J. Plant Biochemistry & Biotechnology Vol. **6**, 119-123, July 1997

Isolation and Characterization of an Oxygen Sensitive Mutant of *Azorhizobium caulinodans*

A Das[1] and M L Lodha*
Division of Biochemistry, Indian Agricultural Research Institute, New Delhi 110 012, India

An oxygen sensitive mutant of *Azorhizobium caulinodans* strain IRBG 46 was isolated by NTG mutagenesis. It was defective in N_2 fixation under 3% O_2 level, while under 1% O_2 it was almost as active as the parent strain IRBG 46. The mutant was also found to be a slow grower with reduced respiratory activity, low azide tolerance and no catalase activity. However, it did not differ from its parent strain with respect to nitrate respiration. Under symbiotic condition the mutant formed smaller, light green nodules as compared to bigger, dark green nodules formed by the wild type strain. The mutant was also defective in N_2 fixation under symbiotic condition. Complementation analysis showed that the mutation might be in either *fix*L or *fix*J gene which are involved in O_2 regulation of *nif/fix* gene expression. A possible role of all these factors in conferring a highly O_2 tolerant nitrogen fixing system in the organism, has been discussed.

Key words : oxygen sensitive, *Azorhizobium caulinodans,* azide tolerance, respiration rate, oxygen regulation, nitrogen fixation.

Nitrogenase enzyme consists of two metallo-proteins both of which are essential for its activity. Both the proteins are irreversibly destroyed by O_2. Oxygen also inhibits nitrogenase synthesis *via* regulatory cascades. Various diazotrophs, therefore, have evolved different detoxification and/ or avoidance mechanisms to protect their nitrogenases from O_2 damage. However, the optimum O_2 concentration for nitrogenase activity varies in different diazotrophs. This is also true among the different rhizobia. *Azorhizohium caulinodans* assumes a unique position amongst the nitrogen fixing organisms. In addition to its ability to fix N_2 in aerial stem nodules as well as root nodules while in symbiosis with its host *Sesbania rostrata*, it is also capable of fixing N_2 under free living conditions at a high O_2 (3%) level (1, 2). While a free living culture of this diazotroph has very high nitrogenase activity at 2 µM dissolved O_2 (3), *Bradyrhizohium japonicum* bacteroids have no nitrogenase activity at this O_2 level. Stouthamer *et al* (4), using steady state culture of *A. caulinodans* observed maximum nitrogenase activity at an O_2 concentration of 57.5 µM. *Rhizobium leguminosarum* bacteroids have an O_2 optimum of 800 nM for nitrogenase activity but *B.japonicum* bacteroids are not very active at this O_2 concentration and have an optimum O_2 concentration close to 100 nM (5). Stem nodules have also been shown to harbour chloroplast containing cortical cells besides bacteroid containing cells. Since green cells produce O_2 during photosynthesis, this poses an additional challenge to O_2 protection of nitrogen fixing system in this organism. Extensive studies have been made on the various O_2 protection mechanisms in *Azotobacter.* However, in rhizobia, particularly in *Azorhizobium*, such information is lacking. In this paper we report the isolation and characterization of an oxygen sensitive mutant of *A. caulinodans* for the purpose of studying the mechanism of O_2 tolerance in this diazotroph.

*Corresponding author
[1]Present address : Division of Biochemistry, Central Rice Research Institute, Cuttack 753 006, India

Materials and Methods

Bacterial strains and plasmids — An isolate of *A. caulinodans* (IRBG 46) was obtained from Dr J K Ladha, IRRI, Manila, Philippines. Pure culture of *Escherichia coli* strain HB101 (pRK 2073) was provided by the National Research Centre on Plant Biotechnology, IARI, New Delhi. Plasmid DNA of pRS2001 was kindly provided by Dr P A Kaminski, Pasteur Institute, France. Relevant characteristics of these strains/plasmids are presented in Table 1.

Table 1. Bacterial strain and plasmids used in the study

Strain/Plasmid	Relevant characteristics	Source/Reference
Azorhizobium caulinodans		
IRBG 46	Hup^+, Nd^R, Ap^R	J K Ladha, IRRI, Manila, Philippines
Plasmids		
pRK2073	Mob^+, Tra^+, Sm^R, Sp^R	6
pRS2001	Partial 9 kb *Bam*HI fragment containing *fix*LJ in pSUP202, Cm^R, Ap^R	7

Media and growth conditions — *A. caulinodans* strain IRBG 46 was grown at 28°C in TYM medium (8) supplemented with nalidixic acid (10 μg ml^{-1}) and ampicillin (100 μg ml^{-1}). The *E.coli* strains were cultivated at 37°C in Luria-Bertani (LB) medium supplemented with appropriate antibiotic(s) for respective strain. Antibiotics spectinomycin, amplicillin and chloramphenicol were used @ 50 μg, 100 μg and 25 μg ml^{-1}, respectively. For free living nitrogenase assay LO medium (9) was used.

Isolation of mutant — The mutant was isolated by nitrosoguanidine (NTG) mutagenesis. Log phase cells were treated with NTG @ 100 μg ml^{-1}. Mutagenized cells were spread plated on LO medium. Out of 4000 colonies examined, 70 colonies were picked up on the basis of their smaller size. The individual colonies were again grown on LO broth both under 1% and 3% O_2 in a nitrogen atmosphere. After 5 days growth was measured by taking OD at 600nm. Colonies not showing any detectable growth under 3% O_2 level but still growing under 1% O_2 level were selected. These mutants were further characterized for nitrogenase activity under two O_2 levels (1% and 3%) using gas chromatography. Only one oxygen sensitive mutant showing nitrogenase activity at 1% O_2 but not at 3% O_2 was obtained and named as C48.

Conjugational transfer of plasmid — For mobilising plasmid pRS2001 to the mutant C48 using helper plasmid pRK 2073, triparental mating protocol was followed. The cells were incubated at 28°C for 24 h and then replica plated on RMM containing appropriate antibiotics.

Free living nitrogenase assay — Nitrogenase was assayed by the Stoppered Tube Assay method (10). Cells were grown in TYM broth for 20 h and then pelleted, washed twice in LO medium and again suspended in the same medium in 10:1 ratio. This suspension was used for inoculating 1.5 ml LO medium in 1: 10-15 ratio. Six replicates of each strain or treatment were prepared in 20 ml test tubes capped with serum stoppers. The tubes were evacuated in a vacuum manifold and flushed extensively with N_2 gas. Measured amounts of O_2 gas were then introduced into the tube with the help of a gas tight syringe. From each of these tubes 1 ml gas was withdrawn and replenished with an equal volume of C_2H_2 . The samples were incubated at 30°C for 10 h with vigorous shaking (300 rpm). Nitrogenase (acetylene reduction) activity in each tube was assayed by withdrawing 1 ml gas sample and analysing for its ethylene content using an AIMIL-NUCON gas chromatograph equipped with FID.

Nitrate respiration — Cells were grown to log phase in YEM medium (11). 100 μl of this culture was used for inoculating 30 ml culture tubes completely filled with YEM broth supplemented with 0.2 mM NO_3^-. The tubes were incubated stationary at 28°C for different periods of time for preparing growth curves. The cultures were shaken twice a day by inverting the tubes.

Azide tolerance test — Cultures were grown on TYM, RMM (12) and RMMO (RMM without N-source) media supplemented with different levels of azide. Growth was observed after 2 days in case of TYM and 4 days in case of RMM and RMMO.

Respiratory activity — Respiratory activity of whole cells was measured with a Clarke type O_2 - electrode. For measuring respiration of intact organism, cells were grown in TYM broth to early log phase. 0.5 ml cells was transferred to O_2 electrode chamber which contained LO medium with or without carboxylic acids (10 mM concentration) to reach a final volume of 3.5 ml.

Assay of oxygen metabolizing enzymes — All enzymes were assayed in cell-free extract. Early log phase cells grown in TYM broth were pelleted and washed twice in phosphate buffer (molarity of which varied depending on the enzyme assayed). Washed cells were resuspended in respective phosphate buffers containing phenyl methyl sulfonyl fluoride (1mM) and disrupted with MSE Mullard Ultra Sonicator at 20kc per sec for 3 min with 30 sec intervals at 4°C. The crude homogenate was centrifuged at 5000 × g for 10 min to remove the whole cells. The supernatant obtained was used as cell-free extract for enzyme assays. O-dianisidine peroxidase was assayed according to Page *et al* (13) following the method of Guidotti *et al* (14). Catalase was assayed according to Sinha (15) and superoxide dismutase was assayed by the method of Elstner *et al* (16).

Soluble protein from the bacterial cells was extracted by the method of Stickland (17) and estimated by the procedure of Lowry *et al* (18) using bovine serum albumin as a standard.

Symbiotic test — Undamaged clean seeds of *Sesbania rostrata* selected to a reasonably uniform size were surface sterilized as described by Adebayo *et al* (19). Sterilized seeds were soaked in sterile distilled water for 48 h for germination. Germinated seeds were sown in plastic pots (6" × 6") containing acid washed sterilized sand. Plants were grown during July-Sept under natural conditions and supplied with N- free Hoagland nutrient solution (20) as and when required for the growth of the plants. The inoculum was prepared by growing the bacteria in TGYE broth (21) for 2 days with continuous shaking, harvesting the cells and washing them twice in sterile phosphate buffer saline (0.85% NaCl), pH 7.2. The pellet was resuspended in sterile water to a density of 10^8 cells ml^{-1} and used as inoculum. Root inoculation was done 8 days after sowing (DAS) by applying 2 ml of the inoculum to the base of the seedlings. At 21 DAS, stems were inoculated by coating them with the inoculum mixed with 10% gum arabic. The plants were

harvested at 53 DAS for nitrogenase assay.

Symbiotic nitrogenase assay — For each strain, composite sample containing intact nodules collected separately from stem and roots were used for the assay of nitrogenase (C_2H_2 reduction) activity. Assay was done according to the method of Hardy *et al* (22). Ethylene formed was estimated by using an AIMIL-NUCON gas chromatograph equipped with FID as described before.

Dry weight determination — Plant parts were dried at 70°C in an oven for 48 h or more till a constant weight was obtained. The dry weights for leaf, stem and nodules were determined separately.

Results and Discussion

Oxygen sensitive mutants have previously been used to study the O_2 protection mechanism of N_2 fixing system. However, such mutants have been studied in *Azotobacter* only (23, 24). In the present study, the mutant, C 48 showed a free living nitrogenase activity of about 9% of that of the parent strain IRBG 46 under 3% O_2 level. However, under 1% O_2 level the activity was almost equal to that of IRBG 46 (Table 2). Therefore, we made an attempt to characterize the mutant according to various attributes such as growth rate, respiratory activity, azide tolerance, etc.

Table 2. Expression of nitrogenase activity in free living cells of *A. caulinodans* strain IRBG 46 and its mutant C 48 under two different concentrations* of O_2

Strain	Sp nitrogenase activity (nmol C_2H_4 h^{-1} mg^{-1} protein)	
	1% O_2	3% O_2
IRBG 46	865 ± 56.7	474 ± 55.7
C 48	738 ± 65.3	44 ± 10.2

* O_2 in N_2 atmosphere.

Note : Values reported are mean ± SD of six replicates.

The mutant had a growth rate slower (5 h doubling time) than the parent strain IRBG 46 (3.5 h doubling time) in TYM broth (data not shown). The cell yield of the mutant as determined by turbidity test was about 80% of that of IRBG 46.

Since the mutant was defective in nitrogen fixation, it was thought imperative to see whether there is any difference with respect to azide tolerance or not. Azide is one of the several substrates for nitrogenase but at the same time it is an inhibitor of respiratory electron transport chain. Thus, if nitrogenase catalysed reduction of azide occurs quantitatively *in vivo* then it may not be available in sufficient amounts for inhibitory action on energy generating system. Initial testing indicated that in TYM medium under atmospheric O_2 level IRBG 46 could tolerate upto 10 μg ml^{-1} of azide, whereas the mutant did not show any growth at that concentration of azide (data not shown). The level of tolerance on RMM was quite low (5 μg ml^{-1}). On RMMO, however, the tolerance was higher (20 μg ml^{-1}). In all the three cases the mutant showed decreased tolerance to azide viz. 5, 2 and 10 μg ml^{-1} on TYM, RMM and RMMO, respectively. When diazotrophs form colonies on agar surface, microaerobic environment is created at the centre of the colonies wherein nitrogen fixation genes are derepressed. The higher tolerance level of the parent strain might be because of the expression of an active nitrogenase which leads to dilution of the azide by intracellular reduction. The mutant being defective in nitrogen fixation could not detoxify it and thus tolerance level was lower.

Mutations in genes like *fix*L, *fix*J or *fix*K which are involved in O_2 regulation of nitrogenase expression have been found to affect nitrate respiration (25, 26). However, the mutant has not been found to differ from its parent strain IRBG 46 with respect to nitrate respiration (data not shown).

Respiratory activity was measured in terms of consumption of O_2 in the medium. From the results shown in Table 3 it can be seen that respiratory activity of the mutant was lower than that of IRBG46. However, there is a variation in O_2 consumption with respect to different carbon sources studied. In *A. caulinodans*, lactate and succinate have been shown to be suitable energy sources supporting N_2 fixation (27, 28). The efficiency of lactate as energy source has direct relation to the presence of lactate dehydrogenase in this bacterium (27). Lactate dehydrogenase extracted from this organism exhibited high affinity for lactate. However, in other rhizobia (*R. meliloti, R. leguminosarum* biovar *phaseoli* and *Bradyrhizobium*) the reduction of pyruvate is favoured (27). The lower activity with α-keto glutaric acid is consistent with earlier finding that it blocks oxidation of malate by inhibiting malate dehydrogenase. The inhibition limits NAD^+ reduction and its availability. This, in turn, restricts the pool of NADH reoxidized by the electron transport chain in the bacteria thereby reducing O_2 consumption.

Table 3. Respiratory activity of free living cells of *A. caulinodans* and its mutant C 48

Strain	Respiratory activity (μmol O_2 consumed min^{-1} mg^{-1} protein)			
	LO	LO + succinate	LO+ pyruvate	LO + α-KGA
IRBG46	0.404	0.641	0.468	0.371
C 48	0.300	0.526	0.411	0.381

LO, basal medium containing lactate; α-KGA, α-keto glutaric acid.

The role of O_2 metabolizing enzymes viz. peroxidase, catalase and superoxide dismutase has been well

documented in diazotrophic bacteria. Oxygen sensitivity of the mutants of *Azotobacter* has been shown to be due to either lack of or very low activity of catalase among the other factors studied (13,24). In the present study also the mutant was found to lack catalase activity (Table 4) which again shows the role of O_2 metabolizing enzymes in providing O_2 tolerance.

Table 4. Peroxidase, catalase and superoxide dismutase activity of the free living cells of *A. caulinodans* strain IRBG 46 and its mutant C 48.

Strain	Enzyme activity		
	Peroxidase[a]	Catalase[b]	Superoxide dismutase[c]
IRBG 46	0.48 ± 0.09	59 ± 7.7	71 ± 6.6
C 48	0.49 ± 0.05	nd	68 ± 4.0

Note: Values reported are mean ± SD of six replicates.
[a]Change in OD_{400} mg^{-1} protein h^{-1}.
[b]μmol H_2O_2 degraded mg^{-1} protein min^{-1}.
[c]Units causing reduction in colour formation by 50% under specified condition.

In the symbiotic test, the mutant formed smaller, light green nodules compared to larger, dark green nodules formed by the wild type strain. Plants infected with the mutant were stunted in growth. Regarding nitrogenase (acetylene reduction) activity, stem nodules and root nodules of the plants infected with the mutant showed only about 14% and 36% activity respectively, as compared to those of wild type (Table 5). However, the reason for the difference in the expression of nitrogenase in the stem and root nodules is not known. It may be possible that high O_2 concentration in the stem nodules arising out of photosynthesis by the green cells, is one of the reasons for the lower nitrogenase activity. The biomass produced by the mutant was also much less (1.67 g/plant) than that produced by the wild type IRBG 46 (2.85 g/plant) at 53 DAS.

Table 5. Nitrogenase (acetylene reduction) activity of root and stem nodules of *S. rostrata* infected with *A. caulinodans* strain IRBG 46 and its mutant C 48

Strain	Nodule dry wt (mg $plant^{-1}$)	Nitrogenase activity (μmol C_2H_4 h^{-1} g^{-1} nodule dry wt)
	Root nodules	
IRBG 46	43	44.49 ± 2.485
C 48	19	16.31 ± 2.677
	Stem nodules	
IRBG 46	46	36.00 ± 10.874
C 48	23	5.14 ± 1.501

Note : Values reported are mean ± SD of three replicates. Each replicate consisted of 3 plants for IRBG 46 and 4 plants for the mutant.

The transconjugant, C48(pRS2001) was assayed for nitrogenase activity under both 1% and 3% O_2 levels. From Table 6 it is clear that the plasmid pRS2001 carrying *fix*LJ genes complemented the mutation in C48 and restored the nitrogenase activity to the wild type level at 3% O_2 concentration. However, since pSUP202 from which pRS2001 has been derived, is a narrow host range vector and does not replicate in rhizobia, it may be possible that the pRS2001 got integrated into the C48 genome thus giving Cm^R transconjugants . Thus, the mutation may be either in *fix*L or *fix*J which are involved in O_2 regulation of *nif/fix* gene expression in (azo)rhizobia. The mutant C48 of *A. caulinodans* which did not show characteristic tolerance to O_2 when fixing N_2 at 3% O_2 concentration was distinguished from the Nif^- mutants by its ability to fix N_2 when the environmental O_2 concentration was considerably decreased (1%). Mutants of this kind have previously been reported in *Azotobacter* (23, 24). From the properties studied, it appears that the sensitivity of the mutant might be due to reduced respiratory activity. However, plasmid mobilization shows that the mutation might be either in *fix*L or *fix*J. FixLJ belong to the two component regulatory cascade which activates *nif*A as well as *fix*K. FixK, in turn, positively regulates the expression of *fix* NOQP encoding membrane bound terminal oxidase (29, 30). This oxidase has been identified in *A. caulinodans* and found to be involved in both free living and symbiotic respiration (30). It is thus likely that a mutation in *fix*L/*fix*J will also affect respiratory activity. However, lack of catalase activity in the mutant could not be related to the mutation in *fix*L or *fix*J as information available till now does not suggest any role of FixL or FixJ in the expression or regulation of catalase activity. Since NTG causes point mutation in the genome it may be possible that it has caused a mutation in the gene for catalase also.

Table 6. Nitrogenase (acetylene reduction) activity of the free living cells of *A. caulinodans,* mutant C48 and its transconjugant under two different O_2 concentrations*

Strain/Transconjugant	Sp nitrogenase activity (nmol C_2H_4 h^{-1} mg^{-1} protein)	
	1% O_2	3% O_2
IRBG 46	696 ± 50.2	446 ± 21.5
C 48	533 ± 25.7	29 ± 3.4
C48 (pRS 2001)	579 ± 37.1	383 ± 86.8

* O_2 in N_2 atmosphere.
Note : Values reported are mean ± SD of six replicates.

Thus, the study suggests that the oxygen protection mechanism in diazotrophic *A. caulinodans* strain IRBG 46 comprises not only of respiratory protection, but also of catalase activity combined with molecular tuning of *nif/fix* gene expression by FixLJ cascade.

Acknowledgement

The senior author thanks the Director, Indian Agricultural Research Institute, New Delhi for providing Senior Research Fellowship.

Received 26 May, 1997; revised 7 July, 1997.

References

1 **Dreyfus B, Garcia JL & Gillis M,** *Int J System Bacteriol,* **38** (1988) 89.
2 **deBruijin FJ,** In *Plant microbe interactions* Vol.3 (E Nester, T Kosuge, Editors), Mc Graw Hill, New York (1989) pp 457-493.
3 **Stam H, van Verseveld HW, De Vries W & Stouthamer AH,** *Arch Microbiol,* **139** (1984) 53.
4 **Stouthamer AH, Stam H, De Vries W & van Vlerken M,** In *Nitrogen fixation : Hundred years after* (Bothe H, de Bruijn FJ, Newton WE, Editors), Gustav Fisher, New York (1988) pp 257-261.
5 **Bergersen FJ & Turner GL,** *J Gen Microbiol,* **89** (1975) 31.
6 **Figurski D & Helinski DR,** *Proc Natl Acad Sci,* USA, **76** (1979) 1648.
7 **Kaminski PA & Elmerich C,** *Mol Microbiol ,* **5** (1991) 665.
8 **Khanuja SPS,** *Curr Sci,* **61** (1991) 545.
9 **Dreyfus B, Elmerich CL & Dommergues YR,** *Appl Environ Microbiol,* **45** (1983) 711.
10 **Ditta G, Virts E, Palomares A & Kim CH,** *J Bacteriol,* **169** (1987) 3217.
11 **Daniel RM & Appleby CA,** *Biochim Biophys Acta,* **275** (1972) 347.
12 **Sikka VK & Kumar S,** *Appl Environ Microbiol,* **48** (1984) 1248.
13 **Page WJ, Jackson L & Shivaprasad S,** *Appl Environ Microbiol,* **15** (1988) 2123.
14 **Guidotti G, Colombo JP & Foa PP,** *Anal Chem,* **33** (1961) 151.
15 **Sinha AK,** *Anal Biochem,* **47** (1972) 389.
16 **Elstner FF, Youngman RJ & O'Bwald W,** In *Methods of enzymatic analysis,* 3rd edition, (HU Bergmeyer, Editor), Academic Press. Inc. (London) Ltd., London (1983). pp 293-302.
17 **Stickland LH,** *J Gen Microbiol,* **5** (1951) 698.
18 **Lowry OH, Rosebrough NJ, Farr AL & Randall RJ,** *J Biol Chem,* **193** (1951) 265.
19 **Adebayo A, Watanabe I & Ladha JK,** *Appl Environ Microbiol,* **55** (1989) 2407.
20 **Hoagland DR & Arnon DI,** Circular 347 (Revised Jan., 1950), The College of Agriculture, Univ. of California, Berkeley.
21 **Ladha JK, Miyan S & Garcia M,** *Biol Fertil Soils,* **7** (1989) 191.
22 **Hardy RWF, Holsten RD, Jackson EK & Burns RC,** *Plant Physiol,* **43** (1968) 1185.
23 **Ramos JL & Robson RL,** *J Gen Microbiol,* **131** (1985) 1449.
24 **Iwahashi H, Hachiya Y & Someya J,** *FEMS Microbiol Lett,* **77** (1991) 73.
25 **Anthamatten D & Hennecke H,** *Mol Gen Genet,* **225** (1991) 38.
26 **Batut J, Daveran-Mingot ML, David M, Jocobs J, Garnerone AM & Kahn D,** *The EMBO J,* **8** (1989) 1279.
27 **Trinchant JC & Rigaud J,** *J Gen Microbiol,* **133** (1987) 37.
28 **Trinchant JC & Rigaud J,** *Plant Physiol,* **94** (1990) 1002.
29 **Mandon K, Hillebrand H, Mougel C, Desnouces N, Dreyfus B, Kaminski PA & Elmerich C,** In *New horizons in nitrogen fixation* (R Palacios, J Mora, WF Newton, Editors), Kluwer Academic Publishers, Dordrecht, The Netherlands (1993) p 478.
30 **Mandon K, Kaminski PA & Elmerich C,** *J Bacteriol,* **176** (1994) 2560.