STRESS RESPONSES IN THE NITROGEN-FIXING CYANOBACTERIUM *ANABAENA*

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Abstract

The ancient organisms, cyanobacteria, also thought to be the progenitors of plant chloroplasts, have exhibited various overlapping mechanisms while dealing with myriad environmental stresses. The filamentous nature of the nitrogen-fixing cyanobacterium, *Anabaena*, makes it an interesting model for study of stress responses as several unique features need to be incorporated as against what is observed in the unicellular bacteria. This chapter will take the reader on a journey of what was discovered and established as mechanisms for overcoming heat stress, oxidative stress, nutrient-starvation and DNA damage due to exposure to γ-radiation, in *Anabaena*.

1. Introduction

Having inhabited the Earth billions of years ago, cyanobacteria have learnt to acclimatise themselves to various environmental challenges, be it temperature, radiation, desiccation, salinity, heavy metal exposure etc. The major modes of damage across various stresses are the damage to proteins, membrane and/or production of reactive oxygen species (ROS) leading to damage to DNA and several physiological processes. Many of the stresses have been extensively studied in the filamentous cyanobacterium, *Anabaena* (*Nostoc*) PCC 7120 which has been considered as a model system among the filamentous cyanobacteria owing to its ability to be genetically manipulated. Presence of multiple genes encoding proteins with similar functions, absence of certain genes and pathways,

presence of global transcriptional regulatory proteins, several *cis*-elements contributing to transcriptional and post-transcriptional regulation and protein processing have all contributed to the unique ability of *Anabaena* to combat various stresses. Some of these mechanisms have perpetuated to the modern-day bacteria during the course of evolution, while some have been lost in bacteria, but observed in plants, further indicating the close association of cyanobacteria with plants. A brief glimpse into the world of *Anabaena* while combating the various abiotic stresses is detailed below.

2. Novel Insights into the Heat Shock Response of *Anabaena*

Proteins, be it structural or functional proteins, contribute to the normal functioning of all physiological processes and adaptability when exposed to sudden changes in growth conditions that could be even detrimental to organism i.e. upon perceiving stress. This would require these proteins to maintain their structural and functional integrity, and that's where the magical proteins i.e. chaperones and proteases play an important role. While the chaperones, as the name indicates, guide other proteins to maintain their integrity through assisting in correct folding, proteases, on the other hand, degrade the irreversibly misfolded proteins so that their unwarranted accumulation does not result in cellular toxicity and their degradation also allows for the availability of the basic building blocks i.e. the amino acids for synthesis of new proteins. Since, these proteins were identified in response to heat stress; they have been classified as Heat Shock proteins (HSPs). *Anabaena* has a plethora of HSPs which have been well characterised in other bacteria, but differs in having multiple genes encoding HSPs with similar functions, exemplified by the presence of at least 5 DnaK (Hsp70) encoding genes and two GroEL (Hsp60) encoding genes.

2.1. Multiplicity of Hsp60 proteins in Anabaena

While most bacteria have a single Hsp60 protein with the exception of *Rhizobium* and *Bradyrhizobium*, which have 4-5 genes encoding for this protein, most cyanobacteria possess two Hsp60 encoding genes, the proteins differing in their molecular mass by at least 2 kDa and with distinct C-terminal regions. This difference refers to the presence of a 'GGM' tail which is a signature of all bacterial Hsp60 proteins, but is present only in the larger of the two Hsp60 proteins in cyanobacteria. In general in cyanobacteria, the ~59 kDa GroEL is part of a bicistronic *groESL* operon, while the ~61 kDa Cpn60 is encoded by a monocistronic gene. The 59 kDa GroEL protein exhibited chaperone activity comparable to other bacterial Hsp60 proteins, however, GroES and ATP has not been found to be essential for the chaperone activity. On the other hand, both GroES and ATP aided the chaperone activity of the 61 kDa Cpn60 protein, which was found to be 10-fold lower than that of GroEL. This could also be due to the differences in their substrate specificity. Despite the presence of two *hsp60* gene, deletion mutants were not found to be viable further reiterating that they could be targeting different substrates for folding. Indeed, though the use of recombinant *Anabaena* strains, wherein only one of the two Hsp60 proteins was overexpressed, it was shown that the 59 kDa GroEL was essential under nitrogen-fixing conditions and involved in maintain the integrity of

nitrogenase and photosynthetic apparatus. The 61 kDa Cpn60 protein, on the other hand, was found to be essential under nitrogen-supplemented conditions, contributing to the stability of nitrate reductase and the photosynthetic apparatus. During the course of evolution, the mesophilic bacteria retained only one *hsp60* gene, as part of a bicistronic operon with its co-chaperone *groES* or *hsp10*, with certain essential features of the 2nd Hsp60 protein incorporated in the single *hsp60* gene.

2.2. Regulation of hsp60 genes

Regulation of these *hsp60* genes in cyanobacteria also differed from what was well known in other gram negative bacteria, wherein the *hsp60* genes were under the positive regulation of a special sigma factor (σ32). Cyanobacteria, on the other hand, followed the model established in gram positive bacteria *Bacillus*, wherein they were found to be negatively regulated by the dimeric HrcA protein which bound to an inverted repeat element known as CIRCE. Additionally, several *cis*-regulatory elements in the form of direct and inverted repeats were also found to be involved in the intricate regulation of these genes in *Anabaena* and *Synechocystis*, in response to not only heat stress but also light stress. The intricate regulatory network may have been essential to well regulate the levels of these proteins in response to the environmental conditions.

3. K^+ starvation and the K^+-dependent ATPase (Kdp-ATPase) from *Anabaena*

Potassium (K^+), the chief intracellular cation, is critical for life in all cells. The very high intracellular concentration of K^+ echoes the primeval strategy of living cells to accrue K^+ and exclude the sodium ions. The elevated concentration of K^+ is maintained by a set of K^+ influx or efflux transporters whose activities are modulated in such a manner that there is no wasteful cycling of K^+ across the membranes. When many bacteria are subjected to potassium starvation or osmotic up-stress, the high affinity Potassium-dependent Adenosine triphosphatase (Kdp-ATPase), a P-type ATPase, is synthesized. The *E. coli* Kdp-ATPase (K_m= 2μM) is capable of decreasing the K^+ content in the growth-medium to 100 nM or lower. In effect, the Kdp-ATPase is an efficient potassium scavenging enzyme that is produced when cellular requirement of K^+ cannot be met by other K^+ uptake proteins.

The *E. coli* Kdp-ATPase complex (encoded by the *kdpFABC* operon) is made up of four protein subunits viz. KdpF, KdpA, KdpB and KdpC. The KdpF subunit provides stability, whereas the KdpA polypeptide binds to and carries potassium into the cell. Interestingly, the KdpB subunit, which closely resembles the other characterized P-type ATPases, contains the evolutionarily conserved phosphorylation site, while the KdpC is required for the assembly of the whole complex. Unlike KdpF, KdpA, KdpB and KdpC proteins are indispensable for Kdp-ATPase activity *in vivo* in *E. coli*. The Kdp-ATPase, was the first P-type ATPase to be discovered from any prokaryote. In all other P-type ATPases, the central sub unit that gets phosphorylated is also responsible for the transport of the ion. But in Kdp-ATPase, the ATP hydrolysis is carried out by KdpB, and consequently KdpB is phosphorylated, while the actual transport of K^+ is carried out by the KdpA.

3.1. Identification and structural organization of the kdp operons in Anabaena

In response to potassium starvation, three strains of *Anabaena* (i.e. *Anabaena torulosa*, *Anabaena* sp. strain PCC 7120 and *Anabaena* L-31) produced a 78 kDa polypeptide that cross-reacted with the *E. coli* KdpB antiserum, indicating the presence of Kdp homologs in *Anabaena*. To decipher the structural organisation and regulation of *kdp* genes in *Anabaena*, *kdp* operons from *Anabaena* sp. strain L-31 (henceforth referred to as *Anabaena* L-31) were identified and sequenced. A strategy that employed PCR (employing degenerate primers), followed by Southern blotting and chromosomal walking was utilized to accomplish this objective.

Surprisingly, not one but two, distinct *kdp* operons were found in *Anabaena* L-31 (termed *kdp1* and *kdp2*). The *kdp1* operon (GenBank accession no. AF213466) showed 5 open reading frames (ORFs) viz. *kdpA1*, *kdpB1*, *kdpG1*, *kdpC1* and *kdpD* whereas the *kdp2* operon (GenBank accession no. AY753299) contained 4 ORFs i.e. *kdpA2*, *kdpB2*, *kdpG2* and *kdpC2*. Although *kdpF* was absent from both the *kdp* operons, an additional ORF, *kdp*G (encoding hydrophobic protein), was observed between the *kdpB* gene and the *kdpC* gene in *kdp1* as well as *kdp2*. An unusually truncated *kdpD* ORF was observed downstream of *kdpC1*, while no such ORF was present in *kdp2*. The *kdpD* protein (365 amino acids) showed similarity only to the KdpD-N terminal domain of *E. coli* KdpD; while the critical C-terminal histidine kinase domain, which is responsible for phosphorylation reaction was absent in this KdpD. No *kdpE*-like gene was found downstream of the two *kdp* operons. Thus, analysis of the deduced amino acid sequence suggested that KdpATPase encoded by both the *Anabaena* L-31 *kdp* operons was structurally similar to the other bacterial KdpATPases. However, the genes encoding the regulatory proteins (KdpDE) were distinctly different from those present in other bacteria (such as *E. coli*).

3.2. Induction of the kdp operons in response to different stimuli

When subjected to K^+ limitation (< 50 μM K^+ in medium), expression of only *kdp2* (not *kdp1*) expression was detected as a 5.3-kb transcript on Northern blots, indicating that *kdpA2B2G2C2* genes constituted a polycystronic operon. The *kdp2* expression was seen after 1h of K^+ starvation and maximal expression occurred by 3h of potassium deprivation. When 5 mM potassium was added to the K^+-starved cells, the *kdp2* expression ceased within 30 m. In response to K^+ limitation, a 78 kDa cross-reacting polypeptide, corresponding to *Anabaena* L-31 KdpB, was observed only in K^+-starved cells. The KdpB polypeptide was detected exclusively in the crude membrane fractions while none was observed in the cytosol. When K^+ was re-added to KdpB expressing *Anabaena* L-31 cells, the content of cross-reacting KdpB polypeptide decreased with time. These results demonstrated that neither the *kdp* transcript nor the Kdp-ATPase proteins (KdpB) were stable in presence of K^+. Unlike *E. coli* and *Salmonella typhimurium*, addition of common salt did not induce *kdp* in *Anabaena* L-31. Moreover, pH of the medium, heat-stress or presence/absence of combined nitrogen in the growth medium also did not affect *kdp* expression. Notably, strong induction of the *kdp2* operon was observed in response to desiccation stress. Thus, unlike the enterobacterial *kdp*

operons, the cyanobacterial *kdp* is may not play a role in overcoming salinity, but are likely to enhance survival of *Anabaena* during K^+ starvation or desiccation.

4. Molecular basis of the Oxidative Stress Resistance in *Anabaena*

4.1. Functional aspects of superoxide dismutases from Anabaena

Being a photosynthetic organism, electron transport chain of both photosynthesis and respiration contribute to the generation of intrinsic ROS in cyanobacteria. Additionally, exposure to various environmental stresses induces ROS production and this can cause damage to proteins, lipids, membrane and DNA inducing cell death. The major ROS generated include superoxide radical ($O_2^{\cdot}$), H_2O_2, OH radical etc. Of these, the $O_2^{\cdot}$ generated is accentuated due to photosynthetic activity and needs to be tackled upfront to prevent damage to the photosynthetic apparatus. *Anabaena* has two superoxide dismutases (SODs), which are involved in the dismutation of $O_2^{\cdot}$ to H_2O_2. The need for two SODs, one present solely in the cytosol i.e. FeSOD, and the other distributed in the thylakoid lumen and the cytosol i.e. MnSOD in *Anabaena,* as against only the cytosolic SODs in other bacteria was exemplified when the distribution pattern of these SODs was monitored. Of the two SODs, MnSOD was found to be essential under nitrogen-fixing conditions and FeSOD under N-supplemented conditions. This also explained why the non-nitrogen-fixing cyanobacteria primarily had only the cytosolic FeSOD. The unique feature of SODs which was unearthed in *Anabaena* was the targeted cleavage of MnSOD, its localisation and formation of homo and heterodimers.

The 30 kDa *Anabaena* MnSOD was characterised by a signal peptide (~ 3 kDa) and a linker peptide (~3 kDa) preceding the catalytic unit (~24 kDa). Owing to the signal peptide, the MnSOD could anchor onto the thylakoid membrane, and thereafter could face two fates. Either the signal peptide cleaves off releasing the 27 kDa protein into the cytosol, wherein the linker peptide is further cleaved to generate the 24 kDa protein. Thus, in the cytosol, along with the 22 kDa FeSOD monomer, two monomeric forms of MnSOD i.e. 24 and 27 kDa are available resulting in the formation of 5 homo and heterodimeric species all of which exhibited superoxide activity. The other fate involved translocation of the 30 kDa MnSOD into the thylakoid lumen, wherein the signal and linker peptide get cleaved sequentially to generate the 27 and 24 kDa forms resulting in three dimeric species of active SOD. A similar distribution of SODs was also observed in plants, but the mechanism was unknown, and was demonstrated for the first time in any cyanobacteria. The understanding of the mechanism of SOD processing opened new doors to the possibility of such processing existing for other proteins as well in cyanobacteria, which was earlier thought to be restricted to eukaryotes.

4.2. Role of Mn-catalases and peroxiredoxins from Anabaena

The end product of SOD enzyme activity is hydrogen peroxide (H_2O_2). Although, not very toxic by itself, H_2O_2 gives rise to the most deleterious ROS, the hydroxyl radical, which can damage all biomolecules in its vicinity at diffusion-controlled rates. H_2O_2 is rapidly detoxified by two classes of proteins, catalases and peroxidases. The genome of *Anabaena* PCC 7120 shows the presence of two Manganese-containing, Mn-catalases,

[Alr0998 (named as KatA) & Alr3090 (named as KatB)] while the commonly present iron-containing Heme-catalases are absent. As their name indicates, the Mn-catalases have the metal 'Mn' (instead of Fe-containing heme) at their active sites, and hence, these proteins are also referred to as pseudocatalases or alternative catalases in literature. Along with these two Mn-catalases, 10 genes encoding different classes peroxiredoxins (Prxs, also called as thiol peroxidases) are present in the genome of *Anabaena* PCC 7120. Prxs have a thioredoxin fold-containing thiol–specific antioxidant (TSA) domain, and Prxs in general, have two catalytic cysteine residues at their active site. Incidentally, glutathione peroxidases and ascorbate peroxidases that are present in animal and plant systems respectively, are absent in *Anabaena* PCC 7120.

The catalases dismutate H_2O_2 directly into water and molecular oxygen, whereas peroxiredoxins require the help of a reductant (e.g. thioredoxin, glutaredoxin etc.) to reduce H_2O_2 into water, without the concomitant generation of O_2. Initial experiments with different strains of *Anabaena* showed an inherent lack of catalase activity under control conditions of growth or on exposure to H_2O_2, the substrate of catalase enzyme. However, when overexpressed in *Anabaena* PCC 7120, the KatA was able to defend *Anabaena* from oxidative stress mediated by H_2O_2 or methyl viologen. These results proved that Alr0998 was indeed a functional protein, capable of decomposing H_2O_2 when adequately present in the cyanobacterial cells. Strangely, despite being present in a mesophilic bacterium, the KatA protein was quite thermostable and remained active even after exposure to 80^{o}C.

The absence of catalase activity raised a pertinent question: Do the catalase genes play any role in the physiology of *Anabaena* PCC 7120? Cross-protection experiments with different stresses showed the surprising role of the Mn-catalase Alr3090 (KatB) in overcoming salinity and consequently, other oxidative stresses in *Anabaena*. The salt-treated wild-type *Anabaena* PCC 7120 cells showed remarkable resistance to H_2O_2 as compared to the corresponding cells not pre-treated with NaCl. Subsequent analysis showed induction of the KatB protein in response to NaCl was responsible for this protective effect. The ability of salt to protect *Anabaena* from H_2O_2 was lost in the *katB* mutant, indicating that the KatB protein was indeed responsible for this defensive effect. The *katB* mutant of *Anabaena* was very sensitive to the oxidative effects of salinity, and unlike the wild-type, completely lysed on the subsequent exposure to H_2O_2. These results clearly showed the importance of KatB in the stress physiology of *Anabaena* PCC 7120. Analysis with *katB* promoter-*gfp* fusion showed the absence of *katB* expression in the heterocysts (cells that fix nitrogen), but not vegetative cells of *Anabaena* PCC 7120. It should be noted that, to fix nitrogen, heterocysts maintain a low intracellular concentration of oxygen. Apparently, during the course of evolution, *Anabaena* have ensured that catalases, which liberates O_2 as a bye-product, are not expressed in the heterocysts, thereby maintaining oxygen-free environment for nitrogenase to function.

The his-tagged protein KatB was over-produced in *E. coli* and purified to near homogeneity by affinity chromatography. The KatB protein was an efficient, thermostable catalase, that was insensitive to inhibition by azide. The KatB protein was

crystallized and its structure was determined by X-ray crystallography. Notably, KatB is the only Mn-catalase to be crystallized from any photosynthetic organism. Structural analysis showed KatB to be a compact hexameric protein (i.e. it was a trimer of dimers) whose active site was distinct from that of other previously characterized Mn-catalases. The KatB active site showed Glu_4His_2 coordination geometry with two terminal water ligands, and resembled the active site of the bacterioferritin/ruberythrin proteins. Surprisingly, crystallographic as well as biochemical analysis showed the involvement of the 2nd N-terminal residue of KatB (F-2) in maintaining stability as well as activity of this protein. KatB variants with smaller residues (V/A/G) at the second position were less compact, showed reduced catalytic activity and were more sensitive to denaturation than the corresponding KatB variants with larger residues (Y/W/F) at the same position. The F-2 residue was essential for maintaining intersubunit interactions that provided stability to the hexamer. Also, F-2 interacted with other residues (near the active site) and helped in the formation of the hydrophobic pocket that kept the active site together. Hence, only residues that could sustain activity (i.e., F/Y/W) were naturally selected at the 2nd position in Mn-catalases during the course of evolution.

As catalases are not constitutively expressed in *Anabaena*, which proteins are responsible for detoxification H_2O_2 that is normally produced during photosynthesis or aerobic respiration? Apparently, in the absence of catalases, our research has shown the peroxiredoxins to play a vital role in eliminating the intracellularly produced H_2O_2. Among the different peroxiredoxins present in *Anabaena*, the typical 2-Cys peroxiredoxin (denoted as Alr4641 in *Anabaena* PCC 7120), is the major peroxiredoxin that is constitutively expressed under control conditions of growth. The Alr4641-specific antiserum easily detected this protein the control (i.e. unstressed) cellular extracts of *Anabaena* PCC 7120 on Western blots. Moreover, different stresses such as H_2O_2, methyl viologen, salt and gamma radiation were able to transcriptionally activate expression of this gene over the basal levels. Biochemically, Alr4641 showed dual function i.e. not only it functioned as thioredoxin-dependent peroxidase, but it also showed chaperone function. Interestingly, between the two, only the peroxidase activity was dependent on the presence of the catalytic cysteine residues. The *al4641* promoter was active in vegetative cells as well as heterocysts of *Anabaena* PCC 7120. Overexpression of Alr4641 in *Anabaena* PCC 7120 conferred tolerance to externally added H_2O_2.

To carefully assess the role of Alr4641 in *Anabaena* PCC 7120, the *alr4641* gene was knocked down using the CRISPR-interference approach employing dCas9 and the *alr4641*-specific sgRNA. The knockdown strain (An-KD4641, wherein the Alr4641protein content decreased by ~85%), although viable, grew slower than the corresponding control cells. An-KD4641 displayed inherently higher levels of ROS, suggesting that these filaments were under oxidative duress. The knockdown strain showed disrupted thylakoid structure, diminished photosynthetic parameters, and completely lost its viability when exposed to moderate doses of H_2O_2. Hence, 2-Cys-Prx seems to be the dominant Prx that is required to sustain redox homeostasis in varied photosynthetic systems, ranging from cyanobacteria to chloroplasts, which lack sufficient

catalase expression. Furthermore, as catalase genes are not expressed in heterocysts of *Anabaena*, Alr4641 may play a crucial role in detoxifying H_2O_2 in these specialized cells.

5. DNA Repair and the unusual role of LexA in Multiple Stress Tolerance of *Anabaena*

One of the first proteins, with a probable role in DNA damage to be worked on was a protein, Alr3199 identified as a highly induced heat shock protein. Characterisation of this protein identified it as a hemerythrin DNase with the ability to bind 4 Fe atoms per protein molecule thus playing a role in Fe-homeostasis, and an additional role in regulating DNA degradation in view of its Nickase/ DNase activity. Its neighbouring gene, *Alr3200* was also identified as a DNase involved in radiation tolerance of *Anabaena*. This started the journey for identifying the pathways involved in radiation tolerance and DNA repair in this organism.

5.1. DNA repair genes and mechanisms

Several abiotic stresses, predominantly radiation and desiccation stresses can induce damage to DNA in the form of adduct formation, single and double strand breaks, which if left uncorrected would be lethal to cells. Several cyanobacterial species including *Anabaena*, *Nostoc*, *Chroocodiopsis* exhibit high to both γ-radiation and desiccation. *Anabaena* (*Nostoc*) PCC 7120 exhibits an LD_{50} of 6 kGy and D_{10} of 12 kGy of γ-radiation, making it comparable to the highly radioresistant *Deinococcus*. However, unlike the well-studied mechanisms of DNA repair in *Deinococcus* as well as the radiosensitive *E. coli*, the genes involved in DNA repair mechanisms were unexplored in *Anabaena*. *In silico* analysis revealed the absence of primary proteins (RecB and RecC), SbcA, SbcB required for the RecBCD pathway of Homologous Recombination (HR) and Ku proteins for Non-Homologous End Joining (NHEJ) Pathway. While the absence of RecBCD pathway was also shown for *Deinococcus*, the NHEJ pathway was found to be active in it. Based on the annotated genes in the genome, *Anabaena* was speculated to be dependent on RecF pathway of HR and ESDSA (Extended Synthesis Dependent Strand Annealing), Single Strand Annealing (SSA) and Micro-Homology mediated End Joining (MHEJ) pathway. The major genes in these pathways had to be characterised to know their functional involvement and the feasibility of these pathways of DNA repair in *Anabaena*, which was an unexplored field not just in this organism but in cyanobacteria as a whole.

The very first task in this direction was the identification of a full length Single Strand DNA Binding (SSB) protein, since both the annotated SSB proteins were truncated and lacked the C-terminal region required for interaction with other DNA repair proteins. Based on *in silico* analysis along with biochemical and physiological characterisation, one of the hypothetical proteins, All4779 was identified as the full length SSB, which also contributed significantly to the radiotolerance of *Anabaena*. Ortholog of this protein was found across all cyanobacterial species. The truncated SSB proteins could possibly be involved in DNA replication or recombination processes, as they were not found to

contribute to DNA repair. In the absence of RecBCD complex, the suppressor proteins, SbcC and SbcD were expected to play an important role. Functional characterisation of SbcC and SbcD proteins of *Anabaena* revealed that they could independently contribute to the radiotolerance of *Anabaena*. This is unlike other bacteria, wherein they contribute to DNA repair only when they form a complex (SbcCD) and not independently. It has been speculated that these proteins could be participating in SSA and MHEJ based on their predicted interacting protein partners. The RecA protein which is central to any DNA repair pathway was found to be an essential gene, but expressed at very low levels. Enhancing its expression rendered the *Anabaena* cells radiosensitive.

In general, most DNA repair proteins are expressed at very low levels in *Anabaena*. This was also observed for the proteins central to the RecF pathway, namely RecF, RecO and RecR proteins. Of these, the initiation codon of *recR* was wrongly annotated in the database and with use of *in silico* and expression analysis, the correct initiation codon was identified as 'GTT' present 267 bases upstream of the annotated initiation codon. 'GTT' is a rare initiation codon and has been mentioned as a probable initiation codon only for one gene in *Streptomyces*, though not proven. Presence of the anticodon for 'GTT' in the anticodon arm of fMet-tRNA of *Anabaena* 7120 led to the speculation, that though rare, 'GTT" may function as an initiation codon for more number of genes and not just *recR*, but this needs to be extensively explored.

All three genes (*recF*, *recO* and *recR*) were found to be essential as they could not be deleted; however, knockdown mutants of *recF* and *recR* were viable. Functional analysis of these proteins through modulation of their levels revealed that individual changes in their levels affected the radiotolerance of *Anabaena*, and these proteins would need to interact in a correct proportion to enhance the radiotolerance, which is currently being investigated. All three genes were found to be regulated through an array of elements, which included negative regulation by LexA, positive regulation by NtcA at transcriptional level, role of DNA heptamer repeats in post-transcriptional and translational regulation and that of non-canonical Shine-Delgarno sequence in translational regulation. Involvement of DNA heptamer repeats in gene regulation was established for the first time in bacteria through the studies on their role in regulation of *recF* and *recO* gene expression.

5.2. The global regulator LexA

LexA, which has been extensively studied in *E. coli*, was always considered as a SOS-response regulator in bacteria, involved in the negative regulation of DNA repair genes. However, in cyanobacteria, LexA was first identified as a positive regulator of certain C-metabolism genes and later shown to be involved in negative regulation of two DNA repair genes. However, no specific binding LexA-binding box was identified and showed variation based on the genes regulated. This could have been because of the small set of genes identified to be regulated by LexA in cyanobacteria. Detailed studies on LexA in *Anabaena* 7120 in our laboratory showed that its autoproteolytic activity was independent of activated RecA, unlike that in other bacteria, and was dependent on the availability of nucleophile as in alkaline pH conditions. Physiological evaluation of LexA

overexpressing strain of *Anabaena* revealed its involvement in regulating tolerance to various abiotic stresses, such as γ-radiation, oxidative, C-starvation and heavy metal stress through regulation of genes involved in alleviation of these stresses. While majority of the genes were found to be negatively regulated by LexA, ~10% of the genes were found to be positively regulated, indicating that LexA could function both as a repressor and as an activator protein, and also the consensus LexA-binding box was identified which could cater to all genes identified to be regulated by it. Further analysis of the presence of the AnLexA-Box across the genome identified its presence upstream of several photosynthetic genes and involved in its regulation, and was though to mediate the redox poise. Thus, LexA was identified as a global regulator of stress response in *Anabaena*, adding to the tally of other identified global regulators NtcA and FurA.

6. Future Perspectives

Over three decades of research on stress biology in *Anabaena* revealed cross-talk between stress proteins across various abiotic stresses, intricate linking of some of these proteins with major physiological processes, presence of global regulators and several *cis*-acting regulatory elements. This knowledge is being further expanded to fully understand the role of nucleoside kinases, protein kinases, resolves, and proteins with disordered structures (IDPs) through a mix of omics approaches and relating them not only to their growth but to the major physiological processes, whose functioning is crucial. Moreover, how most of the oxidative stress-responsive genes or the *kdp* operons are regulated is not completely understood, and this aspect can be a fruitful line of research in the future. The deeper understanding of these physiological processes and protein functions would also throw some light into the course of evolution of these proteins from the ancient cyanobacteria to modern day bacteria and plants.

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