Unusual radioresistance of nitrogen-fixing cultures of *Anabaena* strains

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Nitrogen-fixing cultures of two species of the filamentous, heterocystous cyanobacterium *Anabaena,* namely *Anabaena* sp. strain L-31 and *Anabaena torulosa* were found to be highly tolerant to ^{60}Co gamma radiation. No adverse effect on diazotrophic growth and metabolism were observed up to a dose of 5 kGy. At higher doses, radiation tolerance showed a correspondence with the inherent osmotolerance, with *Anabaena* L-31 being the more radiation tolerant as well as osmotolerant strain. In *Anabaena* L-31, exposure to 6 kGy of gamma rays resulted in genome disintegration, but did not reduce viability. Irradiation delayed heterocyst differentiation and nitrogen fixation, and marginally affected diazotrophic growth. All the affected parameters recovered after a short lag, without any discernible post-irradiation phenotype. The radiation tolerance of these Gram-negative photoautodiazotrophs is comparable with that of the adiazotrophic photoautotrophic cyanobacterium *Chroococcidiopsis* or adiazotrophic heterotroph *Deinococcus radiodurans.* This is the first report of extreme radioresistance in nitrogen-fixing *Anabaena* cultures.

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1. Introduction

Radioactivity and ionizing radiations were more prevalent in the primitive atmosphere of the earth than at present. In conformity with this, several ancient bacteria exhibit higher radioresistance than eukaryotes, which evolved later. For example, the Gram-positive bacterium *Deinococcus radiodurans* survives exposure to 6 kGy ^{60}Co-gamma rays without any loss of viability (Battista 1997). Most other bacteria show a D_{10} (dose required for 1 log cycle reduction in survival) of only 0.1–0.5 kGy (Sommers 2003). An intermediate level of radioresistance is seen in the unicellular cyanobacterium *Chroococcidiopsis* with a D_{10} dose of 3–5 kGy (Billi *et al*. 2000) and in the halophilic archaeon *Halobacterium* sp. strain NRC1, which exhibits a D_{10} dose of 5 kGy (Kottemann *et al*. 2005). In comparison, animal cells exhibit extreme radiosensitivity, e.g. mice show 50% lethality to 6–8 Gy in 30 days (Morton and Siegel 1971).

The aforesaid high levels of radiation have not existed on our planet. In the present time, the highest natural background radiation recorded is only about 400 mGy per year and is restricted to only a few sites in the world (Elisabeth 2005). This raises the interesting question as to how such extreme radioresistance developed in some microbes. Both desiccation and gamma radiation cause extensive DNA damage. The fact that several of the ionizing radiation sensitive (*irs*) mutants of *D. radiodurans* isolated earlier were also desiccation sensitive led to speculation that the phenomenal DNA repair proficiency of such microbes may have evolved to combat one of the more common stresses such as desiccation (Mattimore and Battista 1996). Similar arguments have been made for the high DNA repair proficiency and radioresistance of the desert isolate of the cyanobacterium *Chroococcidiopsis* (Billi *et al*. 2000). The radioresistance of these microbes may therefore be incidental, i.e. a consequence of acquired desiccation tolerance. However, the validity of such a hypothesis has, at times, been questioned (Shirkey *et al.* 2003; Billi 2009).

Photosynthesis and nitrogen fixation, the two most vital metabolic activities that sustain life on this planet, originated quite early in evolutionary history, when higher doses

Keywords. *Anabaena*; heterocyst development; nitrogen fixation; radiation tolerance

Abbreviations used: CFU, colony forming unit; LB, Luria–Bertani; OD, optical density; PIR, post-irradiation recovery; ROS, reactive oxygen species

of ionizing radiation apparently prevailed (Brock 1973). Fossil records (*stromatolites)* of filamentous heterocystous cyanobacteria, which harbour both these processes, are estimated to be >3 billion years old. Ionizing radiations generate reactive oxygen species (ROS) in aqueous media, oxygen and oxidative stress are highly inimical to nitrogen fixation in all bacteria, including cyanobacteria (Fay 1992). Oxygen represses the synthesis of nitrogenase proteins and inactivates and degrades them (Robson and Postgate 1980). Ionizing radiations (X-rays, γ-rays), should therefore be detrimental to nitrogen fixation. On the other hand, heterocystous cyanobacteria such as species of *Anabaena* and *Nostoc* are known to be highly desiccation tolerant (Potts 1994) and can revive within minutes of re-wetting post desiccation (Scherer *et al.* 1984). They can therefore be expected to be radioresistant. Evaluation of cyanobacterial radiation tolerance is therefore of considerable academic interest.

Among cyanobacteria, the unicellular non-nitrogen fixer (adiazotroph) *Anacystis nidulans* is fairly radiosensitive and shows a D_{10} dose of only 0.2–0.4 kGy for gamma rays (Asato 1971; Dmitriev and Grodzinskii 1973). In comparison, cyanobacteria such as *Chroococcidiopsis* and *Nostoc muscorum* have been shown to tolerate >10 kGy dose of ionizing radiations in combined nitrogen-supplemented or adiazotrophic growth conditions (Kraus 1969; Billi *et al.* 2000). The radiation tolerance of nitrogen-fixing cyanobacterial cultures has, strangely, remained unexplored.

This study was undertaken to investigate radiation tolerance of nitrogen-fixing cultures of two *Anabaena* species, known to be differentially tolerant to osmotic stress (Fernandes *et al.* 1993). Our results reveal that nitrogen-fixing cultures of both *Anabaena* strains tolerated a 5 kGy gamma-ray dose without loss of survival. At higher doses of ^{60}Co-gamma rays, their radiosensitivity exhibited correspondence with their relative osmotolerance. Diazotrophic cultures of *Anabaena* sp. strain L-31 could repair the DNA damage caused by 6 kGy irradiation and displayed high radiation tolerance in terms of viability, growth, heterocyst development and nitrogen fixation.

2. Materials and methods

2.1 *Strains and growth conditions*

Filamentous, heterocystous, nitrogen-fixing cultures of two *Anabaena* species were used in this study. *Anabaena* sp. strain L-31, (hereafter referred to as *Anabaena* L-31), is a fresh-water osmotolerant isolate from paddy fields (Thomas 1970), while *Anabaena torulosa* is a brackish water strain (Fernandes and Thomas 1982). The strains were grown in BG11 liquid medium at pH 7.2 (Castenholz 1988), without or with combined nitrogen (17 mM $NaNO_3$), and under continuous illumination (30 μE m^{-2} s^{-1}) and shaking (100 ± 5 rpm) at 28° ± 2°C, wherein they showed a doubling time of 20–24 h. *Escherichia coli* MC4100 [F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*] (Tobe *et al*. 1987) was aerobically grown in Luria–Bertani (LB) liquid medium at 37°C under continuous shaking (180 rpm).

2.2 *Irradiation studies*

Mid-exponential phase (three-day-old) *Anabaena* cultures were concentrated to a chlorophyll *a* density of 10 μg ml^{-1} and exposed to ^{60}Co-gamma radiation (dose rate of 6.25 kGy h^{-1}) (Gamma Cell 5000 irradiation unit, Bhabha Atomic Research Centre, Mumbai, India). Irradiated cells were washed twice with BG11 medium, reinoculated into fresh medium (at an initial chlorophyll *a.* density of 1–1.2 μg ml^{-1}) and incubated under usual growth conditions. Radiation tolerance of *Anabaena* strains was assessed in terms of post-irradiation growth measured as chlorophyll *a* content in 95% methanolic extracts of cells, as described earlier (Mackinney 1941). To check the viability, *Anabaena* L-31 cells were exposed to 6 kGy of gamma radiation, stained with Trypan blue (in 0.4% saline) and observed microscopically. Cells were also heat killed and cell death checked by Trypan blue staining (Freshney 1987). For post-irradiation growth studies in *E. coli* MC4100, cells grown overnight in LB medium (Atlas 2004) were exposed to 6 kGy of gamma radiation, washed three times with liquid LB medium and inoculated into fresh medium. Post-irradiation growth was monitored in terms of turbidity (optical density [OD] at 600 nm) or as colony forming units (CFUs).

2.3 *DNA damage studies*

For assessment of genome integrity, *Anabaena* L-31 cells at different times of post-irradiation recovery (PIR) were immobilized in agarose plugs and subjected to *in situ* lysis as described earlier (Mattimore and Battista 1996), followed by electrophoretic resolution of genomic DNA on 0.6% agarose gels. About 6 μg chlorophyll *a* equivalent cells of unirradiated or irradiated (6 kGy) *Anabaena* L-31 cultures were immobilized in 0.7% agarose. Cells in agarose plugs were lysed *in situ* by sequentially treating with lysozyme (2 mg ml^{-1}) and proteinase K (1 mg ml^{-1}). Each treatment was carried out at 37°C for a duration that lasted overnight. Agarose plugs containing cell debris and chromosomal DNA were washed with 1X TBE (89 mM Tris-Cl, 89 mM borate and 2 mM ethylene diamine tetra acetic acid, pH 8.0), embedded in wells on 0.6% agarose gel and electrophoretically resolved at 80 V for 3 h. DNA

was visualized by staining with ethidium bromide post electrophoresis.

2.4 *Heterocyst frequency and determination of nitrogenase activity*

Heterocysts and vegetative cells were counted microscopically. Heterocyst frequency (%) was calculated from a count of at least 1000 cells per sample. Nitrogenase activity was measured by acetylene reduction assay in 2 ml culture aliquots, as described earlier (David *et al*. 1980).

2.5 *Statistical analysis*

Each treatment comprised three replicates and each experiment was repeated at least twice. The results are presented as the average of these with standard error for a representative experiment. Variation between experiments was less than 15%.

3. Results and discussion

Radiation tolerance is conventionally measured in terms of survival or viability of cells immediately after irradiation, or in terms of PIR. Assessment of tolerance in terms of CFUs resulted in erroneous and non-reproducible results for *Anabaena* strains. Due to random fragmentation, lower doses of irradiation in fact increased the CFUs. In multicellular organisms, such as filamentous fungi (Sharma 1998) or *Anabaena* strains, it is rather difficult to accurately estimate CFUs. In such microbes, dry weight, turbidity, protein and chlorophyll *a* content are considered more reliable measures of growth and are generally used for assessing tolerance to radiation and other stress conditions (Kraus 1969; Potts 1994; Kuritz and Wolk 1995; Lehtimaki *et al*. 1997; Huckauf *et al*. 2000; Fiore *et al*. 2005). Chlorophyll *a* in particular shows excellent correlation with cell number and protein content, and is routinely used for assessing growth of cyanobacteria under normal and stress conditions (Stal 1988; Alahari and Apte 1998; Rajaram and Apte 2008).

Based on chlorophyll *a* post-irradiation growth measurements, both *Anabaena* strains were found to display remarkable and comparable radiation tolerance up to 5 kGy (figure 1a). However, at higher doses of 8–11 kGy, a distinct difference was observed between the two strains, with *Anabaena* L-31 showing higher radioresistance. The observed radiation tolerance of *Anabaena* strains contrasted sharply with a wild-type *E. coli* strain MC4100, wherein cells exposed to 6 kGy did not produce any visible colonies (data not included) and did not revive even after 3.5 h of incubation, indicating its high sensitivity to ionizing radiation stress (figure 1b).

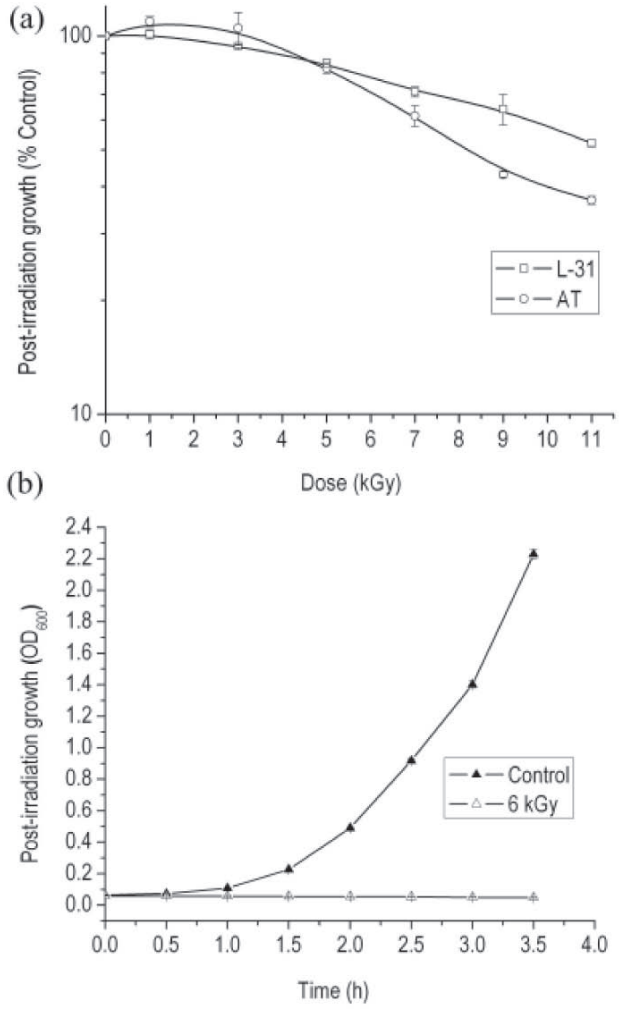

Figure 1. Comparative radiation tolerance of *Anabaena* strains and *E. coli*. **(a)** Post-iradiation growth of *Anabaena* strains. Three-day-old *Anabaena* strains [L-31: *Anabaena* L-31; AT: *Anabaena torulosa*] were exposed to different doses of ^{60}Co-gamma radiation at a dose rate of 6.25 kGy hr^{-1}. Growth was estimated as the content of chlorophyll *a* per ml of culture after 4 days of post-irradiation growth, and shown as per cent chlorophyll *a* content of 4-day-old unirradiated control taken as 100%. The 100% chlorophyll *a* values (in μg ml^{-1}) were 3.0 ± 0.026 (L-31), and 2.6 ± 0.01 (AT). **(b)** Post-irradiation growth of *E. coli.* Overnight grown aerobic cultures of *E. coli* MC4100 were exposed to 6 kGy of ^{60}Co-gamma radiation, washed with and inoculated into fresh liquid LB media and grown. Growth was measured in terms of optical density (OD) at 600 nm.

Possible cell death of *Anabaena* L-31 following irradiation was checked by vital staining with Trypan blue, which stains only dead cells. *Anabaena* L-31 filaments exposed to 6 kGy gamma radiation were not stained by Trypan blue (figure 2) indicating that they remained viable. In comparison, heat-killed *Anabaena* cells readily picked up the stain (figure 2). Genomic DNA of all organisms is highly prone to damage upon irradiation. Post-irradiation genome integrity was

therefore investigated by *in situ* lysis of irradiated (6 kGy) and unirradiated cells, and resolution of genomic DNA by agarose gel electrophoresis (figure 3). This revealed clearly visible damage immediately after irradiation (lane 2) and its notable repair during PIR (lane 3) of *Anabaena* L-31, leading to near complete visual restoration of genome size by day 4 of PIR (figure 3).

Post-irradiation diazotrophic growth was monitored in combined N-free media after exposing exponential phase heterocystous nitrogen-fixing cultures of *Anabaena* L-31 to 6 kGy of ^{60}Co-gamma radiation (figures 4a, c and e). Irradiated cultures showed an extended lag, but recovered quickly thereafter to display growth (figure 4a) and nitrogenase activities (figure 4e), comparable with that of unirradiated controls. The frequency of functional heterocysts (heterocysts attached to the filament) in irradiated cells was however <5% compared with the control (>6.75%). Microscopic examination showed several detached heterocysts in irradiated cultures (figure 5). Only heterocysts attached to filaments can fix nitrogen, since they receive the photosynthate from neighbouring vegetative cells (Wolk 1968). The small decrease in nitrogenase activity of irradiated cultures may be consequent to a decreased number of functional (attached to filaments) heterocysts.

It is possible that the observed nitrogen fixation may be largely due to heterocysts that pre-existed before

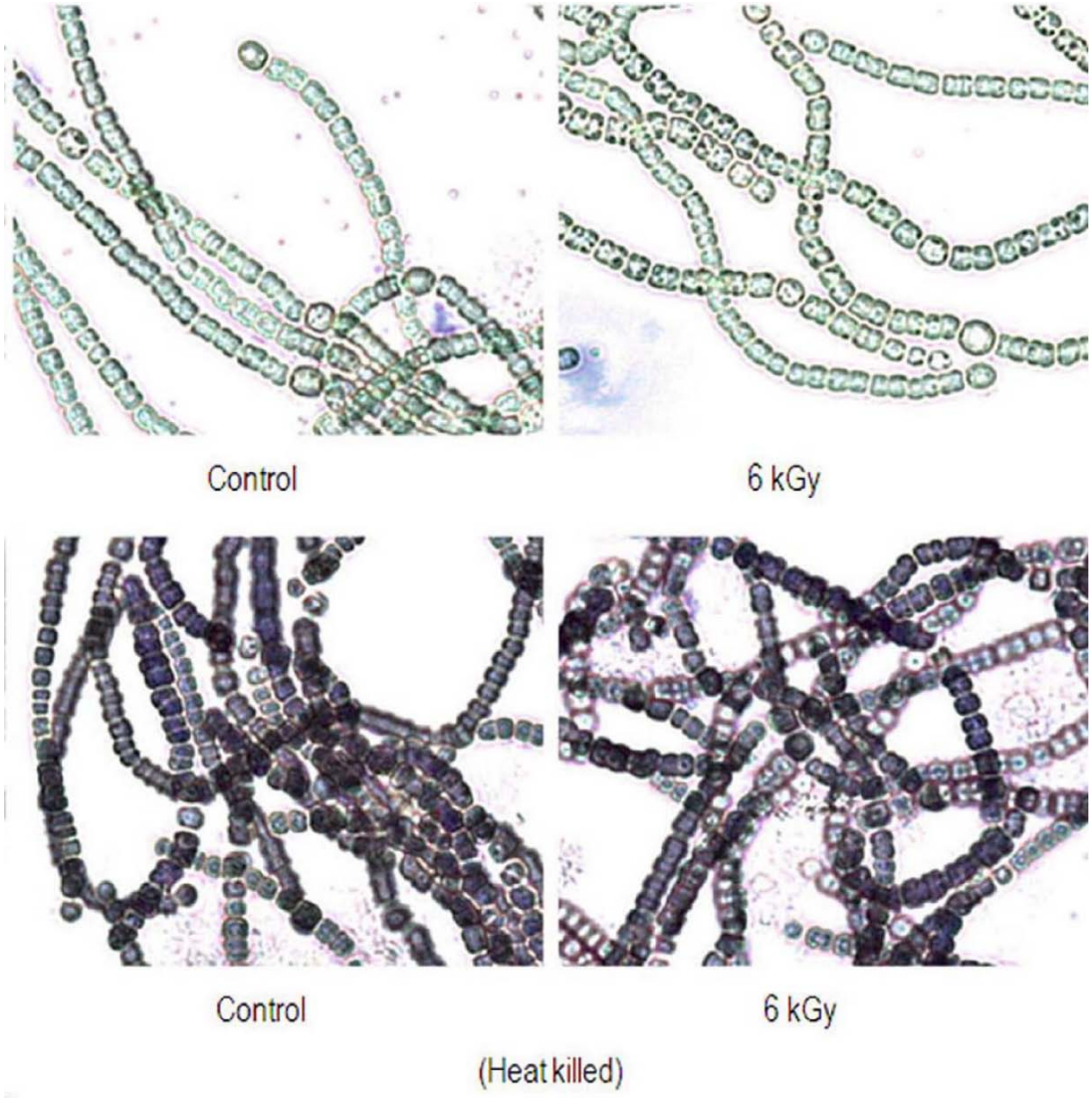

Figure 2. Effect of ^{60}Co-gamma radiation on viability of *Anabaena* L-31. Three-day-old *Anabaena* L-31 culture was exposed to 6 kGy of gamma radiation. The cells were immediately stained with 0.4% of Trypan blue (in saline) for 5 min, and observed microscopically at 400X magnification for vitality staining. The cells were also heat killed by boiling for 30 min, stained, and observed microscopically for cell death.

irradiation, while the ability to differentiate into new functional heterocysts may be adversely affected by γ-rays. To ascertain this, nitrate-grown, heterocyst-free, *Anabaena* L-31 filaments were exposed to a 6 kGy dose of gamma rays, and then allowed to differentiate under nitrogen-fixing conditions (figures 4b, d and f). Such cultures showed a rapid differentiation of normal functional heterocysts (figure 4d) and constitution of high nitrogenase activity (figure 4f), which matched these functions in the corresponding unirradiated controls. These results clearly established that 6 kGy gamma radiation did not seriously interfere with *de novo* heterocyst development and nitrogen fixation in *Anabaena*, but merely enhanced the fragility of existing heterocysts (figure 5b) .

The radiation tolerance of nitrogen-fixing *Anabaena* cultures (figures 1 and 4) contrasts sharply with the radiosensitivity of many Gram-negative and -positive bacteria, which exhibit D_{10} dose values of less than 0.5 kGy (Sommers 2003) and do not recover from exposure to a high dose of radiation (figure 1b). Only spores of *Bacillus* (up to 4 kGy) and *Clostridium* (10 kGy) show radiation tolerance (Sommers 2003) that is comparable with that of *Anabaena* strains (figure 1). It may be argued that our assessment of cyanobacterial radiation tolerance is flawed, since the fraction of surviving cells may perhaps grow to the same chlorophyll levels as controls in 4 days of recovery time. But this is not so. At a 6 kGy dose, the surviving population of most bacteria would be reduced by over 10 log cycles and, given the long generation time of 20–24 h which is typical of *Anabaena* strains, the surviving fraction cannot recover chlorophyll levels equal to 80–90% of unirradiated control levels in just 4 days' time. *E. coli* cells exposed to a 6 kGy dose were not able to revive even after 9–10 generation times post irradiation (figure 1b). Although as expected, the genomic DNA of *Anabaena* cells suffered considerable damage (figure 3), the cells remained viable (figure 2) and could subsequently repair the DNA damage (figure. 3) and recovered (figure 4). The post-irradiation growth seen in figure 1a and figure 4 therefore reflects the genuine radiation tolerance of nitrogen-fixing *Anabaena* cultures.

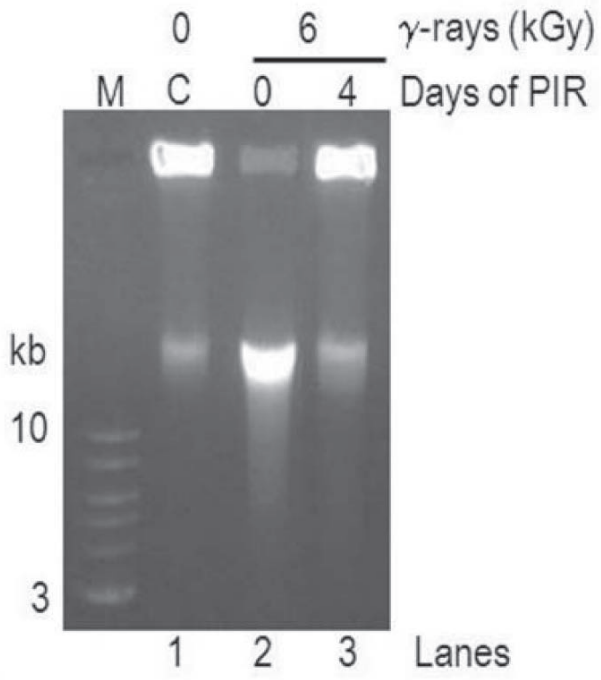

Figure 3. Effect of ^{60}Co-gamma radiation on genome integrity of *Anabaena* L-31.Three-day-old *Anabaena* L-31 cultures were exposed to 6 kGy of ^{60}Co-gamma radiation and allowed to grow under usual growth conditions. Cells were collected at different time points of post-irradiation recovery (PIR) and immobilized in 0.7% agarose plugs, lysed *in situ* sequentially with lysozyme (2 mg ml^{-1}) and proteinase K (1 mg ml^{-1}). The agarose plugs were washed with 1X TBE, embedded in wells on 0.6% agarose gel, and the DNA therein was electrophoretically resolved at 80 V for 3 h. Lane 1(C), DNA of unirradiated control cells; Lanes 2 and 3, DNA of irradiated cells on 0 and 4 days of PIR, respectively; M, 1 kb DNA marker.

Aerobic diazotrophy in *Anabaena* is achieved by execution of an elaborate developmental programme of heterocyst formation, involving an array of structural, biochemical, genetic and physiological changes which culminate in the expression and activity of a nitrogenase complex (Stewart 1980; Haselkorn 1978; Wolk 1996). Heterocyst development is generally a robust process, but synthesis and activity of nitrogenase proteins is highly stress sensitive (Apte 2001). The results presented in figure 4 clearly show, for the first time, that *Anabaena* heterocysts seem to be able to synthesize and protect nitrogenase from severe oxidative stress caused by 6 kGy radiation. Similar ability to protect nitrogen fixation from high osmotic stress was shown earlier in these *Anabaena* strains (Fernandes *et al*. 1993). At high doses (11 kGy) of radiation, *Anabaena* L-31 and *A. torulosa* displayed 52% and 37% growth, respectively, compared with unirradiated controls, which approximately matches the known osmotolerance (GI_{50}) of *Anabaena* L-31 (~350 mM) and *A. torulosa* (~225 mM) reported earlier (Fernandes *et al*. 1993).

It is interesting that diazotrophic growth in *Anabaena* strains shows considerable resistance to ionizing radiations (figure 1), as it does to osmotic stress (Fernandes *et al.* 1993). The similarity between ionizing radiations and osmotic stress/desiccation is unmistakable – both stresses generate ROS, either from water or by heterolytic cleavage of organic compounds which, in turn, are known to damage all major biomolecules, especially DNA, proteins and lipids. Indeed, the damage caused by these stresses has been found to be similar in several microorganisms (Mattimore and Battista 1996; Billi *et al*. 2000; Kottemann *et al*. 2005) and also appears to be repaired similarly (Kottemann *et al.* 2005).

Most radiation/desiccation-resistant microbes are primitive organisms and must have encountered high radiation as well as low water activity environments (drought and hypersaline habitats) during their evolution, where water-generated

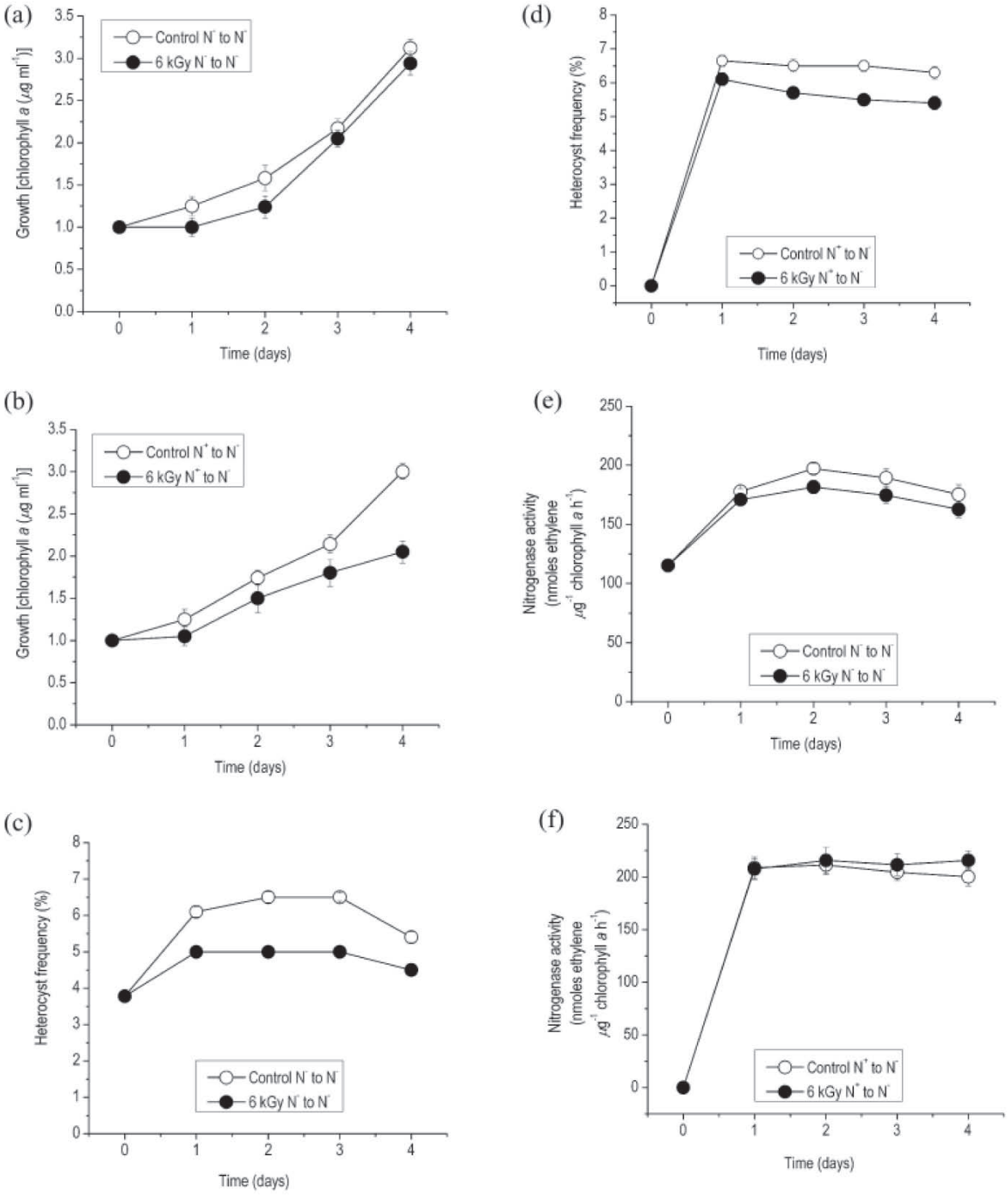

Figure 4. Effect of ^{60}Co-gamma radiation on the diazotrophic growth of *Anabaena* L-31. *Anabaena* L-31 cultures, maintained in nitrogen-free medium (N^-) **(a, c, e)** or nitrogen-supplemented BG11 medium (N^+) **(b, d, f)**, were transferred to nitrogen-free liquid media, either without (controls) or after exposure to 6 kGy of ^{60}Co-gamma rays. Cultures were assessed in terms of growth **(a, b)** heterocyst frequency **(c, d)** and nitrogenase activity **(e, f)** during post-irradiation growth.

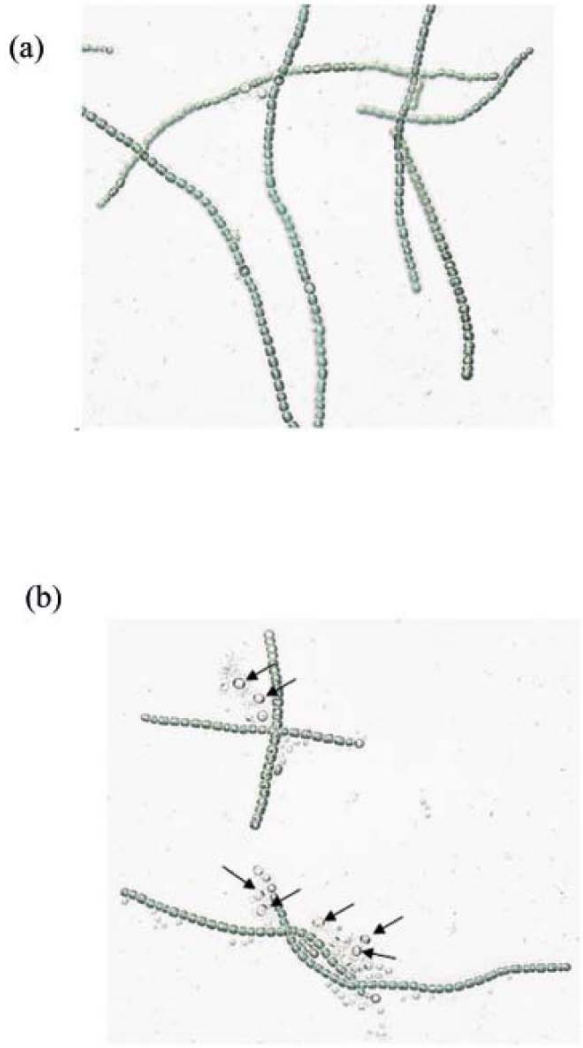

Figure 5. Morphology of *Anabaena* L-31 immediately after irradiation. Three-day-old *Anabaena* L-31 cultures were exposed to 6 kGy of radiation, and immediately observed microscopically (magnification 400X). **(a)** Unirradiated control cells, **(b)** 6 kGy irradiated cells. The detached heterocysts are marked by arrows in (b).

radicals were the main cause of damage (Mattimore and Battista 1996; Kottemann *et al*. 2005). It is not surprising, therefore, that they exhibit exceptional DNA repair capacities and can recover after acute exposure to radiation or prolonged desiccation, without any visible mutation (Mattimore and Battista 1996; Billi *et al.* 2000; Kottemann *et al.* 2005). Based on the facts that (a) very high doses of ionizing radiations have probably never existed on earth, and (b) radiation-sensitive mutants of *Deinococcus radiodurans* are also desiccation sensitive (Mattimore and Battista 1996), it is speculated that radiation resistance of such microbes may be incidental, i.e. a consequence of the ability to survive desiccation stress. The data presented in this paper once again reflect the possible commonality shared by the cellular responses to these apparently different abiotic stresses, as has been observed in *Deinococcus*, *Halobacterium*, *Chroococcidiopsis* and several other microbes.

Radioresistant microbes hold promise for biotechnological applications in high radiation environments. At a 5 kGy dose, radiation tolerance of the *Anabaena* strains appeared to be comparable with that of *D. radiodurans* (Battista 1997) and the cyanobacterium *Chroococcidiopsis* (Billi *et al*. 2000). In recent years, the radioresistant bacterium *D. radiodurans* has been genetically engineered for mercury detoxification (Brim *et al*. 2000), toluene degradation (Lange *et al*. 1998) and uranium bioprecipitation from dilute aqueous waste in high radiation environments (Appukuttan *et al*. 2006). A major limitation of *D. radiodurans* is its heterotrophy and inability to grow in nutrient-limited minimal media (Venkateswaran *et al*. 2000). In this context, the relatively high radiation tolerance and photoautodiazotrophy of *Anabaena* strains offers an attractive potential alternative for bioremediation of radioactive waste.

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