Cyanobacterial Mn-catalase ‘KatB’: Molecular link between salinity and oxidative stress resistance

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ABSTRACT
Catalases are ubiquitous enzymes that detoxify H2O2 in virtually all organisms exposed to oxygen. The filamentous, nitrogen-fixing cyanobacterium, Anabaena PCC 7120, shows the presence of 2 genes (katA and katB) that encode Mn-catalases. We have recently shown that pre-treatment of Anabaena with NaCl causes substantial induction of the KatB protein, which consequently leads to increased oxidative stress resistance in that cyanobacterium. Interestingly, when compared to the wild-type, the katB mutant shows decreased growth and impaired photosynthetic activity in the presence of NaCl. Furthermore, the NaCl-treated katB mutant is extremely sensitive to H2O2. In this study, the ultrastructural changes occurring in the katB mutant and the wild-type Anabaena cells are analyzed to understand the cellular basis of the above-mentioned protective phenomena. Other data show that a wide variety of osmolytes induce katB expression in Anabaena, indicating that katB is a genuine osmo-inducible gene. These results have important biotechnological implications for the development of novel cyanobacterial biofertilizers and transgenic plants with improved resistance to salinity.

The presence of oxygen is a double-edged sword for aerobes. The transfer of electrons through the respiratory chain to oxygen, that drives the production of ATP, also results in the concomitant generation of the deleterious reactive oxygen species (ROS) such as the superoxide radical (O2·¯), hydrogen peroxide (H2O2) or the hydroxyl radical (·OH).1 In living systems, H2O2 forms the veritable connection between O2·¯ and ·OH. Although, H2O2 is produced directly by several oxidases, the dismutation of O2·¯ by superoxide dismutase (SOD) is the major source of H2O2 production in cells.2 However, though not very reactive by itself, H2O2 can very quickly become the key ROS that imperils cell’s survival. In the presence of iron, an essential micronutrient, H2O2 reacts with Fe2+ to generate the ·OH, which can damage all the cellular macromolecules at diffusion-controlled rates.3 Thus, decomposition of H2O2 is very pertinent for cellular existence. Detoxification of H2O2 is primarily brought about by peroxidases and catalases. Peroxidases (e.g. ascorbate peroxidase, peroxiredoxins, etc.) require electron-donating reducing agents as accessors to decompose H2O2; whereas catalases directly detoxify H2O2 by a dismutation reaction, forming H2O and molecular oxygen in the process.4 Catalases, essentially are of 2 types viz; heme catalases or manganese (Mn)-catalases. The typical monofunctional catalase and the catalase-peroxidase (i.e. KatG) contain heme whereas Mn-catalases lack heme, but contain Mn at their active site.5,6 The heme catalases are widespread in both eukaryotes and prokaryotes while Mn-catalases are found exclusively among prokaryotes and archaea.5

Cyanobacteria, widely regarded as the progenitors of plant chloroplasts, were responsible for the early oxygenation of Earth’s atmosphere.7 Due to their close association with O2, they are likely to have developed multiple stratagems to surmount the toxic effects of ROS. Moreover, the nitrogen-fixing strains of cyanobacteria remain the only form life that can harvest solar energy to fix atmospheric nitrogen.8 Incidentally, naturally occurring, filamentous, nitrogen-fixing cyanobacteria (e.g., Anabaena) are widely used as biofertilizers in the paddy fields of Southeast Asia.

Over the last few years, our laboratory has focused on dissecting the mechanisms responsible for overcoming oxidative stresses in the filamentous, heterocystous, nitrogen-fixing cyanobacterium, Anabaena PCC 7120.9-13 This cyanobacterium contains an arsenal of ROS detoxifying enzymes, which include SODs, peroxiredoxins (Prxs), catalases etc.14 Along with the presence of several genes encoding peroxiredoxins (Prxs), this Anabaena has 2 genes that encode Mn-catalase (alr0998 i.e., katA, and
Anabaena cells that were pre-treated with NaCl showed cross-protection experiments with positive stress resistance were obtained while performing high degree of resistance to H₂O₂ and could withstand primary proteins that detoxify H₂O₂ in Anabaena. These observations apparently suggest that, rather than catalases, Prxs may be the primary proteins that detoxify H₂O₂ in Anabaena. However, despite this distinct induction, endogenous production of Prxs is unable to rescue Anabaena from the deleterious effects brought about by exposure to H₂O₂. In fact, treatment with just 0.5 – 1 mM H₂O₂ leads to cell lysis/death in Anabaena.13

Surprising results with wide ramifications for oxidative stress resistance were obtained while performing cross-protection experiments with Anabaena PCC 7120. Anabaena cells that were pre-treated with NaCl showed high degree of resistance to H₂O₂ and could withstand exposure to as high as 3 mM H₂O₂.13 Keeping in mind the presence of katA/B genes in Anabaena and catalase being the known ‘classical’ enzyme that protects organisms from H₂O₂, catalase activity of the above-mentioned cultures was assessed on zymograms. Interestingly, the salt-treated Anabaena cells showed the presence of a distinct catalase activity. When probed with the KatA or KatB antiserum (on native Western blots), the zone of catalase activity matched exactly with the signal obtained from the KatB antiserum, demonstrating that KatB was the catalase induced in response to salt stress in Anabaena PCC 7120.13

Unlike the wild-type Anabaena PCC 7120, the NaCl-treated katB mutant was found to be particularly sensitive to H₂O₂, and exposure to H₂O₂ caused increased formation of lipid peroxides, oxidized proteins and total peroxides in the katB mutant.13 In fact, a considerably higher level of oxidized proteins was found in the culture medium of the mutant at the end of 24 h (Fig. 1A). Under the transmission electron microscope, distinct structural changes were observed between the wild-type cells and the katB mutant (Fig. 2). The wild-type cells appeared to be robust and showed proper thylakoid integrity even after exposure to the oxidizing agent. In contrast, the katB mutant lost the thylakoid ultrastructure, and in some cells, loss of cellular contents (indicative of lysis) was also observed. Carboxysomes were observed in the wild-type cells whereas no such structures were seen in the katB mutant. In filamentous cyanobacteria, the content of chlorophyll a is a good indicator of growth/cellular integrity and this parameter is routinely used to assess resistance to various stresses. Hence, to further substantiate the TEM data, the chlorophyll a content of the NaCl-treated wild-type Anabaena (WT) or the katB mutant (katB−) cells after exposure to H₂O₂ (1mM). These proteins were derivatized with dinitrophenol (DNP), resolved on SDS-PAGE and transferred to nitrocellulose membrane. Subsequently, these proteins were probed with the monoclonal DNP antiserum. A Ponceau S-stained part of the blot is shown in the lower panel as loading control. The oxidized proteins were detected as mentioned in the OxyBlot oxidized protein detection kit (Thermo Scientific, 23280).

Figure 1. Detection of oxidized proteins. Proteins were extracted by TCA precipitation from the culture medium of NaCl-treated wild-type Anabaena (WT) or the katB mutant (katB−) cells after exposure to H₂O₂ (1mM). These proteins were derivatized with dinitrophenol (DNP), resolved on SDS-PAGE and transferred to nitrocellulose membrane. Subsequently, these proteins were probed with the monoclonal DNP antiserum. A Ponceau S-stained part of the blot is shown in the lower panel as loading control. The oxidized proteins were detected as mentioned in the OxyBlot oxidized protein detection kit (Thermo Scientific, 23280).
extreme form of osmotic stress, also induces synthesis of KatB in *Anabaena*. Also, *katB* expression is regulated by the nitrogen status of the medium and there is a distinct lack of *katB* transcription in heterocysts (cells that fix nitrogen). Thus mechanism underlying the transcriptional activation of *katB* is complicated and several environmental cues are integrated in this process. The relatively long (~450-bp) regulatory region between the *katB* promoter and the translational start of KatB is likely to provide ample space for various factors to bind in trans and modulate expression. In the unicellular cyanobacterium, *Synechocystis* PCC 6803, histidine kinases are known to regulate several osmotically induced genes. The presence of these homologs in *Anabaena* lends support to the idea that *katB* too may be regulated by proteins such as histidine kinases.

Our study has provided new insights into the enhancement of cyanobacterial salt tolerance by combined nitrogen that was reported from our laboratory over 25 y ago. In that study, the inhibition of Na\(^+\) influx by combined nitrogen was suggested to be a major mechanism for overcoming salt stress in *Anabaena*. Salt is known to increase ROS (e.g. H\(_2\)O\(_2\)) in several organisms including *Anabaena*. Interestingly, our recent findings show that the presence of combined nitrogen enhances production of KatB in *Anabaena*. Consequently, the increased levels of KatB are likely to help *Anabaena* overcome the oxidative effects of salt stress. So, along with the inhibition of Na\(^+\) influx, reduction in

**Figure 2.** Ultrastructural features of the NaCl-treated wild-type *Anabaena* (WT, upper panel) or *katB* mutant (*katB\(^{-}\), lower panel) after exposure to H\(_2\)O\(_2\) (for 24 h) as seen under the transmission electron microscope. Samples were processed for transmission electron microscopy as described earlier. Thylakoid membranes (Th) and carboxysomes (C) are indicated. Severely disintegrated thylakoid membranes and a distinct loss of ultrastructure are evident in the *katB* mutant filaments exposed to H\(_2\)O\(_2\).

**Figure 3.** The *katB* promoter-gfp fusion construct was transformed into *Anabaena* PCC 7120 and the *katB* promoter activity was monitored in the presence of various osmolytes such as NaCl (150 mM), sucrose (300 mM), glycerol (300 mM), manitol (300 mM), sorbitol (300 mM), and PEG (100 mM). Cells were exposed to the above-mentioned osmolytes for 18h. The green fluorescence (\(\lambda_{\text{ex}} = 490\) nm, \(\lambda_{\text{em}} = 520\) nm) of the reporter GFP is plotted as bar diagram. Standard deviation for 5 independent experiments is shown as error bars.
the levels of ROS brought about by KatB may also contribute to the salinity tolerance of *Anabaena* observed under these conditions.

As far as the future prospects are concerned, 2 lines of research appear to be particularly appealing. One, off course, is to have a detailed understanding of how the katB gene is regulated in *Anabaena*. The other aspect has more practical applications i.e., can the KatB-overexpressing *Anabaena* be employed as a more efficient biofertilizer or can the katB gene transferred to plants in the hope of improving their stress resistance? Once synthesized, KatB persists in cells for several days even when the inducing stimuli (e.g., NaCl) is removed. In our opinion, due to its more robust nature, functional over a wide range of pH or salt concentrations.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**References**

[1] Imlay JA. Pathways of oxidative damage. Annu Rev Microbiol 2003; 57:395-418; PMID:14527285; http://dx.doi.org/10.1146/annurev.micro.57.030502.090938

[2] Collen J, Del Rio MJ, García-Reina G, Pedersén M. Photosynthetic production of hydrogen peroxide by *Ulvaria gida* C. Ag (Chlorophyta). Planta 1995; 196:225-30; http://dx.doi.org/10.1007/BF00201378

[3] Halliwell B, Gutteridge JMC. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. Arch Biochem Biophys 1986; 246:501-14; PMID:3010861; http://dx.doi.org/10.1016/0003-9861(86)90305-X

[4] Berntofter M, Zamocky M, Furthmüller PG, Peschek GA, Obinger C. Occurrence, phylogeny, structure, and function of catalases and peroxidases in cyanobacteria. J Exp Bot 2009; 60:423-40; PMID:19129167; http://dx.doi.org/10.1093/jxb/ern309

[5] Zamocky M, Furthmüller PG, Obinger C. Evolution of Catalases from Bacteria to Humans. Antioxid Redox Signal 2008; 10:1527-48; PMID:18498226; http://dx.doi.org/10.1089/ars.2008.2046

[6] Bihani SC, Chakravarty D, Ballal A. Purification, crystallization and preliminary crystallographic analysis of KatB, a manganese catalase from *Anabaena* PCC 7120. Acta Crystallogr Sect F Struct Biol Cryst Commun 2013; 69:1299-302; PMID:24192374; http://dx.doi.org/10.1107/S1744309113028017

[7] Fay P. Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol Rev 1992; 56:340-73; PMID:1620069

[8] Stewart WDP. Some aspects of structure and function in nitrogen-fixing cyanobacteria. Annu Rev Microbiol 1980; 34:497-536; PMID:6108091; http://dx.doi.org/10.1146/annurev.mi.34.100180.002433

[9] Banerjee M, Ballal A, Apte SK. A novel glutaredoxin domain containing peroxiredoxin ‘All1541’ protects the N2-fixing cyanobacterium *Anabaena* PCC7120 from oxidative stress. Biochem J 2012; 442:671-80; PMID:22150556; http://dx.doi.org/10.1042/BJ20111877

[10] Banerjee M, Ballal A, Apte SK. Mn-catalase (Alr0998) protects the photosynthetic, nitrogen fixing cyanobacterium *Anabaena* PCC7120 from oxidative stress. Environ Microbiol 2012; 14:2891-900; PMID:22897147; http://dx.doi.org/10.1111/j.1462-2920.2012.02847.x

[11] Banerjee M, Chakravarty D, Ballal A. Redox-dependent chaperone/peroxidase function of 2-Cys-Prx from the cyanobacte-rium *Anabaena* PCC7120 from oxidative stress. BMC Plant Biol 2013; 13:60; PMID:25849452; http://dx.doi.org/10.1186/s12870-013-0444-2

[12] Tailor V, Ballal A. Over-expression of Alr4642, a novel Prx-like peroxiredoxin, defends the cyanobacterium *Anabaena* PCC7120 from oxidative stress. J Appl Physiol 2015; 27:2261-70; http://dx.doi.org/10.1107/s10811-014-0503-3

[13] Chakravarty D, Banerjee M, Bihani SC, Ballal A. A salt-inducible Mn-catalase (KatB) protects cyanobacterium from oxidative stress. Plant Physiol 2016; 170:761-73; PMID:26645454; http://dx.doi.org/10.1104/pp.15.01632

[14] Banerjee M, Raghavan PS, Ballal A, Rajaram H, Apte SK. Oxidative stress management in the filamentous, heterocystous, diazotrophic cyanobacterium, *Anabaena* PCC7120. Photosynth Res 2013; 118:59-70; http://dx.doi.org/10.1007/s11110-013-9929-8

[15] Shoumskaya MA, Paithoonrangsarid K, Kanesaki Y, Los DA, Zinchenko VV. Stress sensors and signal transducers in cyanobacteria. Sensors (Basel) 2010; 10:2386-415; PMID:22294932; http://dx.doi.org/10.3390/s100302386

[16] Reddy BR, Apte SK, Thomas J. Enhancement of cyanobacterial salt tolerance by combined nitrogen. Plant Physiol 1989; 89:204-10; PMID:16666516; http://dx.doi.org/10.1104/pp.89.1.204
[18] Choudhury S, Panda P, Sahoo L, Panda SK. Reactive oxygen species signaling in plants under abiotic stress. Plant Signal Behav 2013; 8:e23681; PMID:23425848; http://dx.doi.org/10.4161/psb.23681

[19] Moriwaki T, Yamamoto Y, Aida T, Funahashi T, Shishido T, Asada M, Prodhan SH, Komamine A, Motohashi T. Overexpression of the Escherichia coli catalase gene, katE, enhances tolerance to salinity stress in the transgenic indica rice cultivar, BR5. Plant Biotechnol Rep 2008; 2:41-6; http://dx.doi.org/10.1007/s11816-008-0046-7

[20] Zámocký M, Furtmüller PG, Obinger C. Evolution of structure and function of Class I peroxidases. Arch Biochem Biophys 2010; 500:45-57; http://dx.doi.org/10.1016/j.abb.2010.03.024

[21] Bihani SC, Chakravarty D, Ballal A. KatB, a cyanobacterial Mn-catalase with unique active site configuration: implications for enzyme function. Free Radic Biol Med 2016; 93:118-29; PMID:26826576; http://dx.doi.org/10.1016/j.freeradbiomed.2016.01.022

[22] Kulkarni S, Ballal A, Apte SK. Bioprecipitation of uranium from alkaline waste solutions using recombinant Deinococcus radiodurans. J Hazard Mater 2013; 262:853-861; PMID:24140537; http://dx.doi.org/10.1016/j.jhazmat.2013.09.057