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CHAPTER ONE

Azotobacters as biofertilizer

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Abstract

Azotobacters have been used as biofertilizer since more than a century. *Azotobacters* fix nitrogen aerobically, elaborate plant hormones, solubilize phosphates and also suppress phytopathogens or reduce their deleterious effect. Application of wild type *Azotobacters* results in better yield of cereals like corn, wheat, oat, barley, rice, pearl millet and sorghum, of oil seeds like mustard and sunflower, of vegetable crops like tomato, egg-plant, carrot, chillies, onion, potato, beans and sugar beet, of fruits like mango and sugar cane, of fiber crops like jute and cotton and of tree like oak. In addition to the structural genes of the enzyme nitrogenase and of other accessory proteins, *A. vinelandii* chromosomes contain the regulatory genes *nifL* and *nifA*. NifA must bind upstream of the promoters of all *nif* operons for enabling their expression. NifL on activation by oxygen or ammonium, interacts with NifA and neutralizes it. Nitrogen fixation has been enhanced by deletion of *nifL* and by bringing *nifA* under the control of a constitutive promoter, resulting in a strain that continues to fix nitrogen in presence of urea fertilizer. Additional copies of *nifH* (the gene for the Fe-protein of nitrogenase) have been introduced into *A. vinelandii*, thereby augmenting nitrogen fixation. The urease gene complex *ureABC* has been deleted, the ammonia transport gene *amtB* has been disrupted and the expression of the glutamine synthase gene has been regulated to enhance urea and ammonia excretion. Gluconic acid has been produced by introducing the glucose dehydrogenase gene, resulting in enhanced solubilization of phosphate.

1. Biofertilizers are important

By definition, a biofertilizer comprises live microbes like bacteria, algae or fungi, individually or in combination, which enhances the fertility of soil, thereby benefiting the plants that grow in such soil. Farm yard manure, which is also of biological origin, is not a biofertilizer.

What is wrong with chemically synthesized fertilizers? Chemical synthesis of nitrogenous fertilizers like urea needs expenditure of a huge amount of energy that causes emission of 10-fold or even higher amount of CO_2 equivalent (Zhang et al., 2013). In addition, only 30–40% of the chemical fertilizer applied in the fields is utilized by the plants (Prasad, 2009), while the rest pollutes and causes serious environmental problems. The pollution caused by chemical nitrogenous fertilizers has been estimated to cost the European Union an enormous amount that could be anywhere between euro 70 and 320 billion per year (Sutton et al., 2011).

On the other hand, biofertilizers are environment friendly, contributes nutrients to plants and often counteracts plant pathogens.

2. The genus *Azotobacter*

The genus *Azotobacter* has been used as a biofertilizer since more than a century (Gerlach & Vogel, 1902). This genus was first described in 1901 by *Martinus Beijerinck*. *Azotobacter* belongs to the family *Pseudomonadaceae/Azotobacteraceae* and class *Gammaproteobacteria*, which is common in soils sampled from across the world (Kennedy, Rudnick, MacDonald, & Melton, 2005). Notable species of the genus *Azotobacter* are *A. vinelandii*, *A. chroococcum*, *A. beijerinckii*, *A. paspali*, *A. armeniacus*, *A. nigricans* and *A. salinestri*. The most worked upon species is *A. vinelandii*, the genome sequence of which has been determined by Setubal et al. (2009). Reviews on *Azotobacters* can be seen in Mrkovac-ki and Milic (2001), Paul and Paul (2009) and Sivasakthi, Saranraj, and Sivasakthivelan (2017).

Azotobacters are oval shaped and quite large (1–3 µm wide and 2–10 µm long) compared to other bacteria. An electron micrograph of negatively stained *A. vinelandii* UW that had been grown in modified Burk's nitrogen free medium (Wilson & Knight, 1952) at 30 °C is shown in Fig. 1. The morphology of *Azotobacters* is altered, sometimes drastically, by the medium in which these are grown (Ballesteros et al., 1986; Vela & Rosenthal, 1972). *Azotobacters* are gram negative and some species produce yellow-green, or red-violet, or brownish-black pigments. Naturally occurring *Azotobacters* secrete large quantities of slime around it and sequester water. *Azotobacters* also form small round thick-walled cysts in harsh environment, but cysts cannot fix nitrogen.

Azotobacters are found in neutral to alkaline soils, in rhizosphere of plants and in bodies of fresh water in all the continents irrespective of climate.

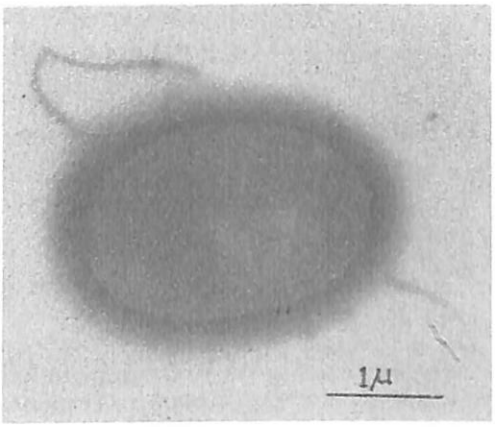

Fig. 1 Electron micrograph of negatively stained *A. vinelandii* ATCC 13705. *Reproduced from Das H.K., (1993). Molecular genetics of nitrogen fixation in Azotobacters, Proceedings of the Indian National Science Academy,* Part B: Biological Sciences 59B, *387–396.*

3. *Azotobacters* can fix atmospheric nitrogen

Beijerinck (1901) had discovered that *Azotobacters* could fix atmospheric nitrogen in the free-living state, without any symbiosis or association with any plant. A review of the early history of nitrogen fixation has been written by Burris (1977). There are other microorganisms that can fix nitrogen, Brill (1977) has reviewed these microorganisms. Elmerich's (2015) article on "One hundred years discovery of nitrogen fixing rhizobacteria" is good reading.

Most of these microorganisms need anaerobic condition to fix nitrogen, but *Azotobacters* have been known to be tolerant to oxygen.

4. *Azotobacters* produce plant hormones

In addition to fixing atmospheric nitrogen that is made use of by the plants, *Azotobacters* also elaborate plant growth hormones. Both *A. vinelandii* and *A. chroococcum* have been found to excrete indole acetic acid, which is enhanced in presence of its precursor, tryptophan (Table 1). Three gibberellin-like compounds in amounts of 0.01–0.1 μg GA_3 equivalent per mL had been detected in cultures of an *A. chroococcum* strain (Brown & Burlingham, 1968). Five cytokinins were identified in an *A. chroococcum* culture filtrate (Nieto & Frankenberger, 1989). Addition of cytokinin precursors like adenine and isopentyl alcohol enhanced cytokinin excretion

Table 1 Production of indole acetic acid (IAA) by *Azotobacter chroococcum* CBD15 and by *Azotobacter vinelandii* UW.

	IAA production (ppm/mg protein)	
	In the absence of tryptophan	**In the presence of tryptophan (50 µg/mL)**
Azotobacter chroococcum CBD15[a]	4.7	9.4
Azotobacter vinelandii UW[b]	9.1	16.5

[a]Isolated from the field of Indian Agricultural Research Institute, New Delhi, India.
[b]Strain from Madison, Wisconsin, USA.
Compiled from Paul, S., Verma, O. P. & Das, H. K. (2005). Evaluation of the modified Azotobacter strains for their performance as biofertilizers in wheat. Report of project funded by the Department of Biotechnology; Bageshwar, U. K., Srivastava, M., Pardha-Saradhi, P., Paul, S., Gothandapani, S., Jaat, R. S., Shankar, P., Yadav, R., Biswas, D. R., Kumar, P. A., Padaria, J. C., Mandal, P. K., Annapurna, K. & Das, H. K. (2017). An environmentally friendly engineered Azotobacter strain that replaces a substantial amount of urea fertilizer while sustaining the same wheat yield. Applied and Environmental Microbiology, 83, e00590–17.

(Nieto & Frankenberger, 1991). Mrkovac-ki and Milic (2001), Kukreja, Suneja, Goyal, and Narula (2004) and Paul and Paul (2009) have reviewed information on this topic.

5. *Azotobacters* can solubilize phosphates

Only the soluble form of phosphates can be assimilated by plants. *Azotobacters* are capable of solubilizing insoluble phosphates in the soil. Kumar and Narula (1999) and Kumar, Behl, and Narula (2001) could isolate *Azotobacter* mutants that were able to release 1.5–1.7 µg phosphate per mL of supernatant from tricalcium phosphate. Similar results were also obtained by Deubel and Merbach (2005). Paul and Paul (2009) have dealt with this area in their review article.

6. *Azotobacters* are capable of suppressing phytopathogens or reduce their deleterious effect

Azotobacters have been reported to control fungal and bacterial diseases and nematode infestation in crop plants. Meshram (1984) observed suppressive effect of *A. chroococcum* on *Rhizoctonia solani* infestation of potatoes. Field experiments of Beniwal, Karwasara, Lakshminarayana, and Narula (1996) revealed that incidence of flag smut was considerably reduced when wheat

seeds were inoculated with *A. chroococcum* strains and mutants. Chakrabarti and Yadav (1991) found that incidence of downy mildew (*Poronospora arborescens*) infestation of opium poppy (*Papavar somniferum*) was much less and yield of opium was much better, when the seeds were inoculated with an *Azotobacter* species. *A. chroococcum* strains evinced fungistatic activity, when tested in the laboratory, against *Sclerotium* sp., *Fusarium* sp., *Cephalosporium maydis, Alternaria brassicola* and *Colletotrichum falcatum* (Pandey & Kumar, 1990).

Chahal-and Chahal (1988) reported inhibition by *A. chroococcum* of hatching of egg masses of the nematode *Meloidogyne incognita* (Kofoid and White) and of penetration of the larvae into the roots of eggplant. *A. chroococcum* strain W-5 was also observed to inhibit hatching of egg masses of the insects *Spodoptera litura* (Fab.), *Spilarctia obliqua* (Walker) and *Corcyra cephalonica* Stainton (Paul, Paul, & Verma, 2002). Paul and Paul (2009) have reviewed this area also.

7. Use of *Azotobacters* as biofertilizers dates back to almost a century and two decades

Gerlach and Vogel (1902) inoculated seeds of buck wheat with *A. chroococcum* and observed considerable increase in dry matter of the plants that were grown in pots. They also attained enhanced yield of mustard by *A. chroococcum* inoculation of seeds. Kostychev, Sheloumova, and Shulgina (1926) had recognized the usefulness of *Azotobacter* inoculation of seeds of crop plants. Since the 1930s *Azotobacter* preparations under the name "Azotobacterin" had been used in the erstwhile U.S.S.R. and the East European countries to treat seeds of wheat, barley, corn, sugar beet, carrot and potato (Lakshminarayana, 1993).

Inoculation of seeds with wild type *Azotobacters* has been reported to result in better yield of cereals like corn, wheat, oat, barley, rice, pearl millet and sorghum. *Azotobacters* have also led to better yield of oil seeds like mustard and sunflower. Vegetable crops like tomato, eggplant, carrot, chillies, onion, potato, beans and sugar beet have also exhibited enhanced yield on inoculation of their seeds with *Azotobacters*. Fruits like mango and sugar cane, fiber crops like jute and cotton and tree like oak also have responded positively on application of *Azotobacters*. All these beneficial effects have been dealt with in details by Mrkovac-ki and Milic (2001) and Paul and Paul (2009).

8. Methods of application of *Azotobacters*

Bageshwar et al. (1997) had applied *A. chroococcum* to wheat seeds as a suspension in BNF medium with 2% sucrose that contained $\sim 1 \times 10^{10}$ cells per mL. The seeds were soaked for 3 h at 25 °C and air dried at 25 °C. Paul and Paul (2009) used 10% jaggery in place of sucrose. Sashidhar and Podile (2009) suspended *A. vinelandii* in 0.5% carboxy methyl cellulose and applied to sorghum seeds. Gum Arabic has also been recommended as an adhesive so that *Azotobacters* can stick to the seeds. For application to roots of trees, *Azotobacters* are often mixed with a carrier like lignite, compost or peat soil. Various formulations of *Azotobacters* alone and of combinations with other microbes are available commercially for use as biofertilizers.

9. The chromosomes of *Azotobacters*

After narration of the beneficial effects of *Azotobacters*, we now have a look at the chromosomes of these useful soil bacteria.

9.1 *Azotobacters* contain multiple copies of the chromosome

A single cell of *A. vinelandii* from mid exponential culture contains $(1.35–1.5) \times 10^{-13}$ g DNA (Nagpal, Jafri, Reddy, & Das, 1989; Sadoff, Berke, & Loperfido, 1971), an amount about 40 times that contained by *Escherichia coli*. Interestingly, the sedimentation coefficient of gently prepared folded chromosomes of both bacteria is comparable, 1700 S for *A. vinelandii* and 1600 S for *E. coli* (Sadoff, Shimel, & Ellis, 1979). Moreover, unique sequence lengths of DNA in both these bacteria have been found to be similar, as revealed by *cot* values of DNA (Sadoff et al., 1979). These results suggested that *A. vinelandii* genome comprised multiple copies of its chromosome. Direct titration of the number of copies of several genes has been carried out with cloned probes to dwell on this point. Indeed, it has been found that the genes *lueB*, *nifH*, *nifD* and *nifK* are present in about 80 copies in the *A. vinelandii* genome in its early stationery phase of growth (Nagpal et al., 1989). Subsequently, the β-lactamase gene isolated from the plasmid pBR322 was tagged with the *leu B* gene of *A. vinelandii* and integrated into the chromosome of *A. vinelandii* by single point cross over and the cells were subcultured 20 times in presence of ampicillin to ensure transfer of the

β-lactamase gene to all the copies of the chromosome. Nearly 80 copies of the β-lactamase gene could be detected per cell of early stationery phase *A. vinelandii* by titration with cloned β-lactamase gene probe (Nagpal et al., 1989). The inference drawn was that *A. vinelandii* genome consisted of 80 copies of the chromosome.

Later Maldonado, Jimenez, and Casadesus (1994) carried out flow cytometry with *A. vinelandii* cells at different stages of growth. The number of chromosomes per cell increased from 4 in early exponential phase to >40 in the late exponential phase, to >80 in the early stationary phase, and to >100 in the late stationary phase. *A. chroococcum* CBD15, a strain isolated from the field of the Indian Agricultural Research Institute in New Delhi, India also has 20 copies of chromosomes at mid exponential phase of its growth (Bageshwar et al., 2017).

Base sequencing of the chromosomes has revealed the presence of 5,365,318 base pairs in *A. vinelandii* (Setubal et al., 2009) and 4,591,803 base pairs in *A. chroococcum* (Robson, Jones, Robson, Schwartz, & Richardson, 2015) as compared to 4,639,221 base pairs in *E. coli* (Blattner et al., 1997).

9.2 Construction of mutants of *Azotobacters*

For construction of mutants of *Azotobacters*, it must be ensured that the mutation occurs in all the copies of chromosomes of a cell, as otherwise the apparent mutants would not really be completely true mutants. For example, Brewin, Woodley, and Drummond (1999) observed while they were inserting the KIXX cassette into the *nifL* gene by transforming *A. vinelandii*, that the "apparent transformants could be shown by DNA blotting to contain wild-type *nifL* as well as the mutant sequence." Another observation of Brewin et al. (1999) was that "the strain in which transcription from KIXX was in the same direction as *nifA* (MV372) could not be isolated free from wild type *nifL*." These observations were the likely consequence of their failure to ensure that the KIXX cassette was transferred to all the copies of the chromosome of *A. vinelandii*. Bageshwar et al. (2017) inserted the kanamycin interposon ΩKm (Fellay, Frey, & Krish, 1987) into the gene *nifL* in a DNA fragment isolated from *A. chroococcum* CBD15 and cloned it in a plasmid which contained a β-lactamase gene but could not replicate in *A. chroococcum*. *A. chroococcum* CBD15 was then transformed by electroporation with this plasmid and kanamycin resistant, but ampicillin sensitive cells were selected. The interposon would be inserted in the chromosomal gene *nifL* by double point cross over and would disrupt the *nifL*

gene and would, therefore, render the downstream gene *nifA* devoid of any promoter. The cells should then be *nif* minus and should not be able to grow in BNF medium without added ammonium acetate. The kanamycin resistant transformed *A. chroococcum* cells could, however, grow well without any addition of ammonium acetate. Bageshwar et al. (2017) assumed that this was because of the presence of multiple chromosomes and incomplete segregation of chromosomes containing ΩKm inserted in the *nifL* gene in the chromosome of *A. chroococcum*. Hence, they continued subculturing the cells in BNF medium in presence of kanamycin and ammonium acetate. The *A. chroococcum* cells after the 18th subculture failed to grow in BNF medium containing kanamycin, but not containing any ammonium acetate. Genomic DNA was isolated from the *A. chroococcum* cells obtained after the 6th subculture and after the 18th subculture and PCR of the *nifL* region was performed. Fig. 2 shows the PCR products after agarose gel electrophoresis. It was obvious that even after the 6th subculture, chromosomes

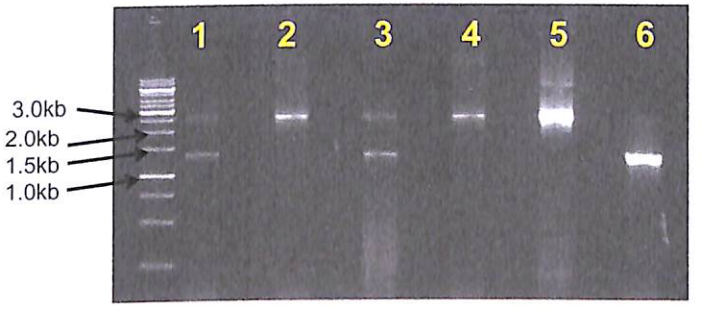

Fig. 2 Photograph of agarose gel after electrophoresis of PCR products from the *nifL* region of the chromosome of *A. chroococcum* CBD15. A modified version of this figure has appeared in Bageshwar et al. (2017). *A. chroococcum* was transformed with a plasmid that was unstable in *A. chroococcum* and that had cloned in it the *nifL* gene disrupted by the kanamycin interposon ΩKm. Lane 1: PCR done with chromosomes of transformed *A. chroococcum* colony no. 1 that was subcultured 6 times in BNF medium containing kanamycin and ammonium acetate; lane 2: PCR done with chromosomes of transformed *A. chroococcum* colony no. 1 that was subcultured 18 times in BNF medium containing kanamycin and ammonium acetate; lane 3: PCR done with chromosomes of transformed *A. chroococcum* colony no. 2 that was subcultured 6 times in BNF medium containing kanamycin and ammonium acetate; lane 4: PCR done with chromosomes of transformed *A. chroococcum* colony no. 2 that was subcultured 18 times in BNF medium containing kanamycin and ammonium acetate; lane 5: PCR done with chromosomes of transformed *A. chroococcum* colony no. 2 that was subcultured 18 times in BNF medium containing kanamycin and ammonium acetate, large excess of DNA electrophoresed; lane 6: PCR done with chromosomes of *A. chroococcum* CBD15 that was not transformed with any plasmid.

containing the interposon were less in number than the chromosomes containing no interposon. Only after the 18th subculture all the chromosomes had the interposon and had been mutated.

10. The enzyme that converts nitrogen into ammonia

Conversion of nitrogen into ammonia is the most important property of *Azotobacters* that makes these soil bacteria qualify as biofertilizer. Nitrogenase is the enzyme that carries out this conversion. In addition to dinitrogen, the isolated enzyme requires a strong reductant and ATP (Burris, 1991) and an anaerobic environment to maintain catalytic activity. Nitrogenase comprises two enzymes, the dinitrogenase and the dinitrogenase reductase. Dinitrogenase, which is also known as component I, is an iron-molybdenum protein. Dinitrogenase reductase, the component II, is an iron protein (Smith, Richards, & Newton, 2004; Yates, 1992). The dinitrogenase comprises two α subunits and two β subunits, each subunit having an average molecular weight of 56 kDa. The dinitrogenase reductase comprises two identical subunits, each subunit being 34 kDa in molecular weight (Yates, 1992). A single 4Fe:4S cluster is bridged between the two subunits of dinitrogenase reductase (Hausinger & Howard, 1980). Dinitrogenase, however, contains two Mo-Fe clusters (M center) and two Fe-S clusters (P-clusters) (Kim & Rees, 1992a). Each M center contains 7 Fe atoms out of 30 present in an iron-molybdenum protein, which participate in the reduction of dinitrogen. Altogether 16 Fe atoms are present in the four 4Fe:4S clusters (P-cluster) which accept 1 electron pair per cluster from dinitrogenase reductase (Kim & Rees, 1992b). The transfer of electrons from dinitrogenase reductase to dinitrogenase requires the mediation of Mg-ATP. Altogether four MgATP molecules are hydrolyzed for each pair of electrons transferred between dinitrogen reductase and dinitrogenase. As 4 pairs of electrons are transferred, the reaction as shown below, requires a minimum 16 MgATP under ideal conditions (Burris, 1991):

$$N_2 + 8e^- + 16MgATP + 8H^- \rightarrow 2NH_3 + H_2 + 16MgADP + 16Pi$$

Under normal physiological conditions, however, the requirement is closer to 20–30 molecules of Mg-ATP (Burris, 1991). The next step is the transfer of electron to the iron-molybdenum cofactor (Fe-Mo-co) which was first isolated by Shah and Brill (1977). Fe-Mo-co is the site where dinitrogen is reduced (Hoover, Imperial, Ludden, & Shah, 1988).

11. Protection of nitrogenase from oxygen in *Azotobacters*

Most of the nitrogen fixing microorganisms need anaerobic conditions to fix nitrogen. Not only the enzyme nitrogenase is inactivated by oxygen in these microorganisms, the genes involved in nitrogen fixation are also repressed by oxygen. In contrast, *Azotobacters* have the unique ability to fix nitrogen aerobically. The most widely accepted theory to explain oxygen tolerance of nitrogen fixation in *Azotobacters* is "respiratory protection" (Robson & Postgate, 1980). *Azotobacters* have one of the highest respiratory quotients among all biological systems examined (Haddock & Jones, 1977). Due to this, oxygen present inside the cells would be consumed at a very rapid rate resulting in very low intracellular oxygen concentration. Ramos and Robson (1985a) isolated mutants of *A. chroococcum* that were defective in aerobic nitrogen fixation and found that these mutants had lesions in citrate synthase (Ramos & Robson, 1985b). The enzyme citrate synthase is an essential component of the tricarboxylic acid cycle, which is the respiration hub of a cell. Cytochrome *d* is the terminal oxygen carrier of the electron transport chain; hence, it should contribute to high respiratory quotient. *Azotobacter* mutants deficient in cytochrome *d* failed to fix nitrogen in air (Kelly, Poole, Yates, & Kennedy, 1990). The level of cytochrome *d* messenger RNA in *A. vinelandii* cells that were actively fixing nitrogen was two- to threefold higher than those not fixing nitrogen (Moshiri, Smith, Taormino, & Mayer, 1991). Poole and Hill (1997) have summarized the evidence that cytochrome *d* indeed plays a crucial role in preventing nitrogenase in *A. vinelandii* from being inactivated by oxygen in air.

Another mechanism of protection of nitrogenase from oxygen appeared to be operative in *Azotobacters* and that involved binding of some proteins to nitrogenase, thus shielding it from oxygen. Such proteins have been characterized (Moshiri, Crouse, Johnson, & Maier, 1995; Moshiri, Kim, Fu, & Maier, 1994; Robson, 1979; Scherings, Haaker, Wassink, & Veeger, 1983; Shethna, Wilson, & Beinet, 1966). Nitrogenase-protective Shethna protein has been found to prevent oxygen-mediated cell death of *A. vinelandii* (Maier & Moshiri, 2000). The transcript mapping to the Shethna protein gene has been found to increase two- to sixfold under conditions of nitrogen fixation (Hamilton et al., 2011).

Interestingly, transcripts of genes that encode type IV pili also exhibit substantial increment during nitrogen fixation by *A. vinelandii*

(Hamilton et al., 2011). One of the many functions of type IV pili is cell aggregation. It has been suggested that cell aggregation could be another way of protecting nitrogenase of *A. vinelandii* from damage by oxygen in air (Hamilton et al., 2011).

12. DNA gyrase is necessary for nitrogen fixation by *Azotobacter vinelandii*

Coumermycin A and novobiocin, which are known to inhibit DNA gyrase (Kranz & Haselkorn, 1986), have been found to inhibit nitrogen fixation in *A. vinelandii* (Raina, Bageshwar, & Das, 1993a), suggesting that repression by oxygen of expression of *nif* genes may be mediated through alteration of super helical status of DNA.

13. Genes coding for the three polypeptides of the enzyme nitrogenase

The three contiguous genes *nifH*, *nifD* and *nifK* coding for the Fe protein (dinitrogenase reductase or component II), as well as the α subunit and β subunit of Mo-Fe protein (dinitrogenase or component I), respectively, of the enzyme nitrogenase were first isolated from the facultative anaerobe *Klebsiella pnumoniae*, taking advantage of the fact that this microorganism was amenable to the techniques of genetic manipulation developed for *Escherichia coli* (Cannon, Riedel, & Ausubel, 1979). The DNA fragment containing the three genes *nifH*, *nifD* and *nifK* of *K. pneumoniae* was nick translated and used as probe to isolate the *nif* genes of *A. vinelandii* from a cosmid library (Medhora, Phadnis, & Das, 1983). Gel electrophoretic analysis of the *Bgl*II digests of the DNA from several cosmids, all eliciting positive response to the *K. pneumonia* probe, revealed the presence of three distinct classes of signaling fragments (Fig. 3), suggesting the presence of three distinct classes of *HDK* genes in *A. vinelandii* (Medhora et al., 1983).

14. Alternative pathways of nitrogen fixation in *Azotobacters*

Until 1980 it was believed that the metal molybdenum comprising the iron-molybdenum protein dinitrogenase was essential for nitrogen fixation. Bishop and coworkers (Bishop, Jarlenski, & Hetherington, 1980, 1982; Bishop & Premakumar, 1992; Bishop et al., 1986) had presented evidence

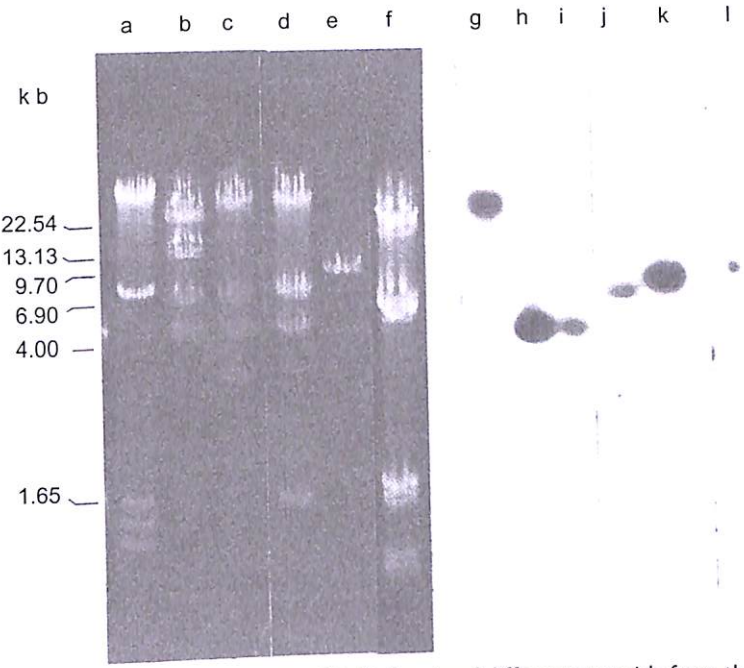

Fig. 3 Agarose gel electrophoresis of *Bgl*II digests of different cosmids from the cosmid library of the *A. vinelandii* UW genome. The positions of the DNA size markers are indicated at the extreme left. Lanes A to F show ethidium bromide stained DNA fragments generated by *Bgl*II digestion of different cosmids. Lanes A to D represent four different cosmids of *A. vinelandii* that contain *nif* genes. Lane E represents a DNA fragment containing the *nif* genes from *Rhizobium meliloti,* which has been used as a positive control. Lane F represents an *A. vinelandii* cosmid that does not contain any *nif* gene and has been used as a negative control. Lanes G to I represent signals generated by hybridization of the ^{32}P-labeled *nif* HDK genes of *K. pneumoniae* with Southern blots of the DNA transferred from lanes A to F. The dot in lane I is an artifact. All the lanes in this figure were taken from the same gel and corresponding Southern blot, which were exposed to the X-ray film for the same period of time after hybridization. *Reproduced from Medhora, M., Phadnis, S. H., & Das, H. K. (1983). Construction of a gene library from the nitrogen-fixing aerobe* Azotobacter vinelandii. Gene 25, *355–360*

that alternative pathways existed in *A. vinelandii* mediated by iron vanadium dinitrogenase and just iron dinitrogenase, the last one containing neither molybdenum, nor vanadium. The three pathways of nitrogen fixation were later named *nif*, *vnf* and *anf*, respectively. *A. chroococcum* has only the *nif* and the *vnf* pathways (Robson, Woodley, & Jones, 1986). The *vnf* nitrogenases have been isolated from both *A. vinelandii* and *A. chroococcum* (Hales, Case, Morningstar, Dzeda, & Maurer, 1986; Robson, Eady, et al., 1986). The *anf* nitrogenase has been isolated from *A. vinelandii* (Chisnell, Premakumar, & Bishop, 1988). Biochemical analysis has, however, revealed that the rate of

conversion of nitrogen to ammonia by molybdenum nitrogenase is much higher than the rates by either of the two alternative nitrogenases (Eady, 1996). The term ammonia has been used in this chapter to mean both free ammonia (NH_3) and ammonium ion (NH_4^+).

15. Genes of the constituents of the molybdenum dependent pathway of nitrogen fixation in *A. vinelandii*

As mentioned earlier, *nifH*, *nifD* and *nifK* are the genes for the three polypeptides of the molybdenum nitrogenase. These three genes are present in the same operon along with and followed by the *nif* genes *T* and *Y* and two open reading frames (*orfs*) of unknown function (Jacobson, Brigle, et al., 1989). The *nifU* gene product is a scaffold protein, while *nifS* gene product is cysteine desulphurase, both being involved in [Fe-S] cluster biosynthesis and assembly of Nif-specific metal clusters associated with the Fe protein and may also be with the MoFe protein (Johnson, Dean, Smith, & Johnson, 2005). The product of the gene *nifM* is thought to be involved in stabilization and activation of the Fe protein (Howard et al., 1986), an increase of six- to eightfold of its transcript being observed during nitrogen fixation (Hamilton et al., 2011). The gene *nifF* codes for flavodoxin (Bennett, Jacobson, & Dean, 1988), which is the reductant of the Fe protein. The product of *nifJ* couples the oxidation of pyruvate to the reduction of flavodoxin.

The genes *nifE*, *nifN*, *nifB* and *nifQ* are required for the biosynthesis of Fe-Mo-co. The genes *nifE* and *nifN* are thought to code for proteins that act as scaffold (Brigle, Weiss, Newton, & Dean, 1987), while the gene *nifB* codes for an S-adenosylmethionine protein that serves as an enzyme for Fe-Mo-co biosynthesis. The gene *nifQ* codes for a protein involved in Mo acquisition for the biosynthesis and maturation of Fe-Mo-co (Rubio & Ludden, 2008). The gene *nifX* codes for the FeMoco precursor carrier protein during the assembly (Rubio & Ludden, 2008). The gene *nifV* codes for homocitrate synthase. Homocitrate is a constituent of Fe-Mo-co in *A. vinelandii* (Zheng, White, Dean, 1997). The products of the genes *nifW* and *nif Z* are also required for full activity of the MoFe protein (Jacobson, Cash, et al., 1989) and their transcript levels were enhanced five- to ninefold during nitrogen fixation (Hamilton et al., 2011). The genes *nifT* and *nifY* may not be essential for nitrogen fixation (Dean & Jacobson, 1992).

In *A. vinelandii* a major *nif* cluster of 28,793 base pairs exists (Jacobson, Brigle, et al., 1989) containing the *nif* genes *H, D, K, T, Y, E, N, X, U, S, V, W, Z, M, F* in this order, interspersed with several *orf*s of unknown functions. The first operon in this cluster comprises the genes, *nifHDKTY orf1 orf2*, while the second operon comprises the genes *nifENX orf3 orf4*. There is an *orf5*, which seems to be the sole constituent of the third operon. The fourth operon contains the genes, $iscA^{nif}$, *nifUSV*, $cysE1^{nif}$, *nifWZM* and *clpX2*. The level of expression of the gene $iscA^{nif}$ was very high during diazotrophic growth (Hamilton et al., 2011), but its deletion did not affect nitrogen fixation (Jacobson, Cash, et al., 1989). The gene $cysE1^{nif}$ is involved in cysteine biosynthesis, but its deletion also was inconsequential to nitrogen fixation (Jacobson, Cash, et al., 1989). The gene *clpX2* was observed to be involved in recycling NifB and NifEN (Martinez-Noel, Curatti, Hernandez, & Rubio, 2011), but again this gene was also not essential for nitrogen fixation. The last operon in the major *nif* cluster contains the gene *nif F* (Jacobson, Brigle, et al., 1989).

In addition to the major *nif* cluster, there is a minor *nif* cluster in *A. vinelandii*. The first operon in this cluster contains the single gene *nafY*, which codes for Fe-Mo-co insertase (Rubio, Rangaraj, Homer, Roberts, & Ludden, 2002). Interestingly, the *nafY* gene was found to be well transcribed even in the presence of ammonia (Poza-Carrion, Jimenez-Vicente, Navarro-Rodriguez, Echavarri-Erasun, & Rubio, 2014). Next is the *rnfABCDGEH* operon. Deletion of the *rnf* gene cluster resulted in slower *nifHDK* gene expression. Deletion also resulted in lower dinitrogenase reductase activity, because of lower rate of incorporation of [4Fe-4S] into dinitrogenase reductase.

Dinitrogenase activity was, however, unaffected (Curatti, Brown, Ludden, & Rubio, 2005). It has been speculated that the control by the Rnf protein complexes is mediated through a redox regulatory mechanism (Curatti et al., 2005). Interestingly, Hamilton et al. (2011) found that expression of the *rnf* genes was enhanced under conditions of expression of the genes of all the three pathways, *nif*, *vnf* and *anf*.

Next is the *nifLA* operon, *nifL* proximal and *nifA* distal to the promoter (Raina et al., 1993a). The direction of transcription of this operon is opposite to the direction of transcription of the *nafY* operon and also of the *rnfABCDGEH* operon. NifA, the product of the gene *nifA*, serves as the positive regulator and must bind upstream of the promoters of all the *nif* operons for enabling their expression. NifL, the product of the gene *nifL*, on activation by oxygen or ammonium, acts as the negative regulator, as it interacts with NifA and neutralizes it.

The last in the minor cluster is the *nifB fdxN nifOQ rhdN grx5*^{nif} operon. Poza-Carrion, Echavarri-Erasun, and Rubio (2015) have reviewed the *nif* genes of *A. vinelandii*.

16. Genes of the constituents of the vanadium dependent pathway of nitrogen fixation in *A. vinelandii*

The gene *vnfH* that codes for the Fe protein (dinitrogenase reductase or component II) of the vanadium nitrogenase is followed in the same operon by a gene coding for a ferredoxin-like protein (Raina, Reddy, Ghosal, & Das, 1988). Site directed mutagenesis of the gene coding for the ferredoxin-like protein, rendered the vanadium dependent pathway of nitrogen fixation in *A. vinelandii* inoperative, establishing its essentiality (Raina, Bageshwar, & Das, 1993b). About 1 kb downstream of the *vnfH-fd* operon of *A. vinelandii*, another *vnf* operon containing *vnfD* coding for the α subunit of dinitrogenase, *vnfG* coding for the δ subunit and *vnfK* coding for the β subunit of dinitrogenase has been detected (Joerger et al., 1990). These four genes have also been detected in *A. chroococcum* (Robson, Woodely, Pau, & Eady, 1989; Robson, Woodley, et al., 1986). There is no counterpart of *vnfG* in the molybdenum dependent nitrogenase system. The gene *vnfY* has been detected downstream of the gene *vnfK* (Ruttimann-Johnson, Rubio, Dean, & Ludden, 2003). An insertion mutation in *vnfY* resulted in 10-fold less *vnf*-dinitrognase activity and substantially decreased level of incorporation of ^{49}V label into the *vnf*-dinitrogenase (Ruttimann-Johnson et al., 2003). It has been speculated that "*vnfY* has a role in the maturation of the V-dependent dinitrogenase, with a specific role in the formation of the V-containing cofactor and/or its insertion into apodinitrogenase" (Ruttimann-Johnson et al., 2003). Some role of the product of *vnfX* gene in Fe-V-co biosynthesis has also been suggested (Hamilton et al., 2011). The gene *vnfA* for the positive regulator has also been found in *A. vinelandii*, but in a separate cluster (Joerger, Jacobson, & Bishop, 1989). The *vnfENX* operon is present immediately downstream of the *vnfA* gene (Wolfinger & Bishop, 1991). Interestingly, mutation in *vnfE* or *vnfN* did not affect the *vnf* pathway of nitrogen fixation. Vanadium dependent nitrogen fixation was, however, abolished if *vnfE* or *vnfN* was mutagenized in an *A. vinelandii* strain which already had deletion in the *nifEN* region, suggesting that the gene products of *nif E* or *nifN*

could substitute gene products of *vnfE* or *vnfN* (Wolfinger & Bishop, 1991), though the latter two could be the preferred ones (Hamilton et al., 2011).

17. Genes of the constituents of the *anf* pathway of nitrogen fixation in *A. vinelandii*

The gene *anfH* coding for dinitrogenase reductase, *anfD* coding for the α subunit of dinitrogenase, *anfG* coding for the third subunit δ and *anfK* coding for the β subunit of dinitrogenase of the *anf* pathway, the pathway that is independent of both molybdenum and vanadium, are present in the same operon (Joerger, Premakumr, Wolfinger, & Bishop, 1989). About 700 bp upstream of *anfH* is present the *anfA* gene, the positive regulator of the *anf* pathway (Joerger, Jacobson, et al., 1989).

It may be mentioned here that neither the *vnf*, nor the *anf* system has all the genes necessary to operate these systems. The products of the genes *nifU*, *nifS* and *nifV* participate in all the three nitrogen fixing pathways (Kennedy & Dean, 1992) and so does the product of the gene *nifB* (Joerger, Premakumar, & Bishop, 1986). On the other hand, protein products of the genes *vnfE*, *vnfN*, *vnfX* and *vnfY* are thought to participate in the *anf* pathway, as transcripts of these genes were produced in much larger quantities during nitrogen fixation by this pathway (Hamilton et al., 2011). Interestingly, mutation in *vnfH* severely affected the *anf* pathway (Joerger, Wolfinger, & Bishop, 1991).

18. Effect of molybdenum and vanadium ions on transcription of the structural genes of nitrogenases of the three pathways

Molybdenum ion has been found to be essential for transcription of the structural genes of nitrogenase of the *nif* pathway, as revealed by *lacZ* fusion analysis (Raina, Bageshwar, & Das, 1992) and Northern analysis (Jacobitz & Bishop, 1992; Luque & Pau, 1991), but vanadium ion has not been found necessary for transcription of the structural genes of nitrogenase of *vnf* pathway. Molybdenum ion inhibited both *vnf* and *anf* pathways, while vanadium ion inhibited the *anf* pathway (Bishop et al., 1982; Chisnell et al., 1988; Jacobson, Premakumar, & Bishop, 1986; Raina et al., 1992). Newton (2015) has reviewed the working of the different nitrogenases.

19. The promoters of the operons containing the genes involved in nitrogen fixation in *Azotobacters*

Beynon, Cannon, Buchanan-Wollaston, and Cannon (1983) had found out that the promoter sequences of the *nif* operons of *K. pneumoniae* were, unlike the most common sigma 70 dependent promoter sequences of Gram-negative bacteria, an invariant GG–N_{10}–GC located −24 and −12 nucleotides upstream from the transcriptional start site +1. Such a sequence is recognized by sigma 54 which is coded for by the gene *ntrA*. Eleven potential *nif* promoters with the same invariant sequence had been identified in the main *nif* cluster of *A. vinelandii* (Jacobson, Brigle, et al., 1989). For example, the *nif HDKTY* operon has the sequence GGCACAGACGCTGC as its promoter, while the *nif ENX* operon has the sequence GGTACAGGCATTGC as its promoter (Jacobson, Brigle, et al., 1989). The consensus sigma 54 promoter sequence of GGCACGNNNNTTGC has been derived after compilation and analysis of 186 published sequences of sigma 54 dependent promoters (Barrios, Valderrama, & Morett, 1999). The sigma 54-RNA polymerase holoenzyme binds to the specific promoter but forms a transcriptionally inactive closed complex. In order to transform the closed complex into an open complex and initiate transcription, interaction with a transcriptional activator bound upstream, with concomitant nucleotide hydrolysis, is essential (Buck, Gallegos, Studholme, Guo, & Gralla, 2000; Xu & Hoover, 2001).

20. Upstream activator binding site

Presence of activator binding site upstream of the promoters of nitrogen fixation gene operons was first reported by Buck, Miller, Drummond, and Dixon (1986). Similar activator binding sequences (invariant TGT–N_{10}–ACA), which are binding sites of NifA, have also been detected about 100 base pairs upstream of the *nif* promoters of *A. vinelandii* (Jacobson, Brigle, et al., 1989). Examples are activator sequence TGTAGCAATTACAACA upstream of the *nif HDKTY* promoter and TGTTGCAAACCTGACA upstream of the *nif ENX* promoter (Jacobson, Brigle, et al., 1989).

21. Expression of the *nifLA* operon of *A. vinelandii* is an enigma

As mentioned earlier, the genes *nifL* and *nifA* of *A. vinelandii* are present in the same operon, *nifA* distal and *nifL* proximal to the promoter. Fig. 4

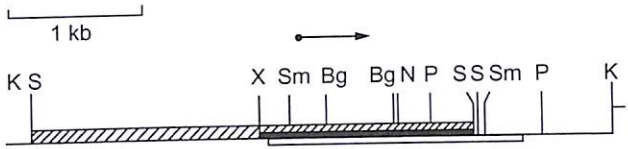

Fig. 4 Partial restriction map of the *nifL* gene of *A. vinelandii* UW and its upstream region. Abbreviations: Bg, *Bgl*II; Bm, *Bam*HI; K, *Kpn*I; N, *Not*I; P, *Pst*I; R, *Eco*RI; S, *Sal*I; Sm, *Sma*I; X, *Xho*I. *Reproduced from Raina, R., Bageshwar, U. K. & Das, H.K. (1993a). The Azotobacter vinelandii nifL-like gene: Nucleotide sequence analysis and regulation of expression.* Molecular and General Genetics 237, *400–406.*

shows the partial restriction map of the *nifL* gene and its upstream region reproduced from Raina et al. (1993a). The open bar below the line represents the region, the base sequence of which has been determined (Raina et al., 1993a). The base A of the translation initiator codon ATG is the 107th base downstream of the last base G of the first *Sma*I site from the left in Fig. 4. The transcription initiation site of the *nifLA* operon has been mapped by S1 nuclease assay to be the base C, the 69th base upstream of the translation initiation codon ATG and the 38th base downstream of the last base G of the first *Sma*I site from the left in Fig. 4 (Mitra, Das, & Dixit, 2005).

There is a potential sigma 54 promoter, GGCACAGGATTTGC (shown by the filled circle in Fig. 4, the arrow indicating the direction in which the translation initiation codon ATG is), the last base C being the 81st base upstream of the translation initiation codon ATG and the first base G being the 13th base downstream of the last base G of the first *Sma*I site from the left. There are 11 bases between the last base of the potential sigma 54 promoter and the transcription initiation site, which is exactly what is expected. There is a potential NifA binding site, TGTGCGCTTTCGCACA, 78 bases upstream of the potential sigma 54 promoter, which also seems normal. The last base A of the potential NifA binding site is the 173th bases upstream of the translation initiation code ATG of the *nifL* gene and the 61st bases upstream of the first base C of the first *Sma*I site from the left in Fig. 4. The *Xho* I site in Fig. 4 is about 220 bases upstream of the first *Sma*I site from the left.

Now let us look at some of the surprising findings. The expression of the *nifLA* operon was found to be unaffected in an *A. vinelandii* strain that had a deletion in the *ntrA* gene (Raina et al., 1993a). The *ntrA* gene codes for sigma 54 (Hirschman, Wong, Sei, Keener, & Kustu, 1985). The expression of the *nifLA* operon was also unaffected in an *A. vinelandii* strain that had

a deletion in the *ntrC* gene (Raina et al., 1993a). In *K. pneumoniae*, the face of the helix dependent upstream binding of NtrC converts the transcriptionally non-productive closed complex between sigma 54 and the *nifLA* promoter into the transcriptionally productive open complex (Minchin, Austin, Dixon, 1989; Popham, Szeto, Keener, & Kustu, 1989). The observations of Raina et al. (1993a) related to *ntrA* and *ntrC* genes of *A. vinelandii* were subsequently confirmed by Blanco, Drummond, Woodley, and Kennedy (1993). Further, the expression of the *nifLA* operon was also unaffected in an *A. vinelandii* strain that had a deletion in the *nifA* gene (Raina et al., 1993a). This was in contrast to earlier observations in *K. pneumoniae* (Drummond, Clement, Merrick, & Dixon, 1983; Ow & Ausubel, 1983). The tentative explanation is that in wild type *A. vinelandii*, the expression of the *nifLA* operon is facilitated by the sigma 54 promoter, but the expression continues during unavailability of sigma 54, aided by a hitherto uncharacterized element present upstream. It has not been ascertained as to whether the transcription initiation site remains unaltered during unavailability of sigma 54. The expression of the *nifLA* operon was determined by making use of *lac* fusion constructs. When the 1.6 kb *Xho*I–*Sal*I fragment (the filled bar in Fig. 4), which contained both the potential sigma 54 promoter and the potential NifA binding site, was fused to the promoterless β-galactosidase gene, the β-galactosidase activity elicited in *A. vinelandii* UW was only ~370 Miller units. This was enhanced to ~1000 Miller units when the 3.2 kb *Sal*I–*Sal*I fragment (the hatched bar in Fig. 4) was fused to the promoterless β-galactosidase gene, suggesting that some element further upstream of the *Xho*I site was necessary for full expression of the *nifLA* operon. Deletion of the 591 base pair *Bgl*II–*Bgl*II fragment (see Fig. 4) from the coding region (+142 to +733 with respect to the transcription initiation site) of the *nifL* gene completely abolished expression of the *nifLA* operon, indicating the presence of a positive regulatory element there (Mitra et al., 2005). Introduction of this fragment in *A. vinelandii* cloned in a stable plasmid, did not restore activity, suggesting that the regulatory element does not function *in trans*. Several protein molecules have been found to bind to this region and this binding appeared to be specific, as excess calf thymus DNA could not affect the binding. The specific binding sites have been mapped by DNase I foot printing. Introduction of four bases just before the binding sites completely disrupted the expression, indicating that interaction of these proteins was face of the helix dependent (Mitra et al., 2005).

22. Interaction of NifL and NifA

As mentioned earlier, NifL in presence of oxygen or ammonium interacts with NifA and neutralizes it. NifL and NifA of *A. vinelandii* have been isolated and purified (Austin, Buck, Cannon, Eydmann, & Dixon, 1994). NifA has been found to be capable of specific binding to a DNA sequence upstream of the *nifH* promoter in an isolated DNA fragment containing the *nifH* gene and of promoting *in vitro* transcription from the *nifH* promoter. NifL was able to counteract this NifA action *in vitro* (Austin et al., 1994). Stoichiometric amount of NifL was necessary to inactivate NifA *in vitro*, suggesting direct protein-protein interaction as opposed to catalytic intervention or phosphate transfer (Dixon, 1998; Hill, Austin, Eydmann, Jones, & Dixon, 1996). NifL of *A. vinelandii* happens to be a flavoprotein with FAD as the prosthetic group. NifL did not inhibit NifA activity *in vitro* in the presence of the reducing agent sodium dithionite even under aerobic conditions, as the bound flavin was then in the reduced state. Interestingly, addition of ADP resulted in NifL regaining its ability to inactivate NifA *in vitro* even in the presence of dithionite. It has been inferred that both energy and redox status of NifL could be important for its interaction with NifA (Dixon, 1998; Hill et al., 1996). An N-terminal domain and a C-terminal domain of NifL have been isolated by partial digestion with trypsin in presence of adenosine nucleotides (Söderbäck et al., 1998). It was the N-terminal domain that was reduced by dithionite, suggesting that flavin was bound to this domain. The precise seat of the redox response of NifL has been traced to be in the first 146 amino acids, where indeed a conserved S-motif (PAS-like domain) has been found (Söderbäck et al., 1998; Taylor & Zhulin, 1999; Zhulin, Taylor, & Dixon, 1997). The isolated N-terminal domain did not, however, inhibit NifA activity. On the other hand, the C-terminal domain of NifL has been found to bind ADP (Söderbäck et al., 1998) and it was the C-terminal domain of NifL that interacted with the N-terminal domain of NifA (Money, Jones, Dixon, & Austin, 1999). A complex of purified NifA and NifL formed in presence of MgADP, when subjected to limited proteolysis, revealed protection of the N-terminal region of NifA close to the Q-linker (Money, Barrett, Dixon, & Austin, 2001). Interestingly, NifL devoid of the first 146 amino acids could counteract NifA activity *in vitro* in response to ADP and also in response to fixed nitrogen, but not in response to oxygen. It thus appeared that the domain of

NifL that sensed redox status was distinct from the domain that sensed presence of fixed nitrogen or ADP (Söderbäck et al., 1998). Nitrogen sensing ability of NifL *in vitro* was found to be dependent on a PII regulatory protein like GlnK, and also GlnD, which uridylylates GlnK in absence of fixed nitrogen, but de-uridylylates GlnK in presence of fixed nitrogen (Little, Colombo, Leech, Dixon, 2002; Schmitz, Klopprogge, & Grabbe, 2002). *In vivo* studies with specific mutants of *glnK* is in conformity with the view derived from *in vitro* experiments (Little et al., 2002) that the de-uridylylated form of GlnK generated in presence of fixed nitrogen was involved in interaction with the C-terminal kinase-like domain of NifL promoting its inhibition of NifA, while the uridylylated form of GlnK generated in absence of fixed nitrogen is unable to mediate inhibition by NifL of NifA (Rudnick, Kunz, Gunatilaka, Hines, & Kennedy, 2002). De-uridylated form of GlnK has been found to form a GlnK-NifL-NifA ternary complex in presence of fixed nitrogen (Martinez-Argudo, Richard, & Dixon, 2004). The GlnK-NifL-NifA ternary complex was dissociated when GlnK was uridylylated as a result of unavailability of fixed nitrogen (Little, Reyes-Ramirez, Zhang, van Heeswijk, & Dixon, 2000; Martinez-Argudo, Little, Shearer, Johnson, & Dixon, 2005). Inhibition of NifA *in vitro* by NifL, however, was relieved by 2-oxoglutarate. Experiments using isothermal titration calorimetry have suggested that when fixed nitrogen is limited, binding of 2-oxoglutarate to the GAF domain of NifA, might bring about a conformational change in NifA, that makes it resistant to inhibition by NifL (Little & Dixon, 2003). All functions of NifA, namely, DNA binding, interaction with sigma 54-RNA polymerase holoenzyme and catalyzing initiation of transcription are inhibited by NifL (Barrett, Ray, Sobczyk, Little, & Dixon, 2001). An arginine residue at position 306 of NifL of *A. vinelandii* has been found to be essential for alteration of conformation of NifL under fixed nitrogen limiting conditions, that results in dissociation of the NifL–NifA complex and allows NifA to function as the positive regulator of nitrogen fixation.

Interestingly, the central domain of NifL has been found to be involved in bringing about the change in the conformation of NifL that decides whether NifL would be active or inactive in blocking NifA function, in response to the status of fixed nitrogen or oxygen (Little, Martinez-Argudo, & Dixon, 2006). An arginine residue has been identified in the central domain also that serves as the conformation switch (Little et al., 2006; Martinez-Argudo et al., 2004).

In addition, site-directed mutagenesis of the H domain of NifL has revealed that this domain plays an important role in transmission of signals leading to attainment of proper conformation of NifL suitable for inhibition of NifA (Little, Martinez-Argudo, Perry, & Dixon, 2007). On the other hand, both the N-terminal and central domains of NifA have been observed to be involved in its interaction with NifL (Barrett et al., 2001). Interestingly, it is the central AAA+ domain of NifA that has been found to activate the sigma 54-RNA polymerase holoenzyme to initiate transcription with concomitant hydrolysis of ATP, while the C-terminal domain of NifA would bind to the activator binding sequence of DNA upstream of the *nif* promoters (Buck et al., 2000). Poza-Carrion et al. (2015) have reviewed information on the NifA-NifL-GlnK system of *A. vinelandii*.

23. Maximizing nitrogen fixation and excretion of ammonia by *Azotobacters*

23.1 Insertion *in vivo* of the kanamycin resistance cassette into the *nifL* gene of *A. vinelandii*

Kennedy and coworkers (Bali, Blanco, Hill, & Kennedy, 1992) inserted *in vivo* the kanamycin resistance cassette KIXX from the plasmid pUC4-KIXX into the *nifL* gene in the chromosome of *A. vinelandii*, thereby removing the C-terminal quarter of the protein product NifL. The acetylene reduction activity (ARA) exhibited by this strain was 48 nmol of ethylene produced per minute, per mg of protein, which was marginally better than the ARA elicited by the wild type strain. The engineered strain could, however, elicit ARA in presence of 15 mM ammonia almost the same as that in the absence of ammonia. This was in contrast to the wild type strain whose ARA was almost completely abolished by 15 mM ammonia. The wild type *A. vinelandii* excreted very little ammonia, while excretion by the engineered strain was perceptible from around 8 h of growth which resulted in 6.5 mM ammonia in the medium by 13 h that remained about the same till 26 h. Interestingly, this engineered strain was found to contain the aminoglycoside phosphotransferase promoter within the inserted KIXX cassette directing transcription in the direction away from *nifA*, but not toward *nifA*. Bali et al. (1992) speculated that the *nifA* gene in the engineered mutant was possibly being expressed from an "unexpected promoter activity in the oppositely oriented cassette or from promoter like sequences generated by the KIXX insertion in the *nifL* region." Brewin et al. (1999) had

subsequently constructed a similar mutant strain using the same kanamycin resistance cassette and confirmed the findings of Bali et al. (1992). Later, Barney, Eberhart, Ohlert, Knutson, and Plunkett (2015) also constructed a similar mutant strain, but by inserting a kanamycin resistance cassette from a different source (pBBR1MCS2) (Kovach et al., 1995). Even though this insertion was exactly at the same location and direction in the *nifL* gene of *A. vinelandii* as was done by Bali et al. (1992), this mutant could not reduce nitrogen or acetylene. Insertion of the cassette at a slightly upstream location did not improve matters. Interestingly, Barney et al. (2015) could recover spontaneous mutants from both their *nif* negative mutants, which were not only *nif* positive, i.e., reduced nitrogen, but also excreted ammonia in the medium resulting in accumulation of 8.5 mM ammonia after 4 days and 35 mM after more than a week. Base sequence determination by Barney et al. (2015) revealed a point mutation exactly at the same location inside the kanamycin resistance cassette in both the spontaneous *nif* positive mutants. Base sequence determination also revealed that there was indeed a small difference in the base sequence of the kanamycin resistance cassette used by Bali et al. (1992) and Brewin et al. (1999) from the one used by Barney et al. (2015). The base sequence of the specific region of the kanamycin resistance cassette used by Bali et al. (1992) and Brewin et al. (1999) was the following:

CCCAGTAGCT**CGAGAAGCTTCC**CGGGCATTCCGCCCG,

while the base sequence of the same region of the kanamycin resistance cassette used by Barney et al. (2015) was the following:

CCCAGTAGCT**GACATTCATCCGGATCAT**CGGGCATTCC GCCCG.

The bases that were different are shown in bold letters. The base sequence of the same region of both the spontaneous *nif* positive mutants obtained by Barney et al. (2015) was the following:

CCCAGTAGCT**GA**T**ATTCATCCGGATCAT**CGGGCATT CCGCCCG, the point mutation to T from C being shown by a larger font letter. No comment is possible to offer to explain why such small change in base sequence upstream of *nifA* results in a *nif* positive or a *nif* negative strain.

23.2 Deletion of the *nifL* gene and continuation of expression of the *nifA* gene from the native *nifLA* promoter

Blanco et al. (1993) deleted the N-terminal region of the *nifL* gene from the chromosome of *A. vinelandii* and observed release of upto 15 mM ammonia

in the medium in stationary phase. Ortiz-Marquez, Nascimento, de los Angeles Dublan, and Curatti (2012) deleted almost the whole of the *nifL* gene and reported 10-fold excretion of ammonia compared to the wild type, but to the extent of only 270 µM after 48 h. Barney et al. (2015) had deleted both *nifL* and *nifA* from the chromosome of their *A. vinelandii ΔureABC* strain resulting in a *nif* minus strain. This strain had the *nifLA* promoter intact. Then they inserted the *nifA* gene in the correct orientation downstream of the *nifLA* promoter. This strain was *nif* plus, but did not excrete ammonia to the extent reported by earlier workers for their mutants with deletion of *nifL* or insertion of antibiotic resistance cassette in the *nifL* gene.

23.3 Deletion of the *nifL* gene and insertion of the *Tet* promoter there

The *nifL* gene of *A. vinelandii* UW (the strain from Madison, Wisconsin, USA) and of *A. chroococcum* CBD15 (a strain isolated from the fields of Indian Agricultural Research Institute, New Delhi, India) were deleted and the constitutive *Tet* promoter from the plasmid pBR322 was inserted there in the correct orientation, thus bringing the *nifA* gene under the control of the *Tet* promoter (Bageshwar et al., 2017 and unpublished work of Umesh Bageshwar). The names given to these engineered strains were *A. vinelandii* HD and *A. chroococcum* HKD15, respectively. ARA exhibited by the engineered strains was three- to fourfold of the wild type strains, when grown in the absence of fixed nitrogen (Table 2). Fixed nitrogen in the form of ammonium acetate in the growth medium reduced ARA activity of the wild type strains by 70–74%, while ARA of the engineered strains was reduced by only 15–18% (Table 2). Ammonia excretion by the engineered strains was close to ninefold of the wild type strains (Table 3). Fixed nitrogen in the form of potassium nitrate in the growth medium reduced ammonia excretion of the wild type strains by 86–87%, while ammonia excretion of the engineered strains was reduced by only 12–15% (Table 3). Ammonia excretion was followed till 60 h of growth of the engineered cells and was found to remain unaffected.

Brewin et al. (1999) had observed up to 35 mM ammonium accumulation in the medium when *nifA* was expressed from the *tac* promoter induced with IPTG.

23.4 Mutagenesis of *nifA* to make NifA resistant to inhibition by NifL

It has been mentioned in an earlier section that NifL interacts with both the N-terminal and central domains of NifA (Barrett et al., 2001). Dixon and

Table 2 Acetylene reduction activity (ARA) exhibited by the engineered *Aztobacter* strains.

Azotobacter strain	Ethylene produced		
	N-free growth medium (nmol/mg protein/h)	Growth medium contains 0.11% ammonium acetate (nmol/mg protein/h)	Decrease in ARA (%)
A. vinelandii UW[a]	526	136	74
A. vinelandii HD[b]	1781	1466	18
A. chroococcum CBD15[c]	755	227	70
A. chroococcum HKD15[d]	3153	2682	15

[a]Wild type strain from Madison, Wisconsin, USA.
[b]Engineered strain, *nifL* gene deleted, *nifA* gene under the control of the constitutive *Tet* promoter.
[c]Wild type strain isolated from the fields of Indian Agricultural Research Institute, New Delhi, India.
[d]Engineered strain, *nifL* gene deleted, *nifA* gene under the control of the constitutive *Tet* promoter.
Compiled from Paul, S., Verma, O. P. & Das, H. K. (2005). Evaluation of the modified Azotobacter strains for their performance as biofertilizers in wheat. Report of project funded by the Department of Biotechnology; Bageshwar, U. K., Srivastava, M., Pardha-Saradhi, P., Paul, S., Gothandapani, S., Jaat, R. S., Shankar, P., Yadav, R., Biswas, D. R., Kumar, P. A., Padaria, J. C., Mandal, P. K., Annapurna, K. & Das, H. K. (2017). An environmentally friendly engineered Azotobacter strain that replaces a substantial amount of urea fertilizer while sustaining the same wheat yield. Applied and Environmental Microbiology, 83, e00590–17.

coworkers (Reyes-Ramirez, Little, & Dixon, 2002) *in vitro* mutagenized the two corresponding regions of the isolated *nifA* gene of *A. vinelandii* by carrying out error-prone PCR using Taq DNA polymerase. The primers for PCR had specific restriction sites at the 5′-ends that were chosen in accordance with the existing sites flanking the regions to be mutagenized of the *nifA* gene. The products of error prone PCR were used to replace the corresponding wild type regions of the *nifA* gene by restriction digestion and ligation. The *nifA* gene was a part of the complete *nifLA* operon (containing the *nifL* gene also), that was cloned in a plasmid. For identifying NifA mutant plasmids which would not be inactivated by NifL, Dixon and coworkers constructed a reporter plasmid containing a fusion of the *K. pneumoniae nifH* promoter with a promoter-less *lacZ* gene. They had earlier observed that NifL expressed in *E. coli* from the *nifL* gene cloned in a plasmid would inactivate NifA expressed in the same *E. coli* from the *nifA* gene in the presence of oxygen and fixed nitrogen. This inactivation would

Table 3 Ammonia excretion by the engineered Aztobacter strains.

	Ammonia excreted		
Azotobacter strain	N-free growth medium (µg/mg protein)	Growth medium contains 0.1% potassium nitrate (µg/mg protein)	Inhibition of excretion (%)
A. vinelandii UW[a]	7	1	86
A. vinelandii HD[b]	208	184	12
A. chroococcum CBD15[c]	31	4	87
A. chroococcum HKD15[d]	267	227	15

[a]Wild type strain from Madison, Wisconsin, USA.
[b]Engineered strain, *nifL* gene deleted, *nifA* gene under the control of the constitutive *Tet* promoter.
[c]Wild type strain isolated from the fields of Indian Agricultural Research Institute, New Delhi, India.
[d]Engineered strain, *nifL* gene deleted, *nifA* gene under the control of the constitutive *Tet* promoter.
Compiled from Paul, S., Verma, O. P. & Das, H. K. (2005). Evaluation of the modified Azotobacter strains for their performance as biofertilizers in wheat. Report of project funded by the Department of Biotechnology; Bageshwar, U. K., Srivastava, M., Pardha-Saradhi, P., Paul, S., Gothandapani, S., Jaat, R. S., Shankar, P., Yadav, R., Biswas, D. R., Kumar, P. A., Padaria, J. C., Mandal, P. K., Annapurna, K. & Das, H. K. (2017). An environmentally friendly engineered Azotobacter strain that replaces a substantial amount of urea fertilizer while sustaining the same wheat yield. Applied and Environmental Microbiology, 83, e00590–17.

be relieved in the absence of oxygen and fixed nitrogen (Reyes-Ramirez, Little, & Dixon, 2001; Söderbäck et al., 1998). They transformed *E. coli* with the plasmid library containing the mutagenized *nifA* stock in the complete *nifLA* operon and also with the reporter plasmid. The transformed *E. coli* cells were plated on minimal agar containing ammonium sulfate, glucose and X-gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) and the antibiotics specific for the plasmids and incubated aerobically. The *E. coli* cells containing the NifA mutant plasmids that were unaffected by NifL even in the presence of oxygen and fixed nitrogen were identified by their deep blue color. The activities of the best mutants were similar to the activity of NifA in the absence of NifL (Reyes-Ramirez et al., 2002). It is important to restate that the above-mentioned experiments were all in *E. coli* and involved only NifA and NifL of *A. vinelandii* and *nifH* promoter of *K. pneumoniae*. The results only reflect NifA activity, not nitrogen fixation.

23.5 Introducing a plasmid containing the *nifH* gene into *A. vinelandii*

Nag and Pal (2013) cloned the *nifH* gene (the gene for the Fe-protein of nitrogenase) of *A. vinelandii* in the wide host range plasmid, pJB654

(Blatny, Brautaset, Winther-larsen, Haugan, & Valla, 1997) downstream of the inducible metatoluic acid pathway promoter Pm and transferred it into *A. vinelandii* UW from *E. coli* by triparental mating using the helper plasmid pRK2013 (Ditta, Stanfield, Corbin, & Helinski, 1980). Using NifH antibody, they found two- to threefold NifH protein in the lysate of the engineered cells that were induced with *m*-toluic acid. They also observed twofold ARA and 70% higher excretion of ammonia by the engineered cells compared to the wild type ones. It would be interesting to point out here that even in wild type *A. vinelandii* the amount of *nifH* transcript and also the Fe-protein had always been found to be higher than the *nifD–nifK* transcripts and the FeMo-protein. Dingler, Kuhla, and Wassink, Oelze (1988) evaluated by Western blotting (immunoblotting) the amounts of Fe-protein and FeMo-protein in nitrogen-fixing growing wild type *A. vinelandii* at different dissolved oxygen and sucrose concentrations and found their ratio to be more or less constant at 1.45:1.0. Hamilton et al. (2011) observed that transcript level of *nifH* increased 143-fold in nitrogen-fixing growing wild type *A. vinelandii* compared to those growing in non-nitrogen fixing condition, while the levels of transcripts of *nifD* and *nifK* increased only 54-fold under the same conditions. Nag and Pal, however, appreciated that their engineered strain would not be suitable for use in the field, as it required application of the chemical agent m-toluic acid for induction of the additional *nifH* gene.

23.6 Engineering of genes other than those associated with the *nif* complex

Sashidhar and Podile (2009) isolated the glucose dehydrogenase gene of *E. coli* and the glutamine synthetase promoter and also the phosphate transport system promoter of *A. vinelandii* by conducting PCR using sequence-based primers. The glucose dehydrogenase gene was cloned downstream of each of the two *A. vinelandii* promoters in the broad host range plasmid vector pMMB206. *A. vinelandii* was transformed with the resultant recombinant pMMB206 plasmids. The transformed *A. vinelandii* strains elicited enhanced production of gluconic acid resulting in enhanced release of inorganic phosphate from tricalcium phosphate. Nitrogen fixation was, however, slightly reduced.

Barney et al. (2015) deleted the urease gene complex *ureABC* from the chromosome of *A. vinelandii* and replaced it with a streptomycin resistance cassette. The streptomycin resistant cells accumulated urea in the medium to the extent of 110 μM in 11 days. They also carried out mutagenesis through

the kanamycin resistant mariner transposon to obtain mutants that excreted more ammonia than the wild type *A. vinelandii*. They used a nitrogen biosensor strain to screen for ammonia excreting mutants. The biosensor strain was constructed by inserting a promoterless β-galactosidase gene downstream of the *scrX* promoter of *A. vinelandii* and replacing the *nifLA* operon by a tetracycline resistance gene cassette. This strain turned blue when grown on agar plates containing Burk's medium plus ammonium acetate but did not grow in a medium containing no nitrogen source. The transposon mutagenized cell stock of *A. vinelandii* was layered on nitrogen free agar medium containing X-gal that was already layered with the biosensor cells. Mutants that excreted ammonia allowed the biosensor cells to grow which turned blue. The transposon containing mutants were isolated and purified from the blue spots on the plate free from the biosensor strain by using selective antibiotics. The mutants were checked again by plating on X-gal medium and those that turned blue even in the absence of the biosensor strain were rejected as false positives. Base sequence determination of the region of transposon insertion revealed that all the mutants had their ammonia transport gene *amtB* disrupted by the transposon. Ammonia estimation in the medium, however, revealed presence of only 5 µM ammonia after 20 h which was reduced to 2 µM after 50 h.

Ambrosio, Ortiz-Marqueza, and Curattia (2017) replaced the native promoter of the glutamine synthase gene of *A. vinelandii* DJ by the *trc* promoter, which must be induced with IPTG to produce the enzyme glutamine synthase. Glutamine synthase converts glutamic acid to form glutamine by the amidation of one of its carboxyl groups in presence of ammonia and ATP. This is one of the two main enzymes that directly consume ammonia in a bacterial cell, the other one being glutamic dehydrogenase. So, in the strain engineered by Ambrosio et al. (2017), no glutamine would be formed, if no inducer like IPTG (isopropyl thiogalactoside) is added and hence considerable amount of ammonia would be saved and excreted. They also constructed a double mutant by deleting the *nifL* gene. Glutamine, however, is a component of all proteins, so protein synthesis would stop if glutamine is not available and the cells would not grow. The strategy adopted by Ambrosio et al. (2017) to circumvent this problem was to use suboptimal amount of IPTG for growing the cells. Using 1 µM IPTG they could detect close to 15 mM ammonia released in the medium by the single mutant, while the double mutant released close to 25 mM.

24. Use of genetically engineered *Azotobacters* as biofertilizers

24.1 *Azotobacter* with enhanced phosphate solubilizing capability

Sorghum seeds inoculated with engineered *A. vinelandii*, that had better phosphate solubilizing capability because of the presence of a broad host range plasmid containing *E. coli* glucose dehydrogenase gene under the control of *A. vinelandii* promoters, produced sorghum seedlings in green house, that were of greater height and higher fresh weight compared to those inoculated with wild type *A. vinelandii* (Sashidhar and Podile, 2009).

24.2 *Azotobacter* with enhanced capacity to excrete fixed nitrogen

The growth of the green alga *Chlorella sorokiniana* was used as a model system to test biofertilizer activity of engineered *A. vinelandii* (Barney et al., 2015). Their engineered *A. vinelandii* cells, in which the urease gene complex *ureABC* was replaced with a streptomycin resistance gene cassette, could support good growth of *Chlorella sorokiniana* in a medium devoid of any fixed nitrogen. Their transposon mutagenized Δ*amtB A. vinelandii* cells also supported good growth of *Chlorella sorokiniana* in a medium devoid of any fixed nitrogen. The double mutant Δ*ureABC* Δ*amtB A. vinelandii* that was resistant to both streptomycin and kanamycin supported very good growth of *Chlorella sorokiniana* in the same medium. Wild type *A. vinelandii* was much less effective.

24.3 *Azotobacter* that consumes less ammonia

Ambrosio et al. (2017) used the microalga *Scenedesmus obliquus* as the test system for evaluating biofertilizer activity of their engineered *A. vinelandii* in which the native promoter of the glutamine synthase gene was replaced by the *trc* promoter that needed induction. Robust growth of the microalgae could be seen when cocultured with the engineered *A. vinelandii* that received 1 μM IPTG as inducer. The beneficial effect of the engineered *A. vinalandii* was, however, lost when 100 μM IPTG was added instead. Ambrosio et al. (2017) also tested the biofertilizer activity of the engineered *A. vinelandii* strain on hydroponic cultures of cucumber plants (*C. sativus*). Weight of leaves and roots were recorded 25 days after germination. Inoculation with *A. vinelandii* DJ had negligible effect. Inoculation with the

engineered strain induced with 1 µM IPTG elicited weight of leaves and roots that was twice to thrice of the non-inoculated. An engineered strain that also had a deletion in the *nifL* gene had no added beneficial effect. The weights of leaves and roots of plants that received 3 mM nitrate were, however, about twice that of those inoculated with engineered *A. vinelandii* cells.

24.4 *A. chroococcum* with its *nifL* gene deleted and its *nifA* gene under the control of the *Tet* promoter from the plasmid pBR322

Wheat seeds were inoculated with *A. chroococcum* cells as described in Bageshwar et al. (2017) and were sown in a mixture of vermiculite and perlite, the nutrient used being Hoagland's solution devoid of any nitrogen source. Dry biomass and nitrogen content were determined 60 days after sowing of seeds. Inoculation of seeds with the wild type strain *A. chroococcum* CBD15 (isolated from the fields of Indian Agricultural Research Institute, New Delhi, India) resulted in dry weight of shoot ~1.5 times and of root ~2.5 times that of uninoculated ones. Nitrogen content (mg/g) of inoculated shoot was ~1.6 times and of root was ~1.5 times that of uninoculated ones. Inoculation of seeds with the engineered strain *A. chroococcum* HKD15 (*nifL* gene deleted and the *nifA* gene under the control of the constitutive *Tet* promoter), resulted in dry weight of shoot ~2.8 times and of root ~5.7 times that of uninoculated ones. Nitrogen content (mg/g) of inoculated shoot was ~3.1 times and of root was also ~3.1 times that of uninoculated ones. The actual results are given in Table 4 (Bageshwar et al., 2017). [*A. chroococcum* HKD15 has the very important property that its nitrogen fixation ability is only marginally affected by the presence of chemically synthesized fertilizers like urea. Another notable feature of this strain is the absence of any antibiotic resistance marker or any foreign gene in it. Also, no inducer is necessary for the activity of this strain].

Wheat seeds inoculated with *A. chroococcum* HKD15 were also sown in the field. In order to find out whether the engineered strain survived in the field soil, the number of cells present in the soil adhering to the roots of the wheat plants was determined by real time PCR and subsequent comparing the threshold cycle (C_T) values of DNA from the soil with C_T values obtained from a precounted number of *A. chroococcum* HKD15 cells. About 10^7 *A. chroococcum* HKD15 cells were found to be present per gram soil till ~50 days after sowing and subsequently about 10^3 cells till about 110 days. The actual results are shown in Fig. 5 (Bageshwar et al., 2017).

Table 4 Average dry biomass and nitrogen content of shoot and root of each wheat (HD 2967) seedling after 60 days of seed sowing in vermiculite—perlite in pots.

Seed inoculant	Shoot biomass[a] (mg)	Shoot nitrogen[a] (mg/g)	Root biomass[a] (mg)	Root nitrogen (mg/g)
None	37.2 (12.5)	4.7 (0.7)	8.2 (2.0)	3.3 (0.6)
CBD15	54.9 (13.9)	7.7 (2.1)	20.7 (4.2)	4.9 (0.9)
HKD15	105.7 (16.3)	14.8 (1.1)	46.5 (16.6)	10.2 (2.0)

[a]Average of four experiments, each experiment comprising four pots, each pot containing one seedling. Figures in parentheses represent standard deviation. CBD15 stands for *A.chroococcum* CBD15 (isolated from the fields of Indian Agricultural Research Institute, New Delhi, India), HKD15 stands for *A. chroococcum* HKD15 (engineered strain, *nifL* gene deleted, *nifA* gene under the control of the constitutive *Tet* promoter). Nutrient used was Hoagland's solution devoid of any nitrogen source.
Reproduced from Bageshwar, U. K., Srivastava, M., Pardha-Saradhi, P., Paul, S., Gothandapani, S., Jaat, R. S., Shankar, P., Yadav, R., Biswas, D. R., Kumar, P. A., Padaria, J. C., Mandal, P. K., Annapurna, K. & Das, H. K. (2017). An environmentally friendly engineered Azotobacter strain that replaces a substantial amount of urea fertilizer while sustaining the same wheat yield. Applied and Environmental Microbiology, 83, e00590–17.

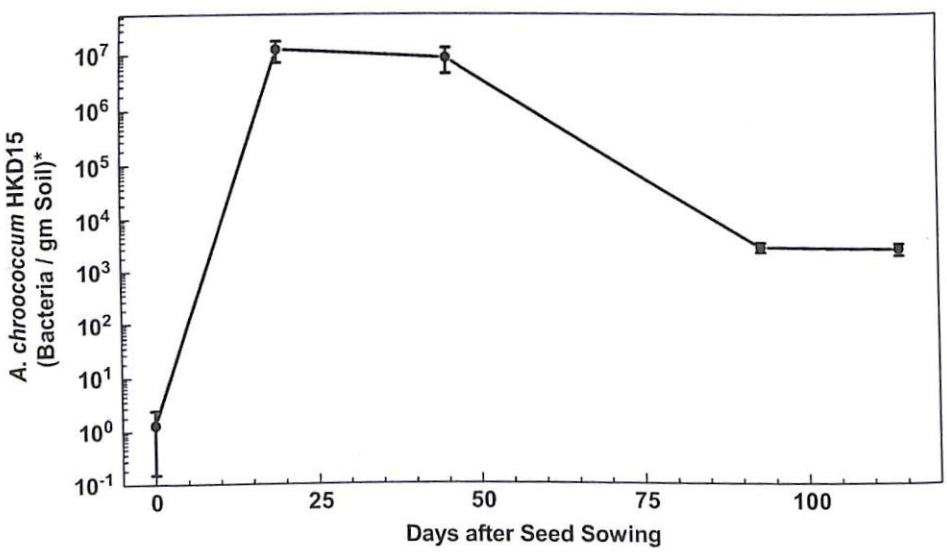

Fig. 5 Survival of *A. chroococcum* HKD15 cells in the rhizosphere of wheat seedlings in the field. The values are average of three field plots, five seedlings per field plot. *Reproduced from Bageshwar, U. K., Srivastava, M., Pardha-Saradhi, P., Paul, S., Gothandapani, S., Jaat, R. S., et al., (2017). An environmentally friendly engineered Azotobacter strain that replaces a substantial amount of urea fertilizer while sustaining the same wheat yield.* Applied and Environmental Microbiology, 83, *e00590–17.*

The *A. chroococcum* HKD 15 cells not only survived in the field soil but were actively fixing nitrogen. Determination of ammonium and nitrate in the soil adhering to the roots of wheat plants that grew out of wheat seeds inoculated with *A. chroococcum* HKD 15, as presented in Fig. 6, established

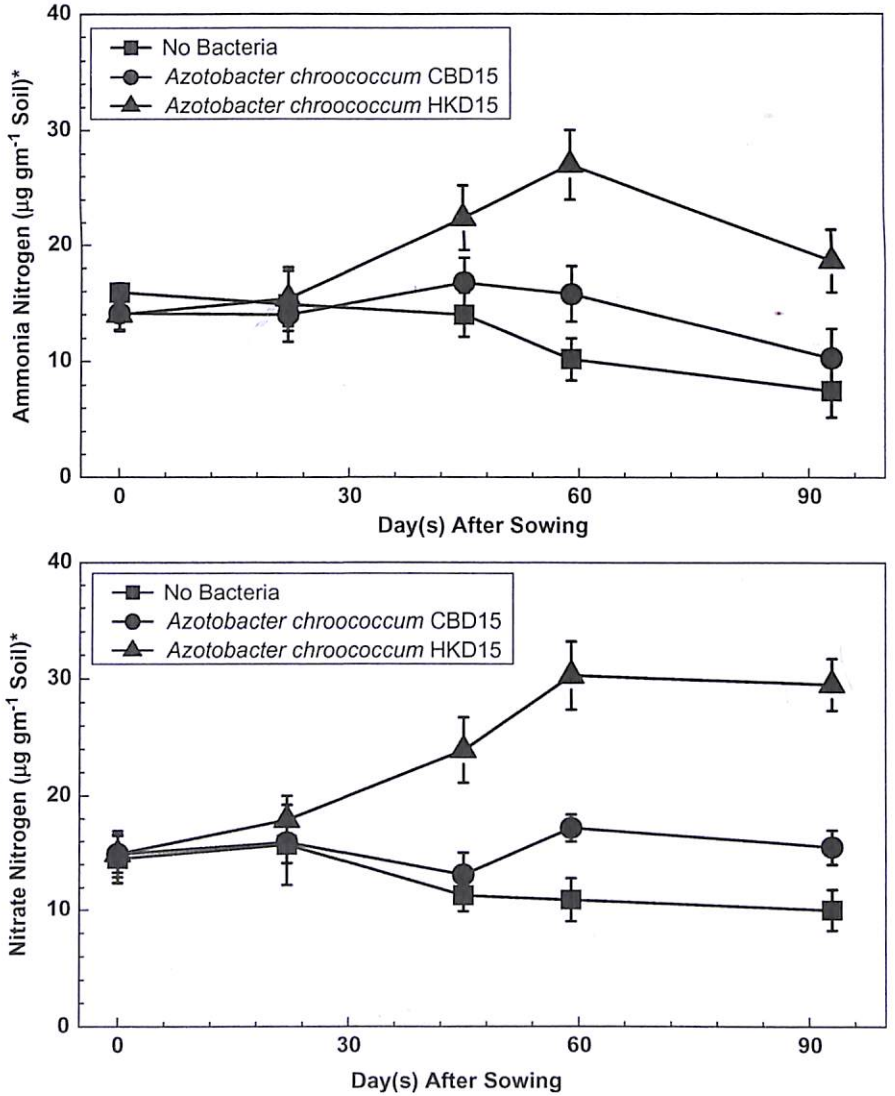

Fig. 6 Ammonia (top) and nitrate (bottom) in soil adhering to roots of wheat plants. No urea was added to soil. Azotobacter chroococcum CBD15 is the wild type and Azotobacter chroococcum HKD15 is the engineered strain. Data presented are averages from three plots, five seedlings per plot. *Reproduced from Bageshwar, U. K., Srivastava, M., Pardha-Saradhi, P., Paul, S., Gothandapani, S., Jaat, R. S., et al., (2017). An environmentally friendly engineered Azotobacter strain that replaces a substantial amount of urea fertilizer while sustaining the same wheat yield,* Applied and Environmental Microbiology 83, *00590–17.*

that the engineered cells were active even after 90 days of sowing (Bageshwar et al., 2017). Ammonium and nitrate in the soil adhering to the roots of wheat plants that grew out of wheat seeds inoculated with *A. chroococcum* CBD 15 and not inoculated with any *Azotobacter* were also determined for comparison (Fig. 6).

Finally, let us have a look at the yield of wheat crop that was achieved. The yield of wheat crop was enhanced by ~60% when no urea was applied, but the seeds were inoculated with the engineered *A. chroococcum* HKD15 before sowing (Bageshwar et al., 2017). This was in contrast to only ~10% yield enhancement by inoculation of the seeds with the wild type *A. chroococcum* CBD15. When urea was also applied, the same wheat crop yield could be attained by applying ~85 kg less urea (~40 kg less nitrogen) per hectare, if the seeds were inoculated with *A. chroococcum* HKD15 before sowing (Bageshwar et al., 2017). These were the results of trials conducted for 3 years in pots and 1 year in the field (Bageshwar et al., 2017). The actual result of the field trial is shown in Fig. 7.

Bageshwar et al. (2017) also looked for any adverse effect on the native population of microbes in the rhizosphere of the wheat plants that arose from seeds inoculated with the engineered *A. chroococcum* HKD15. The population of bacteria, fungi and actinomycetes, that was determined periodically by plating on specific agar medium, was practically unaffected during the one-year field trial.

25. Concluding remarks

Azotobacters are being used as biofertilizers since 1902. *Azotobacters* can fix atmospheric nitrogen, can exude plant hormones, can solubilize phosphates and can counteract plant pathogens. Wild type *Azotobacters* have been found beneficial for all types of plants including cereals, oil seeds, fruits, vegetables, fiber crops and trees. *Azotobacters* have been engineered for better phosphate solubilizing activity, better excretion of fixed nitrogen, lesser consumption of ammonia, enhanced fixation of atmospheric nitrogen and for sustained nitrogen fixation even in the presence of chemically synthesized nitrogenous fertilizers. For better acceptability by environmentalists, engineered *Azotobacter* has been developed that does not need any inducer, does not have any antibiotic resistance marker and does not have any foreign gene. It has to be appreciated, however, that presently available biofertilizers, whether wild type or engineered, cannot match the ability of chemically synthesized fertilizers to augment crop yield. So, some amount of chemically

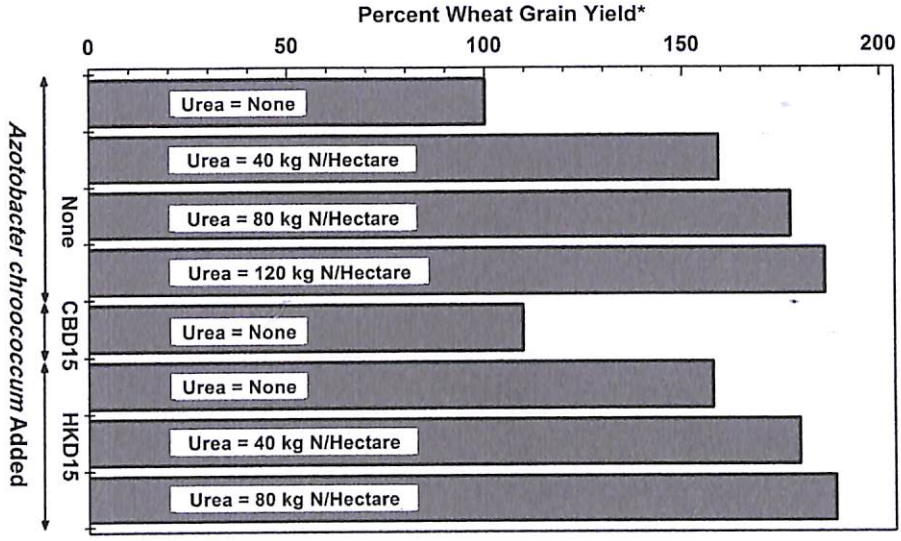

Fig. 7 Effect of inoculation of wheat seeds with the engineered *A. chroococcum* HKD15 on yield of wheat crop in the field. Each plot was 1.2 m by 3 m and the next plot was 3 m away in all directions. Three replicate plots were used for each treatment and distributed in the field by randomized block design. The amounts of urea applied in the field, as mentioned in the figure, are in kg of nitrogen per hectare. Crop neither fertilized with urea, nor the sown seeds inoculated with any *Azotobacter* was considered control (100%). The actual wheat crop yield (average of 3 plots) for the control treatment was 291 g per square meter. The critical difference (equivalent to the least significant difference) at 5% was 69.2, the standard error of the mean was 23.1, and the coefficient of variance was 8.4. *Reproduced from Bageshwar, U. K., Srivastava, M., Pardha-Saradhi, P., Paul, S., Gothandapani, S., Jaat, R. S., et al., (2017). An environmentally friendly engineered Azotobacter strain that replaces a substantial amount of urea fertilizer while sustaining the same wheat yield,* Applied and Environmental Microbiology 83, *00590–17.*

synthesized fertilizers would have to be added along with biofertilizers to achieve the desired yield. The problem is that nitrogen fixation by the wild type fertilizers is completely inhibited by chemically synthesized nitrogenous fertilizers. All chemically synthesized nitrogenous fertilizers produce ammonia and as explained under Section 22, ammonia activates NifL which inactivates NifA and without NifA no *nif* gene is expressed. Farmers would always strive for maximum yield and so would only apply biofertilizers that retain their activity, even when some amount of chemically synthesized fertilizers are also added along with. Farmers are also likely to avoid biofertilizers that contain antibiotic resistance genes or need an inducer to activate the biofertilizer.