetic elements**. *Nat Methods* 2013, **10**:347-353.
22. Mutalik VK, Guimaraes JC, Cambray G, Lam C, Christoffersen MJ, Mai Q-A, Tran AB, Paull M, Keasling JD, Arkin AP *et al.*: **Precise and reliable gene expression via standard transcription and translation initiation elements**. *Nat Methods* 2013, **10**:354-360.
23. Lou C, Stanton B, Chen Y-J, Munsky B, Voigt CA: **Ribozyme-based insulator parts buffer synthetic circuits from genetic context**. *Nat Biotechnol* 2012, **30**:1137-1142.
24. Chen Y-J, Liu P, Nielsen AAK, Brophy JAN, Clancy K, Peterson T, Voigt CA: **Characterization of 582 natural and synthetic terminators and quantification of their design constraints**. *Nat Methods* 2013, **10**:659-664.
25. Davis JH, Rubin AJ, Sauer RT: **Design, construction and characterization of a set of insulated bacterial promoters**. *Nucleic Acids Res* 2011, **39**:1131-1141.

26. Nielsen AA, Segall-Shapiro TH, Voigt CA: **Advances in genetic circuit design: novel biochemistries, deep part mining, and precision gene expression**. *Curr Opin Chem Biol* 2013, **17**:878-892.

27. Sharon E, Kalma Y, Sharp A, Raveh-Sadka T, Levo M, Zeevi D, Keren L, Yakhini Z, Weinberger A, Segal E: **Inferring gene regulatory logic from high-throughput measurements of thousands of systematically designed promoters**. *Nat Biotechnol* 2012, **30**:521-530.

28. Curran KA, Crook NC, Karim AS, Gupta A, Wagman AM, Alper HS: **Design of synthetic yeast promoters via tuning of nucleosome architecture**. *Nat Commun* 2014, **5**:4002.

29. Farasat I, Kushwaha M, Collens J, Easterbrook M, Guido M, Salis HM: **Efficient search, mapping, and optimization of multi-protein genetic systems in diverse bacteria**. *Mol Syst Biol* 2014, **10**:731.

30. Morton T, Petricka J, Corcoran DL, Li S, Winter CM, Carda A, Benfey PN, Ohler U, Megraw M: **Paired-end analysis of transcription start sites in *Arabidopsis* reveals plant-specific promoter signatures**. *Plant Cell* 2014, **26**:2746-2760.

31. Bayer TS, Widmaier DM, Temme K, Mirsky EA, Santi DV, Voigt CA: **Synthesis of methyl halides from biomass using engineered microbes**. *J Am Chem Soc* 2009, **131**:6508-6515.

32. Czar MJ, Anderson JC, Bader JS, Peccoud J: **Gene synthesis demystified**. *Trends Biotechnol* 2009, **27**:63-72.

33. Kosuri S, Church GM: **Large-scale de novo DNA synthesis: technologies and applications**. *Nat Methods* 2014, **11**:499-507.

34. Brophy JAN, Voigt CA: **Principles of genetic circuit design**. *Nat Methods* 2014, **11**:508-520.

35. Lu TK, Khalil AS, Collins JJ: **Next-generation synthetic gene networks**. *Nat Biotechnol* 2009, **27**:1139-1150.

36. Morey KJ, Antunes MS, Barrow MJ, Solorzano FA, Havens KL, Smith JJ, Medford J: **Crosstalk between endogenous and synthetic components – synthetic signaling meets endogenous components**. *Biotechnol J* 2012, **7**:846-855.

37. Endy D: **Foundations for engineering biology**. *Nature* 2005, **438**:449-453.

38. Chan LY, Kosuri S, Endy D: **Refactoring bacteriophage T7**. *Mol Syst Biol* 2005, **1** [2005.0018].

39. St-Pierre F, Endy D: **Determination of cell fate selection during phage lambda infection**. *Proc Natl Acad Sci U S A* 2008, **105**:20705-20710.

40. •• Wang X, Yang J-G, Chen L, Wang J-L, Cheng Q, Dixon R, Wang Y-P: **Using synthetic biology to distinguish and overcome regulatory and functional barriers related to nitrogen fixation**. *PLOS ONE* 2013, **8**:e68677.

This paper replaces the native promoters of *nif* operons with synthetic promoters to allow controlled expression of nitrogenase with relatively low loss of nitrogenase activity.

41. Udvardi M, Poole PS: **Transport and metabolism in legume-rhizobia symbioses**. *Annu Rev Plant Biol* 2013, **64**:781-805.

42. Poole R, Hill S: **Respiratory protection of nitrogenase activity in *Azotobacter vinelandii*-roles of the terminal oxidases**. *Biosci Rep* 1997, **17**:303-317.

43. Sabra W, Zeng A-P, Lünsdorf H, Deckwer W-D: **Effect of oxygen on formation and structure of *Azotobacter vinelandii* alginate and its role in protecting nitrogenase**. *App Env Mirobiol* 2000, **66**:4037-4044.

44. Moshiri F, Kim JW, Fu C, Maier RJ: **The FeSII protein of *Azotobacter vinelandii* is not essential for aerobic nitrogen fixation, but confers significant protection to oxygen-mediated inactivation of nitrogenase in vitro and in vivo**. *Mol Microbiol* 1994, **14**:101-114.

45. Hamilton TL, Ludwig M, Dixon R, Boyd ES, Dos Santos PC, Setubal JC, Bryant DA, Dean DR, Peters JW: **Transcriptional profiling of nitrogen fixation in *Azotobacter vinelandii***. *J Bacteriol* 2011, **193**:4477-4486.

46. Kitts CL, Ludwig RA: **Azorhizobium caulinodans respires with at least four terminal oxidases**. *J Bacteriol* 1994, **176**:886-895.

47. Kelly M, Poole R, Yates M, Kennedy C: **Cloning and mutagenesis of genes encoding the cytochrome *bd* terminal oxidase complex in *Azotobacter vinelandii*: mutants deficient in the cytochrome *d* complex are unable to fix nitrogen in air**. *J Bacteriol* 1990, **172**:6010-6019.

48. •• Setten L, Soto G, Mozzicafreddo M, Fox AR, Lisi C, Cuccioloni M, Angeletti M, Pagano E, Díaz-Paleo A, Ayub ND: **Engineering *Pseudomonas protegens* Pf-5 for nitrogen fixation and its application to improve plant growth under nitrogen-deficient conditions**. *PLOS ONE* 2013, **8**:e63666.

This work reports the successful transfer of the nitrogen fixation island from *P. stutzeri* to *P. protogens* Pf5 and shows that this transgenic strain has significant growth enhancement effects for plants grown in nitrogen-limited conditions.

49. Yan Y, Yang J, Dou Y, Chen M, Ping S, Peng J, Lu W, Zhang W, Yao Z, Li H: **Nitrogen fixation island and rhizosphere competence traits in the genome of root-associated *Pseudomonas stutzeri* A1501**. *Proc Natl Acad Sci U S A* 2008, **105**:7564-7569.

50. Shah VK, Stacey G, Brill WJ: **Electron transport to nitrogenase. Purification and characterization of pyruvate: flavodoxin oxidoreductase. The nifJ gene product**. *J Biol Chem* 1983, **258**:12064-12068.

51. Edgren T, Nordlund S: **The *fixABCX* genes in *Rhodospirillum rubrum* encode a putative membrane complex participating in electron transfer to nitrogenase**. *J Bacteriol* 2004, **186**:2052-2060.

52. Schmehl M, Jahn A, zu Vilsendorf AM, Hennecke S, Masepohl B, Schuppler M, Marxer M, Oelze J, Klipp W: **Identification of a new class of nitrogen fixation genes in *Rhodobacter capsalatus*: a putative membrane complex involved in electron transport to nitrogenase**. *Mol Gen Genet* 1993, **241**:602-615.

53. Curatti L, Brown CS, Ludden PW, Rubio LM: **Genes required for rapid expression of nitrogenase activity in *Azotobacter vinelandii***. *Proc Natl Acad Sci U S A* 2005, **102**:6291-6296.

54. Desnoues N, Lin M, Guo X, Ma L, Carreño-Lopez R, Elmerich C: **Nitrogen fixation genetics and regulation in a *Pseudomonas stutzeri* strain associated with rice**. *Microbiology* 2003, **149**:2251-2262.

55. Herrmann G, Jayamani E, Galina M, Buckel W: **Energy conservation via electron-transferring flavoprotein in anaerobic bacteria**. *J Bacteriol* 2008, **190**:784-791.

56. Scott JD, Ludwig RA: **Azorhizobium caulinodans electron-transferring flavoprotein N electrochemically couples pyruvate dehydrogenase complex activity to N_2 fixation**. *Microbiology* 2004, **150**:117-126.

57. Triplett EW: **Diazotrophic endophytes: progress and prospects for nitrogen fixation in monocots**. *Plant Soil* 1996, **186**:29-38.

58. Bali A, Blanco G, Hill S, Kennedy C: **Excretion of ammonium by a *nifL* mutant of *Azotobacter vinelandii* fixing nitrogen**. *Appl Environ Mirobiol* 1992, **58**:1711-1718.

59. Brewin B, Woodley P, Drummond M: **The basis of ammonium release in *nifL* mutants of *Azotobacter vinelandii***. *J Bacteriol* 1999, **181**:7356-7362.

60. Van Dommelen A, Keijers V, Wollebrants A, Vanderleyden J: **Phenotypic changes resulting from distinct point mutations in the *Azospirillum brasilense glnA* gene, encoding glutamine synthetase**. *Appl Environ Mirobiol* 2003, **69**:5699-5701.

61. Van Dommelen A, Croonenborghs A, Spaepen S, Vanderleyden J: **Wheat growth promotion through inoculation with an ammonium-excreting mutant of *Azospirillum brasilense***. *Biol Fertil Soils* 2009, **45**:549-553.

62. Zhang T, Yan Y, He S, Ping S, Alam KM, Han Y, Liu X, Lu W, Zhang W, Chen M: **Involvement of the ammonium transporter *AmtB* in nitrogenase regulation and ammonium excretion in *Pseudomonas stutzeri* A1501**. *Res Microbiol* 2012, **163**:332-339.

63. Ortiz-Marquez JCF, Do Nascimento M, Curatti L: **Metabolic engineering of ammonium release for nitrogen-fixing multispecies microbial cell-factories**. *Metab Eng* 2014, **23**:154-164.

64. Savka MA, Dessaux Y, Oger P, Rossbach S: **Engineering bacterial competitiveness and persistence in the phytosphere**. *Mol Plant-Microbe Interact* 2002, **15**:866-874.

65. Dessaux Y, Petit A, Farrand SK, Murphy PJ: **Opines and opine-like molecules involved in plant-Rhizobiaceae interactions**. *The rhizobiaceae*. Springer; 1998:: 173-197.

66. Oger P, Petit A, Dessaux Y: **Genetically engineered plants producing opines alter their biological environment**. *Nat Biotechnol* 1997, **15**:369-372.

67. Savka MA, Farrand SK: **Modification of rhizobacterial populations by engineering bacterium utilization of a novel plant-produced resource**. *Nat Biotechnol* 1997, **15**:363-368.

68. Mondy A, Lenglet A, Beury-Cirou A, Libanga C, Ratet P, Faure D, Dessaux Y: **An increasing opine carbon bias in artificial exudation systems and genetically modified plant rizospheres leads to an increasing reshaping of bacterial populations**. *Mol Ecol* 2014, **23**:4846-4861.

69. Reinhold-Hurek B, Hurek T: **Living inside plants: bacterial endophytes**. *Curr Opin Plant Biol* 2011, **14**:435-443.

70. Turner TR, James EK, Poole PS: **The plant microbiome**. *Genome Biol* 2013, **14**:209.