An environment friendly engineered Azotobacter can replace substantial amount of urea fertilizer and yet sustain same wheat yield

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BIOTECHNOLOGY

An Environmentally Friendly Engineered *Azotobacter* Strain That Replaces a Substantial Amount of Urea Fertilizer while Sustaining the Same Wheat Yield

AQ: au

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AQ: aff

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AQ:A

ABSTRACT In our endeavor to improve the nitrogen fixation efficiency of a soil diazotroph that would be unaffected by synthetic nitrogenous fertilizers, we have deleted a part of the negative regulatory gene *nifL* and constitutively expressed the positive regulatory gene *nifA* in the chromosome of *Azotobacter chroococcum* CBD15, a strain isolated from the local field soil. No antibiotic resistance gene or other foreign gene was present in the chromosome of the engineered strain. Wheat seeds inoculated with this engineered strain, which we have named *Azotobacter chroococcum* HKD15, were tested for 3 years in pots and 1 year in the field. The yield of wheat was enhanced by ~60% due to inoculation of seeds by *A. chroococcum* HKD15 in the absence of any urea application. Ammonium only marginally affected acetylene reduction by the engineered *Azotobacter* strain. When urea was also applied, the same wheat yield could be sustained by using seeds inoculated with *A. chroococcum* HKD15 and using ~85 kg less urea (~40 kg less nitrogen) than the usual ~257 kg urea (~120 kg nitrogen) per hectare. Wheat plants arising from the seeds inoculated with the engineered *Azotobacter* strain exhibited far superior overall performance, had much higher dry weight and nitrogen content, and assimilated molecular ^{15}N much better. A nitrogen balance experiment also revealed much higher total nitrogen content. Indole acetic acid (IAA) production by the wild type and that by the engineered strain were about the same. Inoculation of the wheat seeds with *A. chroococcum* HKD15 did not adversely affect the microbial population in the field rhizosphere soil. AQ:B

IMPORTANCE Application of synthetic nitrogenous fertilizers is a standard agricultural practice to augment crop yield. Plants, however, utilize only a fraction of the applied fertilizers, while the unutilized fertilizers cause grave environmental problems. Wild-type soil diazotrophic microorganisms cannot replace synthetic nitrogenous fertilizers, as these reduce atmospheric nitrogen very inefficiently and almost none at all in the presence of added nitrogenous fertilizers. If the nitrogen-fixing ability of soil diazotrophs could be improved and sustained even in the presence of synthetic nitrogenous fertilizers, then a mixture of the bacteria and a reduced quantity of chemical nitrogenous fertilizers could be employed to obtain the same grain yield but at a much-reduced environmental cost. The engineered *Azotobacter* strain that we have reported here has considerably enhanced nitrogen fixation and excretion abilities and can replace ~85 kg of urea per hectare but sustain the same wheat yield, if the seeds are inoculated with it before sowing.

KEYWORDS *Azotobacter*, urea, wheat crop

AQ: C

Inoculation of wheat seeds by different species of the soil diazotroph *Azotobacter* usually results in only about 8% to 10% wheat crop yield enhancement (1). It is, however, not clear if this enhancement is due to biologically reduced nitrogen or due to the plant growth substances elaborated by *Azotobacter* (2). On the other hand, application of 257 kg of urea (120 kg nitrogen) per hectare can enhance wheat grain yield by ~100%. Production of any synthetic nitrogenous fertilizer, however, is highly energy intensive and results in ~10-fold or higher emission of CO_2 equivalent (3). Besides, only 30 to 40% of the applied urea is utilized by the wheat crop (4), and the rest pollutes groundwater and streams, rivers, and lakes. The situation can be severe. On 15 January 2017, taps ran dry in major parts of the city of Delhi, capital of India, because "dangerously high" levels of ammonia in raw water forced suspension of operations in two water treatment plants (5). Delhi receives most of its raw water from two neighboring states, both mainly agriculture oriented. The situation elsewhere may also be problematic. The pollution cost of chemical nitrogenous fertilizers for just the European Union has been estimated to be between €70 and €320 billion per year (6).

The positive regulator for all operons involved in the main nitrogen fixation pathway in *Azotobacter* is NifA. The negative regulator NifL, on activation by ammonia, interacts with NifA and inactivates it (7). In addition, the promoter of the *nifLA* operon is highly regulated (8). In order to achieve constitutive biological production of ammonia, even in the presence of chemically synthesized nitrogenous fertilizer, one has to inactivate or, better, delete *nifL* and simultaneously express *nifA* under a constitutive promoter. Unlike that in *Klebsiella pneumoniae*, the expression of the *nifLA* operon in *Azotobacter vinelandii* UW is not autogenously regulated (9).

The objective of the present study was to develop an improved *Azotobacter* strain from a local field isolate that would reduce nitrogen constitutively and to evaluate its ability to significantly enhance wheat crop yield. Though we had in our hands the engineered *A. vinelandii* UW (10), because of environmental concern we did not consider it prudent to apply to our field the bacterium whose parent was from Madison, WI. We also wanted to develop and test a general protocol by which in future the chromosome of other *Azotobacter* species isolated from fields of different and diverse areas of the world could be easily engineered by others in local laboratories to constitutively fix nitrogen. Again, because of environmental concern, we ensured that the mutant *Azotobacter* strain that we would use to inoculate wheat seeds would have neither any antibiotic resistance gene nor any other foreign gene. Here, we present the results obtained with *Azotobacter chroococcum* HKD15, a constitutive mutant (even in the presence of fixed nitrogen in the form of chemical nitrogenous fertilizers) and a hyper-nitrogen fixer that has been engineered from the wild-type *A. chroococcum* CBD15, which was isolated from the fields of the Indian Agricultural Research Institute, New Delhi, India. This mutant strain, when used to inoculate wheat seeds, enhanced wheat grain yield by about 60% in the absence of any urea application. When urea was also applied, wheat grain yield could be sustained by applying ~86 kg less urea than the usual ~257 kg urea (~120 kg nitrogen) per hectare. Such novel nitrogen-fixing strains can be of great environmental, agronomical, and economic significance. We strongly believe that the research work presented here is a viable and sustainable alternative to the nitrogen-reducing cereals which scientists pursued unsuccessfully for almost 4 decades.

RESULTS

The *nifL* gene of *A. chroococcum* CBD15. Hybridization of the restriction subfragments of the ~7.5-kb BamHI fragment of the genomic DNA of *A. chroococcum* CBD15 with *nifL* and *nifA* of *A. vinelandii* UW suggested that *nifL* was adjacent to *nifA* in *A.*

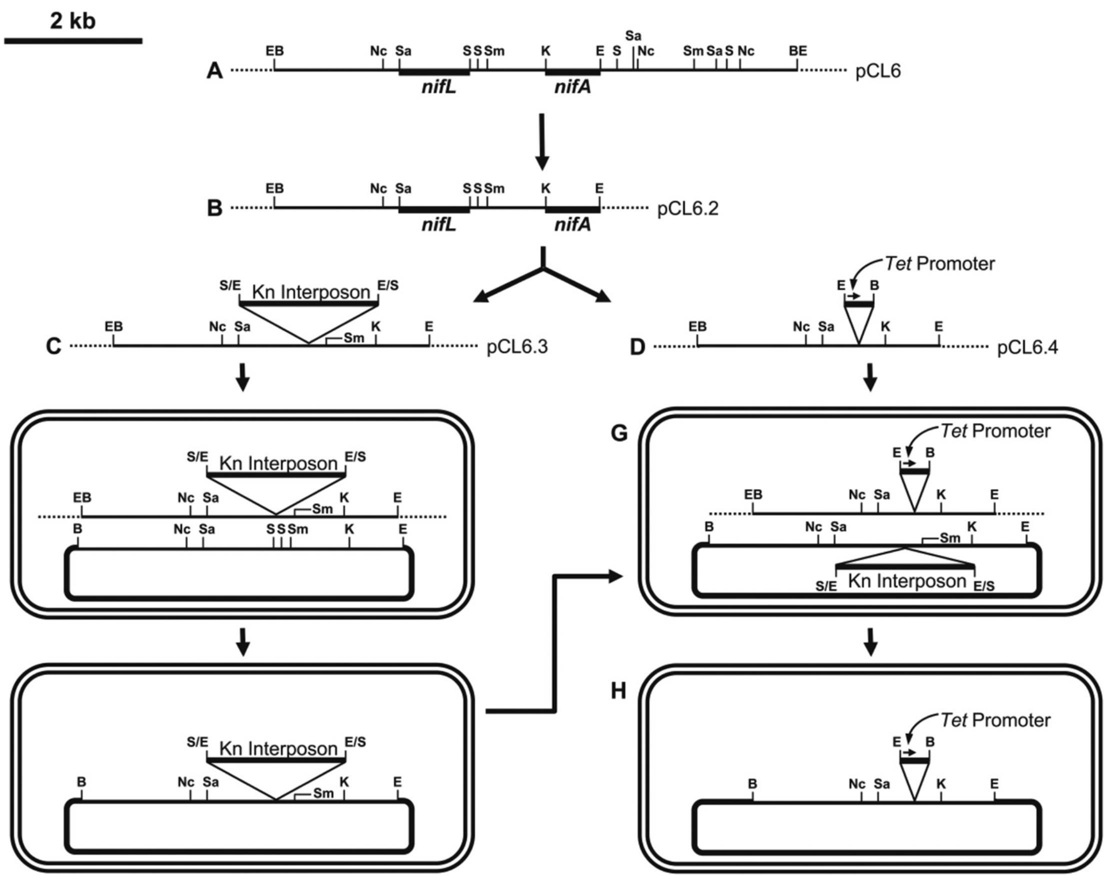

FIG 1 Cloning steps involved for construction of engineered *Azotobacter*. (A) Partial restriction map of the ~7.5-kb BamHI genomic fragment from *A. chroococcum* CBD15, which contains regions homologous to *nifL* and *nifA* of *A. vinelandii* UW, cloned in the BamHI site of pUC7. The construct has been designated pCL6. The restriction subfragments that hybridize with *nifL* and *nifA* of *A. vinelandii* UW are shown by thick lines. (B) The ~4.8-kb EcoRI fragment from pCL6 containing the regions homologous to *nifL* and *nifA* of *A. vinelandii* UW was cloned in pUC7, and this construct has been designated pCL6.2. (C) The ~2.0-kb EcoRI fragment from pHP45ΩKm containing the interposon ΩKm was inserted into the SalI sites of pCL6.2, and this construct has been designated pCL6.3. (D) The construct pCL6.2 was digested with SalI, and deletion of 1,112 bp was achieved by subsequent Bal 31 treatment; the 374-bp EcoRI-BamHI fragment from pBR322 containing the *Tet* promoter was cloned there in the correct orientation. This construct has been designated pCL6.4. (E) The construct pCL6.3 was introduced into *A. chroococcum* CBD15 by electroporation. (F) Insertion of the kanamycin interposon into the *nifL* gene in the genome of *A. chroococcum* CBD15, as a result of homologous recombination between pCL6.3 and the *A. chroococcum* CBD15 genome. (G) The construct pCL6.4 was introduced by electroporation into *A. chroococcum* CBD15, which already had the kanamycin interposon inserted into the *nifL* gene in its genome. (H) Replacement of the region of the *nifL* gene comprising 1,112 bp around the two SalI sites and the kanamycin interposon in the genome of *A. chroococcum* CBD15, as a result of homologous recombination with pCL6.4. Abbreviations: E, EcoRI; B, BamHI; Nc, NcoI; S, SalI; Sa, SacII; Sm, SmaI; K, KpnI.

F1 AQ: D

chroococcum CBD15 (pCL6, Fig. 1A). The base sequence of *nifL* of *A. chroococcum* CBD15 (see Fig. S1 in the supplemental material) (GenBank accession no. KY781893) has 99% homology with the base sequence of *nifL* of *A. vinelandii* UW.

AQ: E

Insertion of a constitutive promoter upstream of *nifA* after deletion in *nifL* in the chromosome of *A. chroococcum*. Direct single-step insertion was ruled out because of the lack of an easy selection procedure. The strain with a deletion in *nifL* and the strain with subsequent insertion of the constitutive promoter are both likely to be *nif*$^+$. A two-step approach was therefore chosen. In the first step, the interposon ΩKm (11) was inserted into *nifL* (Fig. 1C, E, and F). The cassette KIXX (12, 13) was not used instead, as it would have provided a promoter to *nifA* downstream, and hence, the kanamycin-resistant *A. chroococcum* CBD15 would have been *nif*$^+$. Insertion of ΩKm indeed resulted in a *nif* mutant derivative. This validated our assumption that *nifL* and *nifA* were in the same operon, *nifL* being proximal and *nifA* distal to the promoter, a structure similar to that in *A. vinelandii* UW. In the second step, the interposon was replaced by a deleted *nifL* containing the *Tet* promoter (Fig. 1D, G, and H) (the base sequence of the restriction fragment containing the *Tet* promoter is shown in Fig. S2),

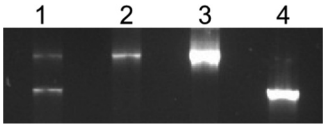

FIG 2 Photograph of agarose gel after electrophoresis of PCR products. Genomic DNA was isolated from kanamycin-resistant cells obtained after transformation by electroporation of *A. chroococcum* CBD15 with pCL6.3 (Fig. 1) and subcultured on BNF agar plus kanamycin and ammonium acetate. PCR of the *nifL* region was conducted by using the primers 5′-ACGAATCGATCCTCTCCAAC-3′ and 5′-TTCGCGATAGTCGGTGTCTAC-3′. The PCR products were electrophoretically separated on an agarose gel. Lane 1, PCR products of chromosomal DNA from cells subcultured six times; lane 2, PCR products of chromosomal DNA from cells subcultured 18 times; lane 3, large excess of PCR products of chromosomal DNA from cells subcultured 18 times; lane 4, PCR products of chromosomal DNA from *A. chroococcum* CBD15.

which resulted in a *nif*$^+$ strain. This strain has been named *Azotobacter chroococcum* HKD15 (the base sequence of the *nifL* region is shown in Fig. S3).

Multiple copies of chromosomes in *A. chroococcum* CBD15. The kanamycin-resistant cells obtained after the first step mentioned above, however, could also grow without ammonium acetate on Burk's nitrogen-free (BNF) medium and kanamycin. This could mean the presence of multiple copies of chromosomes in *A. chroococcum* CBD15, similar to *A. vinelandii* UW (14, 15), and incomplete segregation of chromosomes containing ΩKm. We continued subculturing on BNF medium containing kanamycin and ammonium acetate and examined *nifL* in the chromosome by PCR. Even after the sixth subculture, wild-type chromosomes containing no ΩKm were predominant compared to chromosomes containing ΩKm (lane 1, Fig. 2). A single PCR band representing *nifL* with the inserted ΩKm was visible only after the 18th subculture, with no sign of *nifL* devoid of any ΩKm (lane 2), and even with a large excess of PCR product (lane 3). The cells after 18 subcultures could no longer grow on BNF medium containing kanamycin in the absence of ammonium acetate. Nitrogen fixation had been abolished.

Similarly, after the second step, in which we attempted to replace ΩKm with a deleted *nifL* containing the *Tet* promoter, the cells could grow in the absence of ammonium acetate but were still resistant to kanamycin, again possibly because of incomplete segregation of the chromosomes containing the *Tet* promoter. Subculturing was continued 20 times on BNF medium without ammonium acetate and kanamycin. The cells then became sensitive to kanamycin and could grow without ammonium acetate.

Multiple copies of chromosomes were indeed found to be present in *A. chroococcum* CBD15. The DNA content of *A. chroococcum* CBD15 was determined to be 1.18×10^{-13} g per mid-exponential-phase cell when grown in a shake flask in BNF medium containing ammonium acetate. The size of the chromosome of *A. chroococcum* M4 was earlier estimated to be ∼5,300 kb (16). Assuming that the size of the chromosome of *A. chroococcum* CBD15 would be similar to that of *A. chroococcum* M4, we have calculated that each mid-exponential-phase cell of *A. chroococcum* CBD15 has ∼20 copies of the chromosome.

Confirmation of the deletion of a part of the *nifL* gene and the insertion of the *Tet* promoter there in the chromosome of *A. chroococcum* HKD15. The deletion of a part of *nifL* and the insertion of the *Tet* promoter there in the chromosome of *A. chroococcum* HKD15 have been confirmed by PCR analysis (Fig. 3, top). Complete segregation of the *Tet* promoter containing copies of chromosomes was confirmed by the absence of any wild-type band at ∼1.4 kb (Fig. 3, bottom). Both primer sequences used for the PCR product shown in the bottom panel were chosen from the *nifL* sequence flanking the *Tet* promoter fragment, and only one band of ∼650 bp representing the *Tet* promoter-containing copies of chromosomes appeared.

Comparison of DNA sequence of *nifL* region of *A. chroococcum* HKD15 (Fig. S3) with that of *nifL* of *A. chroococcum* CBD15 revealed the deletion of 1,112 bases from *nifL* and the insertion of the 378-bp fragment containing the *Tet* promoter in the correct orientation. No PCR product was seen when primers (forward primer 5′-CAGCGAATT

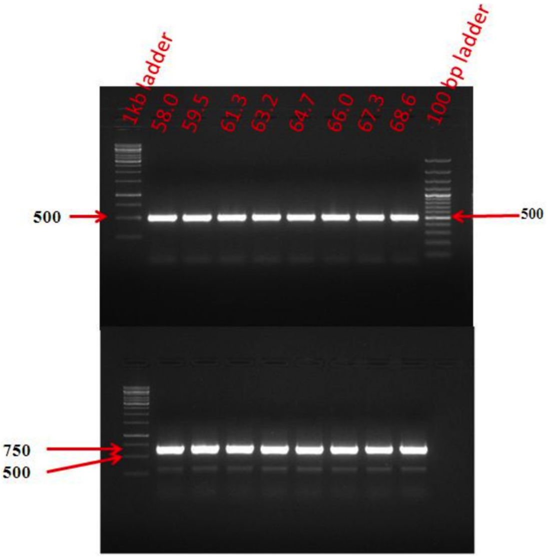

FIG 3 PCR analysis to check whether the DNA fragment containing the *Tet* promoter has indeed been integrated into the *nifL* gene in the genome of *A. chroococcum* HKD15. The PCR patterns have been obtained with genomic DNA of *A. chroococcum* HKD15. The annealing temperature (degrees Celsius) is shown on top of each lane. Primer sequences used for the reaction represented by the top panel were (i) 5′-CCGCACCATCACCGGCTACGGCAGC-3′ and (ii) 5′-GACGGGTGTGGTCGCCATGATCGCG-3′, the first one being from the 5′ end of the *nifL* sequence (see Fig. S1), while the second one was from the 3′ end of the *Tet* promoter fragment (see Fig. S2). Primer sequences used for reaction represented by the bottom panel were (i) 5′-CCGCACCATCACCGGCTACGGCAGC-3′ and (ii) 5′-GCTCGACGACGATCCGGCAGCCTTC-3′, the first one being from the 5′ end of the *nifL* sequence, while the second one was from the 3′ end (see Fig. S1).

GCACGAACTGGAACA-3′; reverse primer 5′-TTGAGGTTGACCGGCATCTTGGA-3′) were chosen from the deleted region of *nifL* in DNA from *A. chroococcum* HKD15. The absence of a wild-type band at ~700 bp was the final evidence of complete segregation of the deleted copies of the chromosome in *A. chroococcum* HKD15.

Fate of the NifL protein. The *nifL* gene of *A. chroococcum* CBD15 has 1,560 bases excluding the chain-terminating codon; hence, the NifL protein has 520 amino acids. The deletion took place after the first 290 bases, and so only the first 96 amino acids of NifL remained. Altogether, 1,112 bases were deleted and 378 bases were added by the fragment containing the *Tet* promoter. Thus, a net 734 bases were lost. So, the bases downstream of the deletion are not in frame any more. Thus, all amino acids beyond the first 96 amino acids of *A. chroococcum* HKD15 NifL would be different from the amino acids of *A. chroococcum* CBD15 NifL. Study of the NifL-NifA interaction has revealed involvement of only the COOH-terminal half of NifL (17). NifA activity of *A. chroococcum* HKD15 would, therefore, not likely be affected even in the presence of fixed nitrogen.

Characteristics of the mutant *Azotobacter*. Acetylene reduction by *A. chroococcum* HKD15 was more than 4 times that by *A. chroococcum* CBD15 (Table 1). Ammonium acetate reduced acetylene reduction by *A. chroococcum* CBD15 by ~70% but

T1

TABLE 1 Acetylene reduction by *A. chroococcum* CBD15 and *A. chroococcum* HKD15

	Ethylene produced[a] (nmol/mg protein/h)		
A. chroococcum strain	N-free growth medium	Growth medium containing 0.11% ammonium acetate	Inhibition by ammonium acetate (%)
CBD15	755 (75)	227 (13)	70
HKD15	3,153 (67)	2,682 (126)	15

[a] Average from 3 experiments; standard deviations in parentheses.

TABLE 2 Ammonium excretion by *A. chroococcum* CBD15 and *A. chroococcum* HKD15

A. chroococcum strain	Ammonium excreted[a] (μg/mg protein) In absence of KNO_3	In presence of KNO_3 (0.1%)	Inhibition of excretion (%)
CBD15	31 (3)	4 (1)	87
HKD15	267 (8)	227 (4)	15

[a]Average from 3 experiments; standard deviations in parentheses.

reduced that by *A. chroococcum* HKD15 by only ~15%. Ammonium excretion by *A.* T2 *chroococcum* HKD15 was more than 8-fold that by *A. chroococcum* CBD15 (Table 2). KNO_3 drastically reduced ammonium excretion by *A. chroococcum* CBD15, while that by *A. chroococcum* HKD15 was marginally affected. Production of indole acetic acid by *A. chroococcum* HKD15 was about the same as that by *A. chroococcum* CBD15 (Table S1).

Pot experiment with soil. The average available nitrogen in the soil of the pots, as determined (18) in 12 random samples, was 1.56 × 10^{-4} kg N per kg soil (standard deviation, 0.145 × 10^{-4}). Until about 2 months after sowing, the dry weight of wheat seedlings that arose from the seeds inoculated with *A. chroococcum* HKD15 but did not receive any urea application was comparable to the dry weight of seedlings that arose from seeds that were not inoculated but received 257 kg of urea (120 kg N) per hectare. F4 Figure 4 shows the yield of wheat crop in the pot experiments. Inoculation of wheat seeds with *A. chroococcum* HKD15 enhanced wheat grain yield by ~60% in the absence of any urea application. When urea was also applied, a savings of ~86 kg urea (40 kg N) per hectare could be achieved on inoculation of wheat seeds with *A. chroococcum* HKD15.

Field experiment. Average available nitrogen in the soil of the field, as determined (18) in 9 random samples, was 1.64 × 10^{-4} kg N per kg soil (standard deviation, 0.157 × 10^{-4}). Each plot was 1.5 m by 3.0 m. Three replicate plots were there for each treatment.

Did the mutant *Azotobacter* thrive in the field soil? Because of our concern for the environment, we did not want to add to the field any bacteria containing any marker or any foreign gene. Hence, the number of *A. chroococcum* HKD15 cells in soil adhering to roots of wheat plants was determined by quantitative real-time PCR (19)

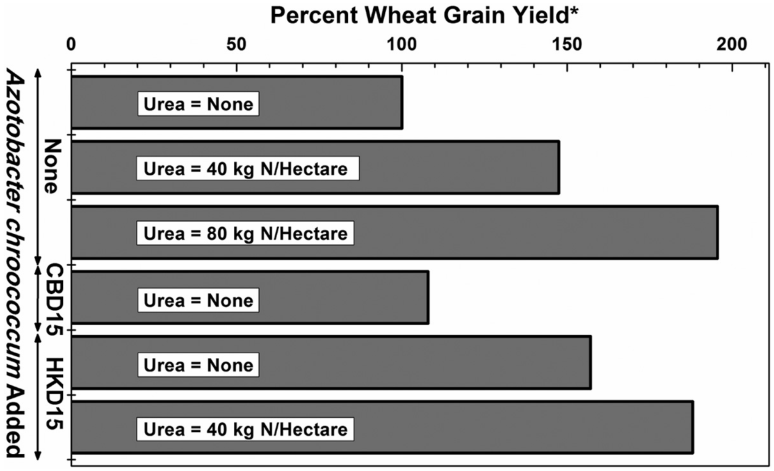

FIG 4 Effect of *A. chroococcum* on wheat (HD 2967) grain yield in pots. The pot experiment with soil was spread over 3 years, and 75 plants were cultivated per treatment per year. The amounts of urea mentioned are in kilograms of N per hectare. *, plants neither fertilized with urea nor inoculated with any bacteria were considered control (100%). The actual average wheat crop yield for this treatment per plant per year was 2.01 g. The standard errors of the means for the 1st, 2nd, and 3rd years are 0.05, 0.07, and 0.11, respectively. The critical differences (equivalent to least significant difference) at 5% for the 1st, 2nd, and 3rd years are 0.17, 0.20, and 0.31, respectively. The coefficients of variance for the first, second, and third years are 5.50, 6.90, and 8.10, respectively.

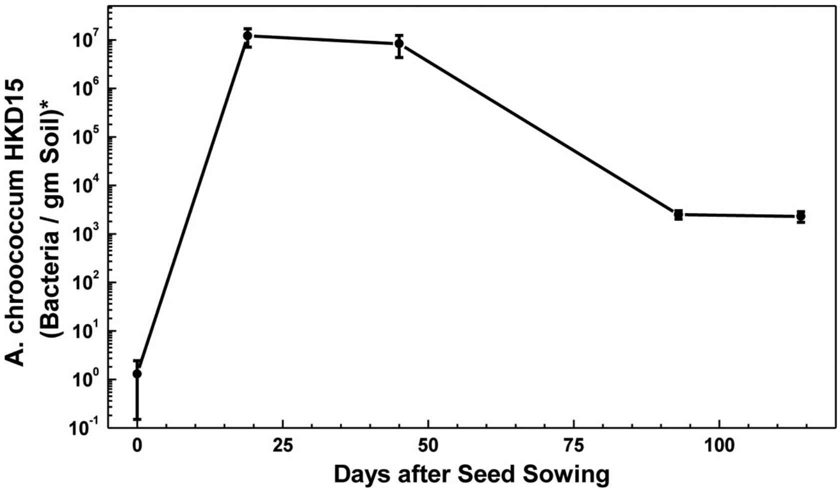

FIG 5 *Azotobacter chroococcum* HKD15 cells (average of three field plots, five seedlings per field plot) in the rhizosphere of wheat seedlings. Values represent means ± standard deviations.

and comparing the threshold cycle (C_T) values of DNA from the soil with C_T values obtained with DNA from a precounted number of *A. chroococcum* HKD15 cells. The forward primer chosen from the *nifL* region was 5′-AGGAAGTGCTCGGCAAGAACGAAT-3′, while the reverse primer from the inserted *Tet* promoter region was 5′-CGATGATA AGCTGTCAAACATGAG-3′. The results are presented in Fig. 5. We conclude from the data in Fig. 5 that *A. chroococcum* HKD15 cells did multiply rapidly until about 20 days after sowing, survived until at least 45 days after sowing, and continued subsequently to inhabit the rhizosphere but in a smaller number. The reduction after 45 days of sowing in the number of *A. chroococcum* HKD15 cells in the rhizosphere (Fig. 5) was possibly a consequence of various ecological factors, including other soil microorganisms that maintain the balance of the diverse microbial population in the soil. It was indeed necessary to inoculate the seeds every year with the engineered *Azotobacter* in order to be able to replace ~85 kg urea and yet sustain the same wheat yield. F5

Was the native population of microbes in the rhizosphere of the wheat plants in the field adversely affected by the inoculation of the seeds? The population of bacteria, fungi, and actinomycetes in the rhizosphere of the wheat plants was determined periodically (Tables S2A, B, and C), but no adverse effect was observed. We concede that it might take several years for any untoward effect to be apparent, and many nonculturable microorganisms would be present in the soil.

Were the *A. chroococcum* HKD15 cells present in the rhizosphere of wheat plants in the field truly active? The content of ammonium and nitrate nitrogen in the soil adhering to the roots of the wheat plants sown in three different locations in the field was determined. Data from seedlings arising out of wheat seeds not inoculated with any *Azotobacter*, inoculated with *A. chroococcum* CBD15, and inoculated with *A. chroococcum* HKD15 are presented in Fig. 6. It is obvious that the mutant strain was indeed active and was presumably fixing nitrogen. F6

F7 **Crop yield in field experiment.** Figure 7 shows the yield of the wheat crop in the field experiment. Inoculation of seeds with *A. chroococcum* HKD15 enhanced the yield of wheat grains by ~60% in the absence of any urea application. When urea was also applied, a saving of 86 kg urea (40 kg N) per hectare was achieved on inoculation of the wheat seeds with *A. chroococcum* HKD15.

Replacement of soil with vermiculite and perlite in the pot experiment. We have presumed that the presence of enhanced ammonium and nitrate in the rhizosphere of the wheat plants in the field experiment and the enhanced crop yield from wheat seeds inoculated with *A. chroococcum* HKD15 in both pot and field experiments were because of enhanced nitrogen fixed by *A. chroococcum* HKD15. Soil, however,

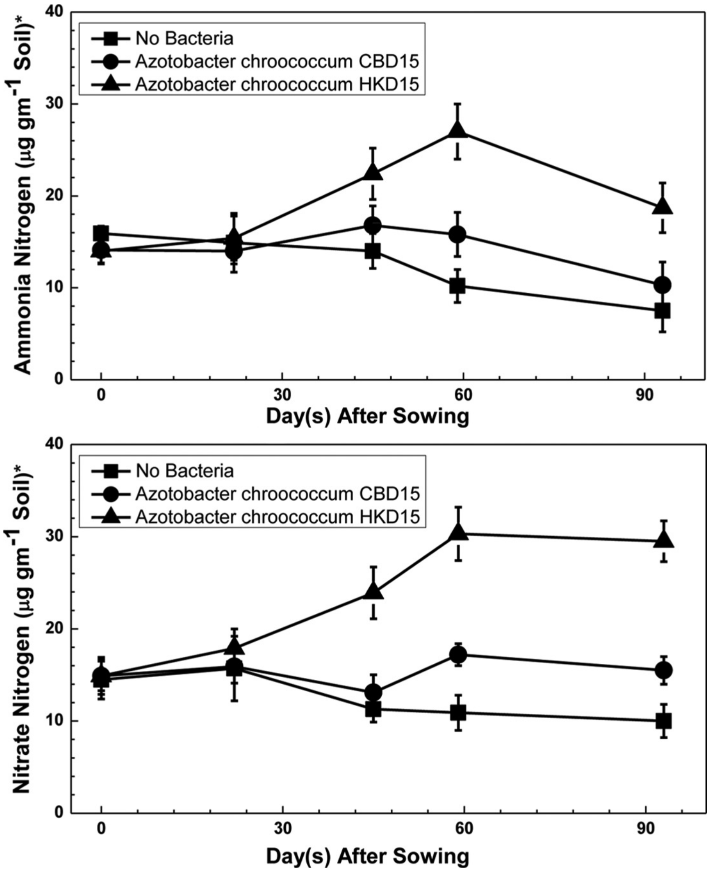

FIG 6 Ammonia (top) and nitrate (bottom) in rhizosphere postsowing in the absence of urea. ■, wheat seeds not inoculated (control); ●, wheat seeds inoculated with wild-type *A. chroococcum* CBD15; ▲, seeds inoculated with the engineered mutant, *A. chroococcum* HKD15. *, data presented are averages from three plots, five seedlings per plot.

contains nitrogen that is unavailable to plants. Could *A. chroococcum* HKD15 be only making the unavailable nitrogen available to the wheat seedlings? A pot experiment was, therefore, conducted using vermiculite and perlite (2:1) instead of soil with Hoagland's solution (20) devoid of nitrogen as nutrient. Vermiculite and perlite contained no detectable nitrogen as determined by Kjeldahl digestion. The enhanced dry weight and nitrogen content of the root and shoot of wheat seedlings arising out of seeds inoculated with *A. chroococcum* HKD15, as shown in Table 3, must be because of enhanced nitrogen that was fixed. T3

Nitrogen balance experiment in pots. The vermiculite-perlite experiment could not be continued until the maturity of the plants. Wheat seedlings that arose from seeds not inoculated with any *Azotobacter* started wilting ~10 days postsowing. Even the ones that arose from seeds inoculated with *A. chroococcum* HKD15 did not survive beyond 70 days. The nitrogen fixed by *A. chroococcum* HKD15 could probably sustain the wheat seedlings but was not enough to nourish the full-grown plants. We wanted to conduct a nitrogen balance experiment with mature plants, so we replaced vermiculite-perlite with soil in the pots. Table 4 shows the results. We again conclude T4/AQ:F from the data in Table 4 that the enhanced combined total nitrogen content of soil plus those of inflorescence, shoot, and root after harvest of the wheat plants arising out of seeds inoculated with *A. chroococcum* HKD15 compared to the total nitrogen content of soil before sowing was because of enhanced nitrogen fixed by *A. chroococcum* HKD15. We, however, observed that the effect of inoculation of the wheat seeds with *Azotobacter* was less pronounced if soil was not autoclaved before sowing of the seeds. For example, the corresponding values in column vi of Table 4 were None, 1.302; *A.*

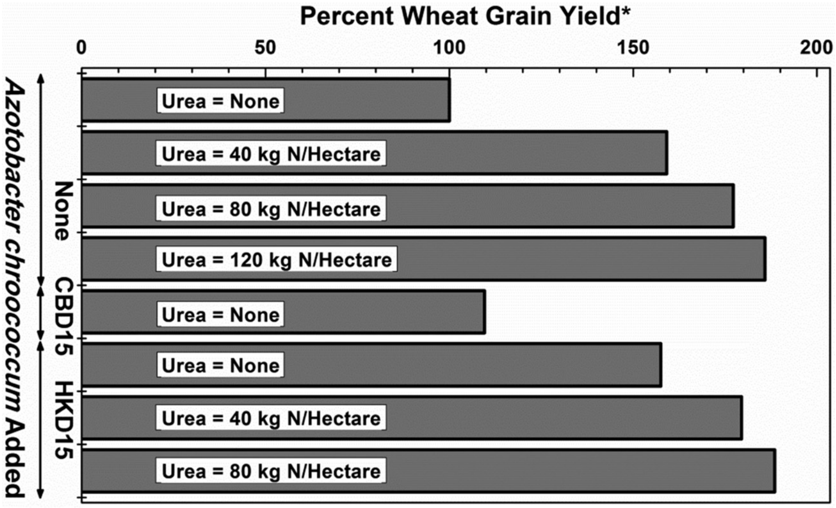

FIG 7 Effect of inoculation of wheat HD 2967 with the engineered mutant, *A. chroococcum* HKD15, on yield of wheat grains in the field. The amounts of urea mentioned are in kilograms of N per hectare. Crop neither fertilized with urea nor inoculated with any bacteria was considered control (100%). *, the actual wheat crop yield (average of three plots) for this treatment was 291.0 g per m^2. The critical difference (equivalent to 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.

chroococcum CBD15, 1.965; and *A. chroococcum* HKD15, 2.798, when soil was not autoclaved. We presume that this was because of the presence of other microorganisms in soil that interfered with the unhindered growth of *Azotobacter*. We had indeed seen a drop in the population of *A. chroococcum* HKD15 in the rhizosphere of the wheat plants after 45 days of sowing of the inoculated seeds (Fig. 5).

Experiment with ^{15}N to conclusively prove that nitrogen fixed by *Azotobacter* was being utilized by the wheat plants. Wheat seedlings in pots were exposed to air containing ^{15}N gas, and ^{15}N abundance in dried seedlings was determined. A small amount of ^{15}N was present even in seedlings that arose from seeds which were not inoculated with any *Azotobacter*, possibly because of ^{15}N fixed by nitrogen-fixing bacteria present in soil that was not autoclaved. Seedlings that arose from seeds inoculated with *A. chroococcum* CBD15 had twice as much ^{15}N, while seedlings that arose from seeds that were inoculated with *A. chroococcum* HKD15 had four to six times as much ^{15}N (Table 5). T5

DISCUSSION

The presence of multiple chromosomes in *A. vinelandii* is a well-established fact (14, 15, 21–23). *A. chroococcum* CBD15, reported in this paper, has also revealed the presence of 20 copies of chromosomes at mid-exponential phase of its growth. Mutation of a gene in *Azotobacter* would, therefore, be inconsequential and liable to reversion, unless it is spread to all the chromosomes by culturing the cells for a large number of generations under selection pressure. Undefined mutants of *Azotobacter* which could reduce nitrogen even in the presence of fixed nitrogen (ammonium and

TABLE 3 Average dry biomass and nitrogen content of shoot and root of each wheat (HD 2967) seedling after 60 days of sowing in vermiculite-perlite in pots[a]

Seed inoculant	Shoot biomass (mg)	Shoot nitrogen (mg/g)	Root biomass (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 from four experiments, each experiment comprising four pots, each pot containing one seedling. Values in parentheses are standard deviations. The nutrient used was Hoagland's solution devoid of any nitrogen source.

TABLE 4 Nitrogen balance[a]

Seed inoculant	Total nitrogen in: (i) Soil before sowing any seed (g)	(ii) Inflorescence (mg)	(iii) Shoot (mg)	(iv) Root (mg)	(v) Soil after harvest (g)	(vi) Inflorescence, shoots, root, and soil (g)
None	1.410 (0.23)	15 (1.3)	27 (3.5)	2 (0.29)	1.36 (0.25)	1.404 (0.26)
CBD15	1.410 (0.23)	95 (6.4)	93 (8.8)	8 (0.44)	2.772 (0.76)	2.968 (0.77)
HKD15	1.410 (0.23)	184 (9.8)	199 (27.3)	47 (6.1)	3.906 (0.58)	4.337 (0.62)

[a] Total nitrogen content of (i) soil (9 kg) before sowing of wheat HD 2967 seeds (the soil was autoclaved, nitrogen content was determined from 3 samples picked up at random, and then each of the 3 autoclaved pots was filled up with 9 kg of soil), (ii) wheat (HD 2967) inflorescence of 3 plants (each pot had 3 plants) 135 days after sowing, (iii) shoots of 3 plants, (iv) roots of 3 plants, (v) soil after harvest of the plants 135 days after sowing, and (vi) inflorescences plus shoots plus roots plus soil. Values are averages (standard deviations).

nitrate), which were obtained in the past through standard mutagenesis techniques (24–31), were not of much practical use, as it was not recognized then that unless the mutation was transmitted to all the copies of the chromosome of *Azotobacter*, the mutant could not be stable.

The construction of the mutant in the present work has been carried out in two steps for ease of selection. The first step resulted in the insertion of the kanamycin interposon (11) in *nifL*, leaving *nifA* promoterless. The selection pressure was applied here by the addition of kanamycin to the culture medium along with ammonium. When the cells were cultured only 6 times, the persistence of the wild-type chromosomes was distinctly discernible. The cells needed culturing 18 times to achieve insertion of the kanamycin interposon into all the chromosomes. The second step has been aimed at the replacement of the kanamycin interposon by the *Tet* promoter in the correct orientation after deletion of a part of *nifL*. The selection pressure was applied here by omitting ammonium and also kanamycin, and the cells were cultured 20 times. The results presented in Fig. 2 confirmed that the kanamycin interposon has been replaced by the *Tet* promoter from all the chromosomes of *A. chroococcum* HKD15.

We had chosen a constitutive promoter in preference to an inducible promoter (cf. reference 32) because we realized that addition of the inducer to the field could be an environmental hazard. We also preferred to overexpress *nifA* and thus enhance nitrogen fixation so as to maximize the availability of ammonia, rather than reduce ammonia assimilation (cf. reference 32), as we appreciated that the two main products of ammonia assimilatory reactions, glutamine and glutamate, are both essential components of proteins.

In India, wheat is cultivated on ~27 million hectares. If wheat seeds are inoculated everywhere in India with *A. chroococcum* HKD15, or similarly engineered *Azotobacter* bacteria derived from strains isolated from other wheat fields, there could be a savings of 2,100 million kg of urea per sowing season of wheat, assuming that the projected linear upscaling is valid.

Azotobacter has also been found to be beneficial for other cereal crops, vegetables, and cash crops (33). Engineered *Azotobacter* strains should be of use for these crops also. Not only in India but anywhere in the world where the agroclimatic conditions are conducive for *Azotobacter* to thrive, strains engineered in a similar way should be able to replace a good proportion of chemical nitrogenous fertilizer. The savings thus achieved would be not only in the cost of chemical nitrogenous fertilizers but, more importantly, in the cost of restoring the environment.

TABLE 5 Assimilation by wheat seedlings of ^{15}N fixed by seed inoculant

Seed inoculant	^{15}N abundance (% of total nitrogen) in: Roots	Shoots
None	3.0	4.4
A. chroococcum CBD15	6.8	8.2
A. chroococcum HKD15	20.5	18.7

Fixed nitrogen is a $100 billion global industry annually, but environmental pollution by synthetic nitrogenous fertilizers remains a real problem. The Bill and Melinda Gates Foundation contributed $10 million to support research for producing nitrogen-fixing cereals (34). This is indeed a challenging task involving anywhere between 9 and 20 genes, taking care of the codon preference in the plant and the transcription of all the genes under plant promoters. It may be mentioned here that 9 nitrogen-fixing genes from *Paenibacillus* sp. strain WLY78, which was considered to be the minimum nitrogen fixation cascade, were only 10% active in *Escherichia coli* (35). Finally, even if the mission of producing nitrogen-fixing plants is accomplished, it may or may not be beneficial for the plants. The enzyme nitrogenase is extremely oxygen sensitive, and fixing nitrogen is metabolically expensive, which would be a heavy burden on plants. About 16 ATP molecules are needed to reduce one molecule of nitrogen (36). On the other hand, only 36 to 38 ATP molecules are released due to hydrolysis of one molecule of glucose (37). Cellulose is a universal backbone of plants, while starch is the most common stored nutrient in seeds and fruits. Both cellulose and starch are polymers of glucose. It will take about 1 mol of glucose (~180 g) to reduce 2.4 mol (~34 g) of nitrogen, which is equivalent to >5 g glucose per gram of reduced nitrogen. The energy requirement may be much higher when expressing nitrogenase in plants. This enormous energy load is likely to adversely affect the foliage and fruit development of plants.

Developing a biofertilizer with enhanced nitrogen-fixing ability, which is sustained even in the presence of synthetic nitrogenous fertilizers, is thus a practical and environmentally friendly answer to the growing demands for nitrogenous fertilizers for agriculture.

MATERIALS AND METHODS

Azotobacter chroococcum CBD15 was obtained from the Division of Microbiology, and the wheat variety HD 2967 was obtained from the Division of Genetics, Indian Agricultural Research Institute, New Delhi, India. HD 2967 has the following genealogy: ALDES/COC//URES/HD2160(M)//HD2278.

General methods. Growth of bacteria, isolation of plasmids, isolation of genomic DNA, acetylene reduction assay, and labeling of DNA fragments were done as described earlier (38). Glucose in Burk's nitrogen-free (BNF) medium was replaced by 2% sucrose. DNA was estimated according to the method of Burton (39), indole acetic acid was determined according to the method of Hartmann et al. (40), and ammonium in culture medium was assayed according to the method of Chaney and Marbach (41). When both ammonium and nitrate were to be determined in soil, the method of Keeney and Nelson (42) was used. "Available nitrogen" in the soil of the field was determined by digestion of the soil samples with alkaline permanganate (18). Bacterial cells were counted in a hemocytometer.

Determination of base sequence of *nifL* of *A. chroococcum* CBD15. The *nifL* region of *A. chroococcum* CBD15 was isolated by PCR using Phusion high-fidelity DNA polymerase and primers based on the sequence of *nifL* (9) of *A. vinelandii* UW. PCR was programmed with an initial denaturing at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 64°C for 45 s, and extension at 70°C for 90 s and a final extension at 72°C for 10 min. The base sequence of the PCR product was determined commercially.

Construction of *A. chroococcum* HKD15. A ~7.5-kb BamHI fragment of *A. chroococcum* CBD15 genomic DNA was hybridized with both *nifL* (9) and *nifA* (43) of *A. vinelandii* UW. This fragment was cloned in pUC7 and named pCL6 (partial restriction map shown in Fig. 1A). The ~4.7-kb EcoRI fragment from pCL6, which contained the entire *nifL* gene and a major part of *nifA*, was cloned in pUC7 and was designated pCL6.2 (Fig. 1B). The plasmid pCL6.2 was digested with SalI, and the 5′ overhangs were filled up. The 5′ overhangs of the EcoRI fragment from pHP45ΩKm (11) were also filled up. These two DNA fragments were then ligated. *E. coli* DH5α was transformed, and kanamycin-resistant colonies were selected. The plasmid isolated from the kanamycin-resistant colonies was designated pCL6.3 (Fig. 1C). The construct pCL6.2 has a single BamHI site and a single SmaI site, while the EcoRI fragment containing ΩKm has two BamHI sites and three SmaI sites. These were used to confirm the insertion of ΩKm into *nifL* in pCL6.3.

The 5′ overhangs of the 381-bp EcoRI-BamHI fragment from pBR322 (base sequence in Fig. S1 in the supplemental material) containing the *Tet* promoter were filled up. The plasmid pCL6.2 was digested with SalI and treated with Bal 31 to obtain a deletion of ~1 kb to ~1.2 kb. The deleted DNA was consecutively treated with mung bean nuclease, Klenow fragment, and all four deoxynucleoside triphosphates (dNTPs) and ligated with the DNA fragment from pBR322. We called the new construct pCL6.4 (Fig. 1D). The success of the ligation operation was confirmed by the appearance in pCL6.4 of a HindIII site and the disappearance of the SmaI site compared to pCL6.2.

The *A. chroococcum* CBD15 cells were transformed with pCL6.3 by electroporation, and the cells (Fig. 1E) were plated once on BNF agar containing ammonium acetate (0.11%). The cells were scraped off and plated again on BNF agar containing ammonium acetate and kanamycin (50 μg/ml). The kanamycin-resistant cells (Fig. 1F) were subcultured 20 times in the same medium and rechecked for their inability to grow on both BNF agar and BNF agar containing ammonium acetate and ampicillin (100 μg/ml).

The kanamycin-resistant derivative of *A. chroococcum* CBD15 (Fig. 1F) was next subjected to electroporation with pCL6.4, and the cells (Fig. 1G) were plated on BNF agar containing ammonium acetate. The cells were scraped off and plated again on BNF agar with no ammonium acetate and no kanamycin in it. The colonies were grown in BNF medium containing no kanamycin and no ammonium acetate, and the cells (Fig. 1H) were subcultured 20 times in the same medium and rechecked for their inability to grow on BNF medium containing ammonium acetate and kanamycin and also on BNF medium containing ammonium acetate and ampicillin.

PCR analysis. PCR analysis was used to confirm the deletion of part of the *nifL* gene, the insertion of the *Tet* promoter there in the chromosome of *A. chroococcum* HKD15, and the achievement of complete segregation of the *Tet* promoter containing copies of the chromosomes. *Taq* DNA polymerase was used, and the operational steps were as follows: (i) initial denaturation for 4 min at 95°C; (ii) 30 cycles of amplification with 1 min of denaturation at 95°C, 1 min of annealing at 58°C to 68.5°C, and 1 min 30 s of extension at 72°C; and (iii) final extension for 10 min at 72°C.

Treatment of wheat seeds with bacteria. *Azotobacter* strains were grown (38) to an A_{600} of 1.5, harvested, washed three times with sterile BNF medium containing sucrose but no ammonium acetate (38), and suspended in the washing medium but at 1/10 the volume of the culture. The concentrated bacterial suspension contained $\sim 1 \times 10^{10}$ cells per ml. The wheat seeds were soaked in the concentrated bacterial suspension for 3 h at 25°C and air dried at 25°C. Seeds that were not intended to be treated with *Azotobacter* were treated exactly the same way in the same medium lacking bacteria. The number of *Azotobacter* cells adhering to each seed was on the order of 10^7.

Urea application. Half of the desired dose of urea was applied immediately prior to sowing. The remaining urea was applied in two installments at successive intervals of 40 days.

Application of other fertilizers. Potash was applied at 60 kg K per hectare and superphosphate was applied at 60 kg P per hectare in a single dose to all the pots and all the plots in the field a few days prior to sowing.

Pot experiments. Pot experiments for crop yield were performed in a net house for 1 year at Jawaharlal Nehru University and for 2 years at the Division of Microbiology of the Indian Agricultural Research Institute, New Delhi. Each pot of 35-cm diameter contained 12 kg soil. Five plants were maintained per pot. Fifteen pots were used per treatment. The pots were distributed in the net house by randomized block design. The amount of fertilizer to be applied per pot was determined on the basis of the weight of soil per pot (12 kg), assuming that 1 hectare is equivalent to 2.24×10^6 kg of soil, which is approximately the weight of the upper 15 cm of the soil in 1 hectare (44).

Field experiment. The field for confined field trials at the Indian Agricultural Research Institute, New Delhi, was used. Each plot was 1.2 m by 3.0 m, and the next plot was 3.0 m away in all directions. Three replicate plots were used for each treatment and distributed in the field by randomized block design. A 3.0-m-wide border of wheat plants was maintained on all four sides 3.0 m away from the experimental plots. Sowing of wheat seeds, 50 g per plot, was performed in six rows per plot occupying 1.2 m at the width of the plot. Each plot was notionally 3.0 m long, seeds falling over a little less or more. The exact length up to which the seeds fell was measured for each plot. The outer two rows of plants were discarded during harvesting, and crop yield was calculated on the basis of yield from the inner four rows of plants that occupied 0.8 m. One irrigation was applied prior to sowing. Six irrigations were applied after sowing. For determination of dry weight, wheat seedlings were uprooted immediately after an irrigation from a 20-cm length of each row chosen at random and washed free of all dust and soil, and the whole seedlings (shoot plus root) were dried at 100°C until they achieved a constant weight. Harvesting of the wheat crop was done manually 135 days after sowing.

Collection of soil samples adhering to roots of wheat plants. Seeds treated with *A. chroococcum* were sown in the field in three different locations. Wheat seedlings were pulled out periodically and gently shaken to get rid of the loose soil from the roots, and the soil sticking to the roots was collected aseptically.

DNA isolation from soil samples. DNA was isolated from 0.5 g soil adhering to roots of the wheat plants sown in three different locations by using the Zymo Research kit ZR soil microbe DNA MiniPrep (catalog no. D6001).

Determination of population of microbes in rhizosphere soil of wheat plants. Soil adhering to roots of uprooted plants was suspended in normal saline and mixed thoroughly, the soil was allowed to sediment, and the supernatant suspension was plated on specific agar medium after appropriate dilution. Nutrient agar (45) was used for bacteria, Ken Knight's medium was used for *Actinomycetes*, and Martin's rose bengal medium (46) was used for fungi.

Determination of ^{15}N assimilated by wheat seedlings. Wheat seedlings in pots, 7 days after sowing, were exposed to ^{15}N in a closed chamber. The gas volume of the chamber was 4.75 liters, and 1 liter of ^{15}N was introduced by replacement of 1 liter of water. Since air has 78% nitrogen, there was 21% ^{15}N in the total nitrogen in the chamber. The experiment was terminated after 20 days, and the roots and shoots were separately collected, dried at 100°C until they achieved a constant weight, and stored in a desiccator. The ^{15}N abundance was determined in an isotope ratio mass spectrometer.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00590-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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H.K.D. conceived the work, designed the experiments, interpreted the results, and prepared the manuscript; U.K.B. assisted in preparing the manuscript and conducted the laboratory experiments; M.S., S.G., and P.S. conducted the laboratory experiments; S.P. performed the characterization of *A. chroococcum* HKD15, conducted the pot experiment for crop yield, and determined the microbial population in the rhizosphere soil; P.P.-S. assisted in preparing the manuscript and conducted the vermiculite-perlite experiment, the nitrogen balance experiment, and the ^{15}N experiment; R.S.J. conducted the field experiment; R.Y. rendered valuable advice on the field experiment and edited the manuscript; D.R.B. helped in chemical analysis of soil; P.K.M. suggested the pot experiment using vermiculite and perlite and facilitated the work; P.A.K., J.C.P., and K.A. facilitated the work.

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