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# Redox interference in nitrogen status via oxidative stress is mediated by 2-oxoglutarate in cyanobacteria

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## Summary

- Reactive oxygen species (ROS) are generated naturally in photosynthetic organisms by respiration and photosynthesis. Therefore, detoxification of these compounds, avoiding oxidative stress, is essential for proper cell function. In cyanobacteria, some observations point to a crosstalk between ROS homeostasis, in particular hydrogen peroxide, and nitrogen metabolism by a mechanism independent of known redox regulators.
- Using glutamine synthetase (GS), a finely regulated enzyme essential for nitrogen assimilation, as a tool, we were able to monitor nitrogen metabolism in relation to oxidative stress.
- We show that hydrogen peroxide clearly alters the expression of different genes related to nitrogen metabolism, both in the wild-type strain of the cyanobacterium *Synechocystis* sp. PCC 6803 and in a mutant strain lacking the catalase-peroxidase encoded by the *katG* gene and therefore highly sensitive to oxidative stress. As cyanobacteria perceive nitrogen status by sensing intracellular 2-oxoglutarate (2-OG) concentrations, the hydrogen peroxide effect was analysed under different nitrogen conditions in the wild-type, the Δ*katG* strain and in a strain able to transport 2-OG.
- The results obtained demonstrate that hydrogen peroxide interferes with signalling of cellular carbon : nitrogen status by decreasing the intracellular concentrations of 2-OG and hence altering the function of the 2-OG-sensing global nitrogen regulator NtcA.

## Introduction

Reactive oxygen species (ROS) are generated in cyanobacteria intracellularly both by oxygenic photosynthesis and by aerobic respiration. Effective scavenging of these products is essential for photosynthetic organisms because they can attack lipids, proteins and DNA, causing severe and irreversible oxidative damage. ROS include singlet oxygen ($^1O_2$), the superoxide anion ($O^{2-}$), hydrogen peroxide ($H_2O_2$) and the hydroxyl radical ($OH^{\cdot}$). Superoxide dismutase produces $H_2O_2$ from $O_2^-$. Although $H_2O_2$ is less reactive than other ROS, it can be reduced to highly reactive hydroxyl radical via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$) (Latifi *et al.*, 2009). On the other hand, recent studies indicate that because ROS can function as signalling molecules, maintaining basal concentrations of these compounds is essential for life (Forman *et al.*, 2010; Mittler, 2017). In particular, $H_2O_2$ has been proposed as a suitable second messenger because it is produced and degraded enzymatically and participates in oxidation of protein thiols, which may be biologically relevant. Living organisms have developed different defence mechanisms against damage caused by ROS. A correct balance between ROS and antioxidant defences allows the correct functioning of the cellular machinery and the ability to adapt to different stimuli (Imlay, 2013).

Cyanobacteria have developed different strategies or mechanisms to cope with oxidative stress. These mechanisms can be divided into enzymatic and nonenzymatic. The nonenzymatic ones involve mainly carotenoids, glutathione or vitamins (Edge *et al.*, 1997). Antioxidant enzymes are responsible for the neutralization or elimination of the superoxide anion and the $H_2O_2$ produced by the photosynthetic chain. $H_2O_2$ is mainly processed by catalases, and these enzymes exclusively dismutate $H_2O_2$, whereas peroxidases use a broad range of peroxides (ROOH) as substrates. In addition to the catalase-peroxidases, other peroxidases are present in cyanobacteria, including the peroxiredoxins or thioredoxin-peroxidases (Latifi *et al.*, 2009; Pérez-Pérez *et al.*, 2009). In *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), two enzymatic mechanisms have been proposed for $H_2O_2$ decomposition. One is catalysed by a catalase-peroxidase (Tichy & Vermaas, 1999) and the other is catalysed by thiol-specific peroxidases (Pérez-Pérez *et al.*, 2009). Peroxidase enzymes have a $K_m$ in the micromolar range, being more efficient eliminating trace concentrations of $H_2O_2$ than catalase enzymes. In this sense it has been proposed that catalases, with low affinity for $H_2O_2$ and high $V_{max}$, are responsible for the elimination of $H_2O_2$ entering the cells from the environment (Tichy & Vermaas, 1999).

In nonphotosynthetic organisms like *Pseudomonas fluorescens*, a role of the tricarboxylic acid cycle in the detoxification of ROS has been described. It appears that 2-oxoglutarate (2-OG) is an

important metabolite in the response to oxidative stress and its concentration is modulated accordingly (Mailloux *et al.*, 2009).

The action of various antioxidant systems and enzymes might not be enough to protect cellular components in cells subjected to drastic changes in environmental conditions. Under these circumstances, organisms detect increases in ROS concentrations and regulate gene expression in order to tolerate oxidative stress. The molecular mechanism of these sensing pathways has been extensively studied in the model bacterium *Escherichia coli* (reviewed by Imlay, 2013). The response to an increase in superoxide production is orchestrated by the SoxRS system, while $H_2O_2$ sensing in *E. coli* and many other Gram-negative bacteria is achieved by the transcription factor OxyR. Other bacteria lack an OxyR homologue and contain a Fur-like peroxide-sensing repressor PerR. In cyanobacteria, no SoxR/SoxS homologues have been reported but a PerR orthologue has been identified. However, a majority of the genes that responded to oxidative stress were PerR-independent. (Latifi *et al.*, 2009). It seems that PerR is not the master regulator of the response to peroxide in cyanobacteria. In *Synechocystis*, several His kinases are involved in $H_2O_2$ sensing, suggesting that the response to this compound is achieved by the action of several regulators (Kanesaki *et al.*, 2007). One of these kinases, Hik33, controls the redox-responsive transcription factor RpaB, whose regulon has been recently predicted (Riediger *et al.*, 2019).

A crosstalk between ROS homeostasis and other cellular networks has been proposed in cyanobacteria. Specifically, some data support the connection between oxidative stress and nitrogen (N) regulatory systems (Fadi Aldehni *et al.*, 2003; Stork *et al.*, 2005; Yingping *et al.*, 2015). In *Synechocystis*, oxidative stress caused by $H_2O_2$ leads to induction of the expression of *gif* genes encoding glutamine synthetase (GS)-inactivating factors (Kanesaki *et al.*, 2007). GS, encoded by the *glnA* gene, occupies a central position in the regulation of N metabolism (Leigh & Dodsworth, 2007). Under N-rich conditions (ammonium), GS activity in *Synechocystis* decreases when inactivating factors are present (IF7 and IF17, of 7 and 17 kDa, encoded by *gifA* and *gifB* genes, respectively). The analysis of mutant strains lacking one or both IFs revealed that each of these proteins contributes to GS regulation by inactivation *in vivo* (García-Domínguez *et al.*, 1999). Expression of *glnA* and *gif* genes, regulated by the N control protein NtcA, depends on N status (Reyes *et al.*, 1997; García-Domínguez *et al.*, 2000; Galmozzi *et al.*, 2010), which is perceived as changes in the intracellular pool of 2-OG (Muro-Pastor *et al.*, 2001). In the present study, we analyse how ROS affect N metabolism and demonstrate that 2-OG, the natural signal transducer of N control in cyanobacteria, is a direct target of $H_2O_2$, provoking a metabolic interference between oxidative stress and N signalling.

## Materials and Methods

### Strains and growth conditions

*Synechocystis* derivative strains were grown photoautotrophically at 30°C on BG11 medium (Rippka, 1988), using nitrate as the N source, supplemented with 1 g $l^{-1}$ $NaHCO_3$ (BG11C) and bubbled with a continuous stream of 1% (v/v) $CO_2$ in air, under continuous illumination (50 μmol of photons $m^{-2}$ $s^{-1}$; white light). In cultures with ammonium as the N source (10 mM $NH_4Cl$) or in the case of treatments with an oxidizing agent ($H_2O_2$), BG11C medium was buffered with 20 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulphonic acid (TES)-NaOH (pH 7.5). To establish N deficiency, the cells were washed twice and resuspended in medium $BG11_0C$, lacking any N source. For plate cultures, BG11C liquid medium was solidified using 1% (w/v) agar. Kanamycin was added to a final concentration of 50 μg $ml^{-1}$, and spectinomycin and streptomycin were added to a final concentration of 2.5 μg $ml^{-1}$ when required. *E. coli* DH5α (Bethesda Research Laboratories, Bethesda, MD, USA) grown in Luria-Bertani medium (Sambrook *et al.*, 1989) was used for plasmid construction and replication. Cultures were supplemented with 100 μg $ml^{-1}$ ampicillin or 50 μg $ml^{-1}$ kanamycin when required.

### GS assay

Glutamine synthetase activity was determined *in situ* using the $Mn^{2+}$-dependent γ-glutamyl-transferase assay in cells permeabilized with mixed alkyltrimethylammonium bromide (Mérida *et al.*, 1991). One unit of GS activity corresponds to the amount of enzyme that catalyses the synthesis of 1 μmol $min^{-1}$ of γ-glutamylhydroxamate.

### Generation of mutant strains of *Synechocystis*

To generate the Δ*katG* strain, two DNA fragments of the *katG* genomic region were amplified by PCR. A 1064 bp fragment was obtained using oligonucleotides katG_1_F and katG_1_R, and a 1086 bp fragment was obtained using oligonucleotides katG_2_F and katG_2_R. Those fragments were ligated to generate a *katG* locus with the 600 bp central region deleted and containing a *Bam*HI site. The resulting fragment was cloned into pBS-SK(+) digested by *Not*I–*Pst*I. An $Sm^r$ $Sp^r$ C.S3 cassette (Prentki & Krisch, 1984) from pRL463 (pUC18/19 containing L.HEH1 and C.S3, nomenclature of Elhai & Wolk (1988), was cloned in the *Bam*HI site in both orientations generating plasmids pΔkatG (+) and pΔkatG(−) (Supporting Information Fig. S1). The resulting targeting plasmids containing the mutant variants of *katG* gene were used to transform the wild-type *Synechocystis* strain.

To generate KGTP and Δ*katG*/KGTP strains, the *kgtP* gene from *E. coli* MC1061, under the control of its own promoter, was introduced into the *Synechocystis* wild-type and Δ*katG* strains, respectively. For this purpose a 1729 bp fragment, containing the *kgtP* gene, was amplified by PCR from the pCW28 plasmid (Seol & Shatkin, 1991), using oligonucleotides kgtP_KpnI_5′ and kgtP_BamHI_3′. This fragment was cloned in a plasmid (pPLAT) containing a 2 kb region of the nonessential *nrsBACD* operon (Galmozzi *et al.*, 2007). Finally a $Km^r$ CK1 cassette from pRL161 (Elhai & Wolk, 1988) was cloned in the *Bam*HI site of pPLAT (Fig. S2). The resulting targeting plasmid

with the *kgtP* gene was used to transform the wild-type and Δ*katG* strains of *Synechocystis*.

All DNA constructs were confirmed by DNA sequencing. Correct recombination was verified by PCR analysis (Figs S1, S2), Oligonucleotides used for strain construction and verification are summarized in Table S1.

### RNA isolation and Northern blot analysis

For Northern blot analysis, total RNA was isolated and extracted as previously described (García-Domínguez & Florencio, 1997). The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm. RNA integrity was confirmed by visualization of intact rRNA under UV light. Northern blots were performed as previously described (Saelices *et al.*, 2011). PCR-synthesized fragments, encompassing the *gln*A, *gif*A or *gif*B, *glnN*, *glnB*, *nblA* and *pgr5* genes were used as probes (oligonucleotides used are shown in Table S1). As a control, the filters were reprobed with a DNA fragment containing the constitutively expressed RNase P RNA gene (*rnp*B) from *Synechocystis* (Vioque, 1992).

### Preparation of crude extracts from *Synechocystis* cells

For the analysis of the abundance of proteins of interest in *Synechocystis* cells grown under different conditions, crude extracts were prepared using glass beads as previously described (Reyes *et al.*, 1995). Protein concentration in cell-free extracts or purified protein preparations was determined by the method of Bradford, using ovalbumin as a standard (Bradford, 1976).

### Western blot analysis

All antisera used were obtained previously according to standard immunization protocols (carried out in the facilities of the University of Sevilla) using proteins purified in our laboratory and used at the following dilutions: anti-IF7 and anti-IF17 (1 : 2000) (Galmozzi *et al.*, 2007), anti-TrxA (1 : 5000) (Navarro *et al.*, 2000), anti-GSI (Marqués *et al.*, 1992) (1 : 250 000) and anti-PII (1 : 4000) (Giner-Lamia *et al.*, 2017). For Western blot analysis, proteins were fractionated on 12–15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis or 6% native-PAGE according to the method of Laemmli (1970), transferred to nitrocellulose membranes and immunoblotted with the required antibody in each case, using a standard protocol. The ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, IL, USA) was used to detect the different antigens with antirabbit secondary antibodies (1 : 25 000) (Sigma-Aldrich).

### Gel retardation assays

NtcA-$His_6$, expressed and purified as previously described (Omairi-Nasser *et al.*, 2014), was used in gel retardation assays. DNA fragments used in the binding reactions were obtained by PCR, with the corresponding oligonucleotide pairs that incorporate a *Not*I site (Table S1). DNA probes were cut with *Not*I restriction enzyme, generating fragments of *c.* 200 bp. These fragments were end-labelled with [α-$^{32}$P]-dCTP using SEQUENASE v.2.0 enzyme (Affymetrix, USB, Santa Clara, CA, USA). The binding reactions and electrophoresis were carried out as previously described (Muro-Pastor *et al.*, 1996). Gels were dried and radioactive signals were quantified with a Cyclone Plus Storage Phosphor System using OPTIQUANT software (PerkinElmer, Waltham, MA, USA).

### Fluorometric determination of 2-OG

Intracellular quantification of 2-OG was carried out using the α-ketoglutarate Assay Kit (Sigma Aldrich) following the manufacturer's instructions. The detection ranges of this kit are between 2 and 10 nmol (colorimetric) and 0.2–1 nmol (fluorimetric). For this purpose, 20 ml of *Synechocystis* cultures (3.5 μg $ml^{-1}$ Chl) were collected by vacuum filtration using MF™ Membrane Filters, 0.45 μm HAWP (Millipore) of diameter 47 mm. Samples were stored at −80°C until use. Cell lysates were obtained by adding 1.5 ml $HClO_4$ (0.3 M) and shaking vigorously. The samples were incubated on ice for 15 min and centrifuged at 15 700 ***g*** for 15 min at 4°C to remove cell debris. The preparations were neutralized by adding 150 μl of 2 M $K_2CO_3$, and the precipitated salts ($KClO_4$) were removed by centrifuging at 15 700 ***g*** for 20 min at 4°C. Then the samples were deproteinized using Amicon® Ultra 0.5 ml with the NMWL membrane of 10 kDa Ultracel-10 (Millipore, Burlington, MA, USA). The concentrations of 2-OG were obtained by determining the fluorescence (RFU) in each sample for Excitation/Emission = 535/587 in a spectrofluorimeter Varian Cary Eclipse (PerkinElmer).

## Results

### Treatment of *Synechocystis* cells with $H_2O_2$ provokes a dose-dependent transitory GS inactivation

Glutamine synthetase is a key enzyme of N metabolism. We checked whether GS activity is affected *in vivo* by oxidizing agents such as $H_2O_2$. For this purpose, different amounts of $H_2O_2$ were added to *Synechocystis* cells growing under standard conditions with nitrate as N source. Addition of $H_2O_2$ caused a partial and transient inactivation of the GS whose magnitude was dependent on the amount of $H_2O_2$ added (Fig. 1a). Northern and Western blots were performed over the same time-course in parallel with GS activity measurements. A rapid and transitory increase in the abundance of *gifB* mRNA and a decrease of *glnA* mRNA upon $H_2O_2$ addition were observed (Fig. 1b). *pgr5* (homologous to Proton Gradient Regulation 5 from *Arabidopsis thaliana*) mRNA level was also monitored as a control of oxidative stress caused by $H_2O_2$ (Kanesaki *et al.*, 2007). Western blot analysis showed that both GS-inactivating factors (IF7 and IF17) accumulated after 15 min of $H_2O_2$ addition, but were almost undetectable 60 min after the $H_2O_2$ treatment (Fig. 1c), concomitant with GS reactivation (Fig. 1a). The transient nature of the observed effect of $H_2O_2$ treatment may be a result of the short residence time of this compound in the cells. In fact,

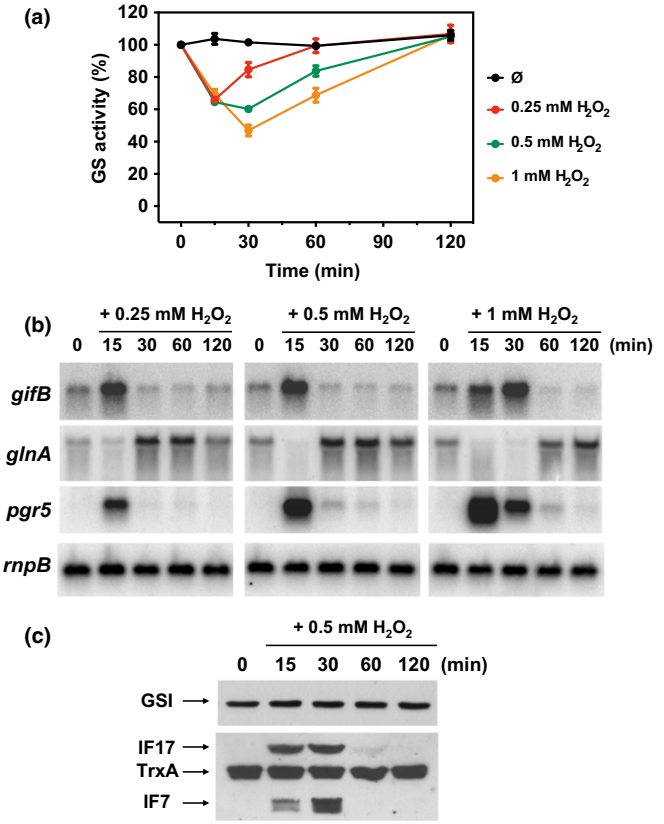


**Fig. 1** Effect of hydrogen peroxide ($H_2O_2$) on glutamine synthetase (GS) activity and gene expression. (a) Kinetics of GS activity after the addition of $H_2O_2$. At time '0' the indicated concentrations of $H_2O_2$ were added to *Synechocystis* cells growing under standard conditions. A control culture was maintained without addition (∅). The GS activity values are relativized to the value at time '0' before the peroxide addition ($64.40 \pm 5.06$ U $mg^{-1}$ Chl). The error bars correspond to the SD of three independent biological experiments. (b) Northern blots of *gifB*, *glnA* and *pgr5* genes following addition of $H_2O_2$. Total RNA was isolated from cells growing under standard conditions (time 0) or at different times after the addition of $H_2O_2$ at the indicated concentrations. Seven micrograms were analysed and gels were blotted and hybridized with *gifB*, *glnA* and *pgr5* probes. The filters were stripped and rehybridized with an *rnpB* probe as a loading control. (c) Western blot of GSI and IFs after addition of 0.5 mM $H_2O_2$. From the same culture used for determination of GS activity and Northern blot analysis, samples were taken before (0) and after $H_2O_2$ addition (0.5 mM) at the indicated times. Total proteins were isolated and resolved on sodium dodecyl sulphate-polyacrylamide gel electrophoresis, blotted and incubated with anti-GSI, anti-IF7 and anti-IF17. As a control for protein loading, membranes were incubated also with anti-TrxA antibodies.

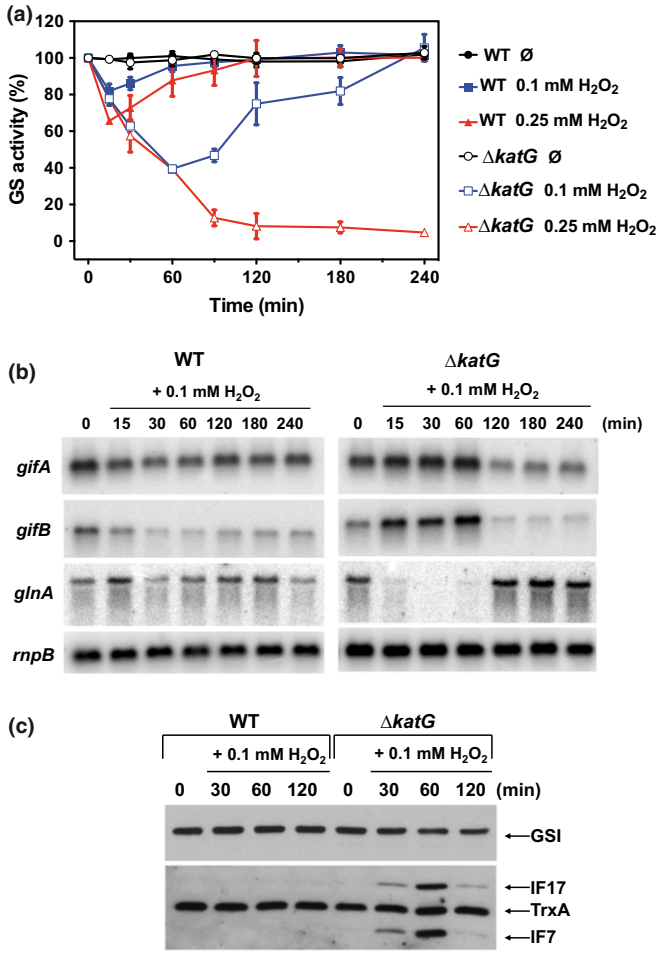


**Fig. 2** Effect of hydrogen peroxide ($H_2O_2$) on glutamine synthetase (GS) activity and gene expression in wild-type (WT) and Δ*katG Synechocystis* strains. (a) Kinetics of GS activity after the addition of $H_2O_2$. At time '0' the indicated concentrations of $H_2O_2$ were added to *Synechocystis* cells growing under standard conditions. A control culture of each strain was maintained without addition (∅). The GS activity values are relativized to the value at time '0' before the $H_2O_2$ addition ($2.31 \pm 0.12$ and $2.12 \pm 0.10$ U $mg^{-1}$ total protein for WT and Δ*katG*, respectively). The error bars correspond to the SD of three independent experiments. (b) Northern blots of *gifA*, *gifB* and *glnA* genes following addition of $H_2O_2$. Total RNA was isolated from cells growing under standard conditions (time 0) or at different times after the addition of 0.1 mM $H_2O_2$. Seven micrograms were analysed and gels were blotted and hybridized with *gifA*, *gifB* and *glnA* probes. The filters were stripped and rehybridized with an *rnpB* probe as a loading control. (c) Western blot of IFs after addition of 0.1 mM $H_2O_2$. From the same cultures used for determination of GS activity and Northern blot analysis, samples were taken before (0) and after $H_2O_2$ addition (0.1 mM) at the indicated times. Total proteins were isolated and resolved on sodium dodecyl sulphate-polyacrylamide gel electrophoresis, blotted and incubated with anti-IF7 and anti-IF17. As a control for protein loading, membranes were incubated also with anti-TrxA antibodies.

*Synechocystis* is equipped with an efficient catalase-peroxidase enzyme that detoxifies $H_2O_2$. This enzyme, encoded by the *katG* gene, has been widely studied (Tichy & Vermaas, 1999; Smulevich *et al.*, 2006). In order to amplify and characterize the effect of $H_2O_2$ on N metabolism, a *Synechocystis* Δ*katG* strain was constructed (Fig. S1; Methods S1). The growth of this strain was drastically affected by $H_2O_2$ treatment at concentrations > 0.1 mM (Fig. S1). Addition of 0.25 mM $H_2O_2$ provoked a drastic and irreversible loss of GS activity in Δ*katG* cells but only a transitory effect in wild-type cells (Fig. 2a). At 0.1 mM the effect was transitory for both strains but was much more pronounced in the Δ*katG* strain. Therefore, 0.1 mM $H_2O_2$ was used hereinafter in the comparative study of the oxidative stress response between wild-type and Δ*katG* strains. In order to further investigate the loss of GS activity after $H_2O_2$ addition, the expression of *glnA* and *gif* genes was analysed by Northern blot in both strains. Whereas in the wild-type strain, 0.1 mM $H_2O_2$ treatment had no

significant effect, the Δ*katG* strain showed transiently altered *gif* and *glnA* genes expression (Fig. 2b). This expression pattern corresponded to the transient inactivation of the GS in the Δ*katG* strain after the addition of 0.1 mM $H_2O_2$ (Fig. 2a). With respect to the inactivating factors, while IF7 and IF17 were not detectable in the wild-type strain, both proteins accumulated transiently in the Δ*katG* strain after $H_2O_2$ treatment (Fig. 2c).

### Hydrogen peroxide interferes *in vitro* with NtcA binding only in the presence of 2-OG

To investigate a possible effect of $H_2O_2$ on NtcA-binding activity, which could explain the observed changes in gene expression, the *glnA* promoter region was used in electrophoretic mobility shift assay (EMSA) assays with purified NtcA protein. As previously described, NtcA binds to *glnA* promoter region *in vitro* in the absence of any effector (Reyes *et al.*, 1997) (Fig. 3a), although its binding affinity increases in the presence of 2-OG (Fig. 3b). On the other hand, when $H_2O_2$ was included in the binding reactions, no effect was observed on the proportion of *glnA* promoter probe bound by NtcA in the absence of 2-OG (Fig. 3c). However, in the presence of a fixed concentration of this effector, NtcA binding to the *glnA* promoter probe was negatively affected by increasing concentrations of $H_2O_2$ (Fig. 3d). The standard concentration of 0.6 mM 2-oxoglutarate (Vázquez-Bermúdez *et al.*, 2002) was used. A similar result was obtained using the *gifB* promoter region in EMSA assays (Fig. S3).

### Nitrogen conditions influence GS inactivation by $H_2O_2$

The observation that $H_2O_2$ only affected NtcA DNA-binding activity (*in vitro*) in the presence of 2-OG led us to analyse whether N conditions, which changed concentrations of this metabolite, influence the effect of $H_2O_2$ on N metabolism. It is known that 2-OG concentration increases when *Synechocystis* cells are subjected to N deprivation (Muro-Pastor *et al.*, 2001). Cells of wild-type and Δ*katG* strains growing under standard conditions using nitrate as N source were washed twice with N-free medium and resuspended in this medium ($BG11_0C$). Then increasing amounts of $H_2O_2$ were added to different aliquots of the culture of each strain, and GS activity was monitored for 4 h (Fig. 4a,b). In line with the results shown in cultures with nitrate (Fig. 2a), concentrations > 0.1 mM of $H_2O_2$ provoke an irreversible GS inactivation in the Δ*katG* strain (Fig. 4b). However, in the absence of combined N, addition of 0.1 mM $H_2O_2$ caused a lower decrease in GS activity than in cultures with nitrate in both wild-type and Δ*katG* strains (compare Figs 2a and 4a,b). This is probably a result of the fact that the negative effect of $H_2O_2$ is partially compensated by an increase in GS activity under conditions of N deficiency already described (Reyes *et al.*, 1997). This increase was observed in the absence of $H_2O_2$ in both strains (Fig. 4a,b).

It is well known that N deprivation triggers a switch in the metabolism of *Synechocystis* from anabolism to maintenance that entails the degradation of photosynthetic pigments. This process, known as chlorosis or bleaching, provokes a change in cell colour from blue-green to yellow and the role of NblA protein is essential (Baier *et al.*, 2014; Klotz *et al.*, 2016). The influence of $H_2O_2$ on bleaching has been analysed in both wild-type and Δ*katG* strains. Simultaneously with the elimination of the N source, $H_2O_2$ was added at different concentrations and a control without addition was included. Also, a control culture was maintained with nitrate. $H_2O_2$ had no significant effect on the bleaching of the wild-type strain (Fig. 4c). However, this process was delayed in the Δ*katG* strain treated with 0.1 mM $H_2O_2$. At higher concentrations (0.2–0.3 mM) bleaching does not take place in this strain, which actually is not viable under these conditions and 24–48 h after treatment it dies. To investigate in more detail the effects produced by the combined treatments of N removal and $H_2O_2$ addition (0.1 mM), the expression of NtcA-regulated genes (*glnA*, *gifA*, *gifB*, *nblA*, *glnN* and *glnB*) was analysed. In the wild-type strain, $H_2O_2$ treatment did not alter the expression pattern of any of genes, but in the Δ*katG* strain, 0.1 mM of $H_2O_2$ altered the dynamics of induction of the expression of the analysed genes (Fig. 4d,e). Induction of *glnA*, *glnB* and *glnN* genes by N starvation was delayed in the Δ*katG* strain, and even they were transiently repressed when $H_2O_2$ was added. Consistent with this, *gif* genes where transiently derepressed. The *nblA* gene is a particular case because, in contrast to other genes induced by N deficiency, it experienced a rapid and transient induction upon peroxide addition followed by a drop in its expression levels that finally increased again but to a lesser extent than in the wild-type strain at the end of the period analysed in the experiment. The rapid and transient induction of this gene probably represents a response to $H_2O_2$ superimposed and independent of the effect of N deficiency (Kanesaki *et al.*, 2007). A parallel analysis was carried out at higher concentrations of $H_2O_2$ (0.2 and 0.3 mM) in wild-type and Δ*katG* strains (Fig. S4). In the case of 0.3 mM, only a 1 h period upon $H_2O_2$ addition was analysed, as oxidative stress provokes RNA degradation in the Δ*katG* strain at that concentration. This effect was already observed in RNA samples obtained 120 min after treatment of cultures of this strain with 0.2 mM $H_2O_2$. In the wild-type strain the addition of $H_2O_2$ led to a moderate delay in the induction by N deficiency of some genes positively regulated by NtcA (namely *glnN and glnB*) (Fig. S4a). No significant effect was observed with respect to *gif* gene expression. In the case of the Δ*katG* strain induction by N starvation of *glnA*, *glnB* and *glnN* is abolished in the presence of $H_2O_2$ (0.2 and 0.3 mM) and *gif* genes were consistently derepressed in these conditions (Fig. S4b). The behaviour of *nblA* expression was similar to that observed with 0.1 mM $H_2O_2$ (Fig. 4e).

The results presented so far show that $H_2O_2$ interferes with N metabolism, affecting the expression of genes regulated by NtcA. It has been observed that the *in vitro* capacity of NtcA for DNA binding is affected by $H_2O_2$ in the presence of its effector 2-OG (Fig. 3d). Moreover, the capacity of $H_2O_2$ to cause GS inactivation was weakened under conditions of N deficiency, when the concentrations of 2-OG are higher. All these data led us to further investigate the role of 2-OG in the mediation of the effects caused by $H_2O_2$.

### Levels of 2-OG determine the effects of $H_2O_2$

First we analysed the effect of addition of 2-OG to the medium on the GS inactivation by $H_2O_2$. Thus, cultures of the Δ*katG*

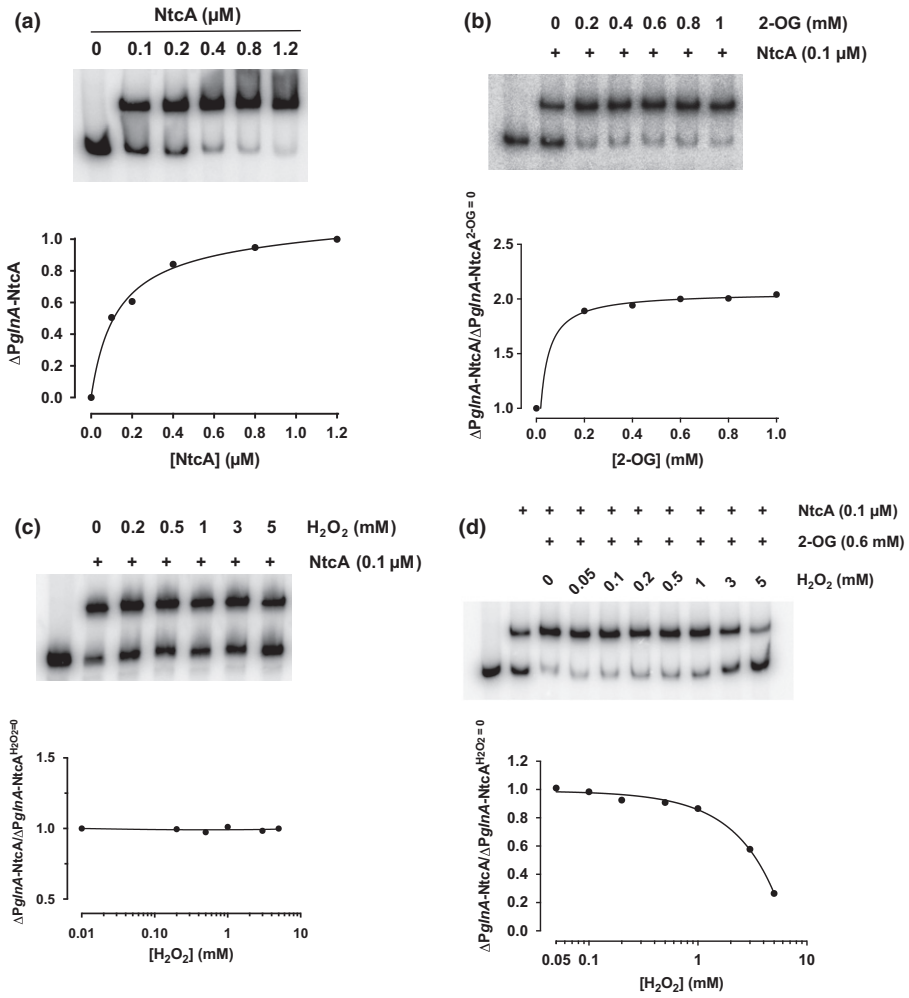


**Fig. 3** Effect of hydrogen peroxide ($H_2O_2$) on NtcA binding to *glnA* promoter region. A PCR-amplified fragment encompassing the *glnA* promoter (Supporting Information Table S1) was used as a probe in gel retardation analysis. (a) The probe was incubated in the presence of purified NtcA protein (0–1.2 μM). (b) The probe was incubated with purified NtcA protein (0.1 μM) and different amounts of 2-oxoglutarate (2-OG) (0–1 mM). (c) The probe was incubated with purified NtcA protein (0.1 μM) and different amounts of $H_2O_2$ (0–5 mM). (d) The probe was incubated with purified NtcA protein (0.1 μM), 2-OG (0.6 mM) and different amounts of $H_2O_2$ (0–5 mM). In each case the increase in radioactive signal corresponding to the fraction of DNA bound relative to the signal without $H_2O_2$ (b, d) or without 2-OG (c) is also represented. In (a) the radioactive signal increase represented is normalized with respect to the maximum signal corresponding to 1.2 μM of NtcA.

strain were treated with $H_2O_2$ alone or in combination with 2-OG. Different combinations of these two compounds were used: equimolar amounts (0.1 mM each) or an excess of 2-OG (1 mM) with respect to $H_2O_2$ (0.1 mM). A control with 2-OG only was also included. Samples were taken during a period of 4 h to determine GS activity and expression of the *glnA* and *gif* genes by Northern blot. The treatment of the Δ*katG* strain with equimolar quantities of 2-OG and $H_2O_2$ (0.1 mM) did not produce any effect on the GS inactivation caused by $H_2O_2$ alone (Fig. 5a). However, addition of an excess of 2-OG (1 mM) leads to a reduction in the degree of GS inactivation provoked by $H_2O_2$. After the transient inactivation of the GS observed at 30 min of treatment, a faster reactivation took place in the presence of 1 mM 2-OG (Fig. 5a).

In agreement with the GS activity data, the combined treatment with 0.1 mM of $H_2O_2$ and 1 mM of 2-OG decreased the expression of the *gif* genes as compared with the treatment with $H_2O_2$ alone. In the case of the *glnA* gene, the decrease in the transcript caused by the addition of peroxide was less intense and more transient in the presence of 2-OG (Fig. 5b). In general, the presence of 2-OG in the medium resulted in a lower effect of $H_2O_2$ on gene expression and GS inactivation, similar to the use of a lower effective concentration of $H_2O_2$ than that actually used. All these data showed the involvement of 2-OG in the GS inactivation process provoked by $H_2O_2$ and pointed to 2-OG as a metabolic quencher of $H_2O_2$. This reduction of the effective concentration of peroxide by the added 2-OG probably takes place extracellularly, as it has been described that cyanobacteria take up this compound very poorly (Vázquez-Bermúdez *et al.*, 2000). To further investigate the role of 2-OG *in vivo*, we decided to incorporate the gene encoding the 2-OG transporter KgtP of *E. coli* in both the wild-type (KGTP) and the Δ*katG* strain (Δ*katG*/KGTP). The functional incorporation of this gene in cyanobacteria has been validated in previous works (Vázquez-Bermúdez *et al.*, 2000; Laurent *et al.*, 2005). Details on the construction of these strains and their characterization are available in Fig. S2 and Methods S2). A deleterious effect of 2-OG (at concentrations ≥ 0.5 mM) was observed on the growth of *Synechocystis* strains bearing KgtP. This effect has already been reported in other cyanobacteria that express this transporter (Fig. S2) (Vázquez-Bermúdez *et al.*, 2000). As the transport carried out through KgtP involves a symport of 2-OG with protons, the experiments with the KGTP and Δ*katG*/KGTP strains were carried out at pH 6.4 (Seol & Shatkin, 1992). We compared the effects of the incorporation of 2-OG on GS inactivation by $H_2O_2$ in both the Δ*katG*/KGTP and Δ*katG* strains at pH 6.4.

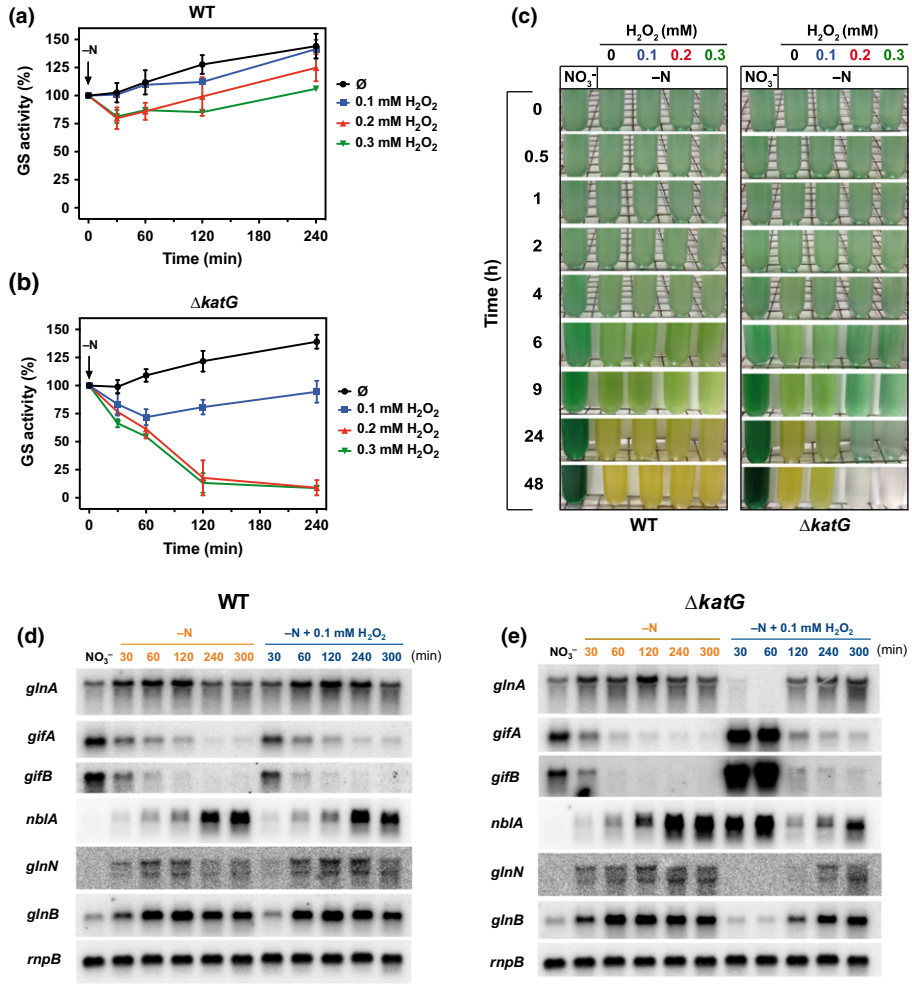


**Fig. 4** Effect of hydrogen peroxide ($H_2O_2$) on glutamine synthetase (GS) activity and N-regulated gene expression in wild-type (WT) and Δ*katG* *Synechocystis* strains under nitrogen starvation. Kinetics of GS activity after the addition of $H_2O_2$ to WT (a) or Δ*katG* (b) cells in the absence of a nitrogen source. Immediately after the elimination of nitrogen, the indicated concentrations of $H_2O_2$ were added. A control culture was maintained without addition (∅). The GS activity values are referred to mg of total protein and relativized to the value at time '0' before nitrogen elimination. The error bars correspond to the SD of three independent experiments. (c) Evolution of growth and bleaching process in WT and Δ*katG* strains. As positive and negative controls of bleaching, cultures of each strain were maintained in nitrogen-free medium without $H_2O_2$ addition and in nitrate-containing medium ($NO_3$), respectively. (d, e) Northern blots of *glnA*, *gifA*, *gifB*, *nblA*, *glnN* and *glnB* genes under standard growth conditions ($NO_3$) or after nitrogen starvation and $H_2O_2$ addition. Total RNA was isolated from cells growing under standard conditions ($NO_3$) or at different times after nitrogen source elimination and addition of 0.1 mM $H_2O_2$. Seven micrograms were analysed and gels were blotted and hybridized with the corresponding probes. The filters were stripped and rehybridized with an *rnpB* probe as a loading control.

Cells of both strains were cultured under standard conditions but at pH 6.4 until they reached the exponential growth phase. At that time, 0.2 mM $H_2O_2$ was added to the culture and, 45 min later, nontoxic concentrations of 2-OG (20 or 100 μM) were added. GS activity was determined throughout the process over 4 h. A control experiment without 2-OG addition was also carried out (Fig. 6c). The addition of 20 μM 2-OG was sufficient to partially reverse the drop in GS activity caused by the previous treatment with 0.2 mM $H_2O_2$ in the Δ*katG*/KGTP strain (Fig. 6a). At higher concentrations (100 μM), not only was the recovery of the GS activity achieved (Fig. 6b), but the cell death caused by the treatment with 0.2 mM of $H_2O_2$ in Δ*katG* strains was also avoided, as evidenced by the appearance of culture. In all cases, treatment with 0.2 mM $H_2O_2$ caused an oxidative damage that turned out to be lethal in the Δ*katG* mutant strain, and particularly in the Δ*katG*/KGTP strain when no 2-OG was added (Fig. 6c).

These results showed that the increase in 2-OG concentrations after their incorporation by the KgtP transporter attenuated the effect of $H_2O_2$ on GS activity. Taking this into account, intracellular concentration of this compound was determined in different N conditions and in the presence of $H_2O_2$ in both wild-type and Δ*katG* strains. Two previously analysed conditions were chosen for this study: addition of 0.1 mM $H_2O_2$ to cells cultured under standard conditions until the exponential growth phase with nitrate as N source (Fig. 7) and, on the other hand, addition of 0.2 mM $H_2O_2$ simultaneously to the withdrawal of the N source from the medium (Fig. 8). As previously shown (Figs 1a, 2a)

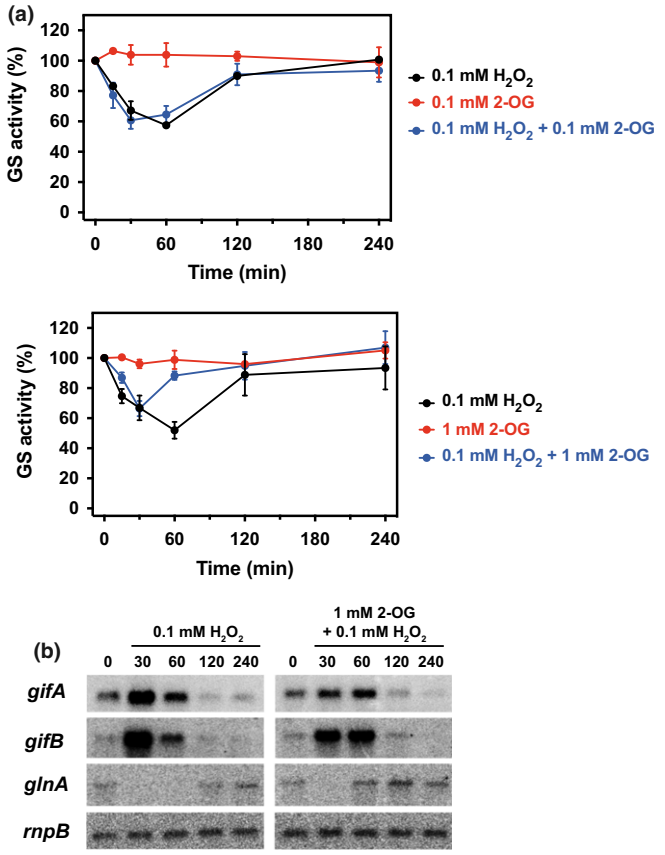


**Fig. 5** Effect of hydrogen peroxide ($H_2O_2$) on glutamine synthetase (GS) activity and gene expression in Δ*katG* strain in the presence of 2-oxoglutarate (2-OG). (a) Kinetics of GS activity after addition of $H_2O_2$ and 2-OG, separately or simultaneously, to cells growing under standard conditions. At time '0' the indicated concentrations of each compound were added. The GS activity values are relativized to the value at time '0' before the additions ($2.29 \pm 0.14$ U $mg^{-1}$ total protein). The error bars correspond to the SD of three independent experiments. (b) Northern blots of *gifA*, *gifB* and *glnA* genes from the same cultures analysed in (a). Total RNA was isolated from cells growing under standard conditions (time 0) or after addition of $H_2O_2$ and 2-OG, separately or simultaneously. Seven micrograms were analysed and gels were blotted and hybridized with the corresponding probes. The filters were stripped and rehybridized with an *rnpB* probe as a loading control.

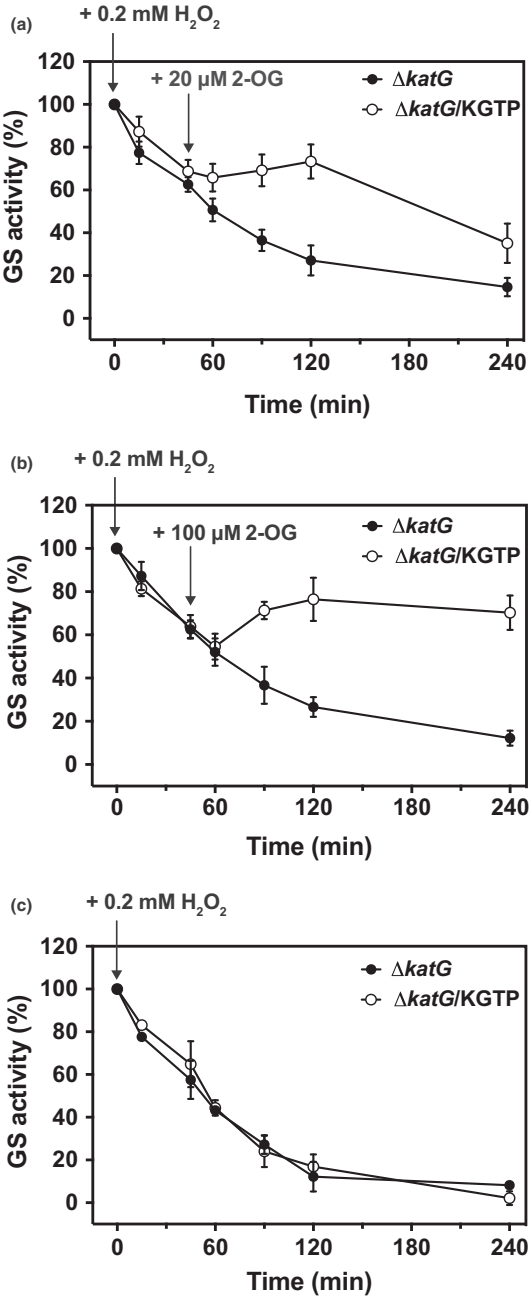


**Fig. 6** Kinetics of glutamine synthetase (GS) activity after the addition of hydrogen peroxide ($H_2O_2$) and 2-oxoglutarate (2-OG) in Δ*katG* and Δ*katG*/KGTP strains. At time '0', 0.2 mM $H_2O_2$ was added to cells of each strain growing in BG11C medium buffered with 100 mM MES-NaOH, pH 6.4. At 45 min after $H_2O_2$ addition, 20 µM (a) or 100 µM (b) of 2-OG was added (time of treatments marked by arrows). A control without 2-OG addition is shown (c). The GS activity values are relativized to the value at time '0' before the additions. The error bars correspond to the SD of three independent experiments.

addition of 0.1 mM $H_2O_2$ to cells growing in standard conditions provoked a transitory GS inactivation in both strains, but it was much more conspicuous in the Δ*katG* strain. As a control of GS inactivation, ammonium-treated cells were included in the analysis (Saelices *et al.*, 2015) (Fig. 7a). As expected, inactivating factor accumulation was in line with the GS inactivation in each strain (Fig. 7b). Furthermore, a correlation was observed between the degree of GS inactivation observed when adding $H_2O_2$ and the decrease in 2-OG concentration, analogous to the decrease of this compound experienced in the case of treatment with ammonium (Muro-Pastor *et al.*, 2001) (Fig. 7c). In fact, the decrease in 2-OG concentration in the Δ*katG* strain caused by the addition of $H_2O_2$ was very similar to that observed after the addition of ammonium. With respect to the withdrawal of the N source, this caused an increase in GS activity together with an increase in the intracellular concentration of 2-OG that was very similar in both strains, wild-type and Δ*katG* (Fig. 8a,c). When 0.2 mM $H_2O_2$ was added to cultures at the same time as N removal, a transitory and small decrease in GS activity, followed by a progressive increase, was observed in the wild-type strain (Fig. 8a). The increase in the concentration of 2-OG caused by N removal was slightly delayed (Fig. 8c). However, in the Δ*katG*, addition of

$H_2O_2$ caused GS inactivation (Fig. 8a) and an immediate and progressive decrease of 2-OG during the total time analysed (Fig. 8c). This corresponded to accumulation of inactivating factors in this strain. After 4 h treatment, protein degradation caused by oxidative damage was observable by Western blot in the Δ*katG* strain (Fig. 8b).

All these results confirmed the alteration of 2-OG homeostasis by the action of $H_2O_2$.

## $H_2O_2$ affects cellular processes dependent on 2-OG

To further investigate the effect of $H_2O_2$, we analysed the possibility that other cellular processes that are influenced by the concentration of 2-OG were also affected by the treatment with $H_2O_2$.

PII signalling proteins constitute one widely distributed family of signal transduction proteins. In cyanobacteria, PII signal perception involves the binding of the effector molecules (ATP, ADP and 2-OG) and also covalent modification by phosphorylation/dephosphorylation of a seryl residue (Ser49). Ligand binding results in conformational changes and thereby controls covalent modification (Forchhammer, 2008). Therefore, given that PII responds to 2-OG concentrations, alteration in the intracellular concentration of this metabolite, produced by the addition of $H_2O_2$, should be reflected in the phosphorylation state of this protein.

As stated earlier, 2-OG concentration increases when *Synechocystis* cells are subjected to N deficiency. Then, to test our hypothesis, cells of the wild-type and Δ*katG* strains were cultured under standard conditions (with nitrate) until they reached the exponential growth phase. At that time they were washed twice with N-free medium and resuspended in this medium. Immediately after washing, 0.2 mM $H_2O_2$ was added to one aliquot of these cells and samples were taken over a period of 4 h. As a control, cells maintained with nitrate, as well as others to which ammonium was added, were analysed. In all these samples, the phosphorylation state of the PII was analysed by nondenaturing electrophoresis and Western blot using specific antibodies. The elimination of the N source caused the progressive appearance of the phosphorylated forms of the PII in a very similar way in both strains under study. However, in the presence of $H_2O_2$, this process was slightly delayed in the wild-type strain (compare samples

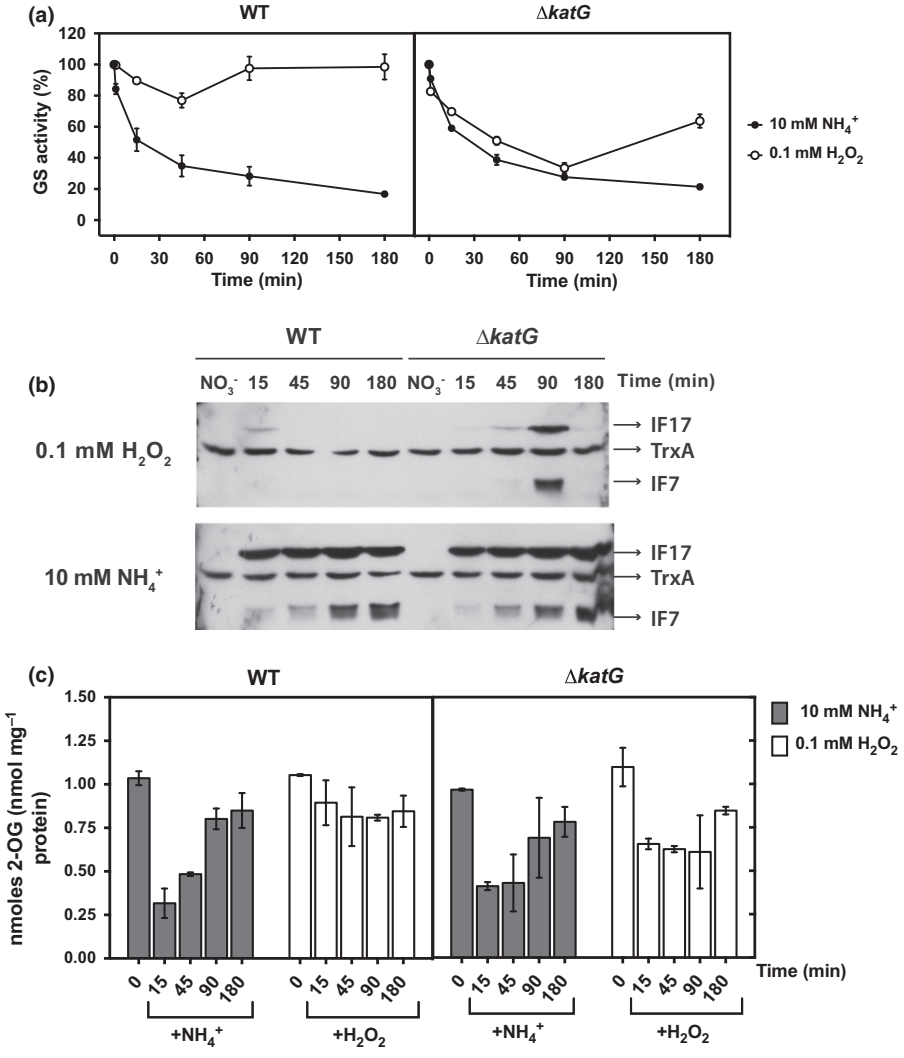


**Fig. 7** Evolution of 2-oxoglutarate (2-OG) intracellular concentrations in wild-type (WT) and Δ*katG* strains following ammonium or hydrogen peroxide ($H_2O_2$) addition. (a) Kinetics of glutamine synthetase (GS) activity after addition of 10 mM $NH_4Cl$ or 0.1 mM $H_2O_2$ to WT and Δ*katG* cells growing under standard conditions. At time '0' the indicated concentration of each compound was added. The GS activity values are relativized to the value at time '0' before the additions. The error bars correspond to the SD of three independent experiments. (b) Western blot of IFs after addition of 10 mM $NH_4Cl$ or 0.1 mM $H_2O_2$. From the same cultures used for determination of GS activity, samples were taken before (0) and after ammonium or $H_2O_2$ addition at the indicated times. Total proteins were isolated and resolved on sodium dodecyl sulphate-polyacrylamide gel electrophoresis, blotted and incubated with anti-IF7 and anti-IF17. As a control for protein loading, membranes were incubated also with anti-TrxA antibodies. (c) Quantification of intracellular concentrations of 2-OG at the indicated times in WT and Δ*katG* cells after the addition of ammonium or $H_2O_2$. Values are calculated as nmol $mg^{-1}$ total protein. The error bars correspond to the SD of three independent experiments.

30 min after N removal) but was completely abolished in the Δ*katG* strain (Fig. 9). After 4 h treatment, protein degradation caused by oxidative damage was observable by Western blot in this strain. These results clearly indicate that $H_2O_2$ interferes with signalling mediated by the PII protein, affecting its covalent modification by phosphorylation. This interference is probably a result of its effect on the intracellular concentration of 2-OG.

## Discussion

Reported evidence indicates that $H_2O_2$ caused the induction of *gif* genes in *Synechocystis* independently of regulators involved in redox signalling such as PerR or different His-kinases. In fact, the *gifB* gene tops the list of genes whose induction by $H_2O_2$ was not abolished in mutants of these regulators (Δ*hik* and Δ*perR*; Kanesaki *et al.*, 2007). This observation suggested that the redox state was interfering with N metabolism through an unknown mechanism. To elucidate how this connection takes place has been the fundamental object of our study. The assimilation of ammonium through the Glutamine synthetase-glutamate synthase (GS-GOGAT) cycle constitutes a central and finely regulated point of N metabolism in cyanobacteria (Muro-Pastor *et al.*, 2005). Here, we have used the modulation of GS activity to monitor N metabolism in relation to the redox state under different metabolic conditions. We have constructed a Δ*katG* mutant in order to increase the residence time of $H_2O_2$ in cells and thus amplify its effects, facilitating the analysis. Despite the efficacy of KatG catalase-peroxidase in the elimination of $H_2O_2$ (Tichy & Vermaas, 1999), its action may not be sufficient to face abrupt changes in its intracellular concentration. Proof of this are the widely studied effects of $H_2O_2$ on gene expression in *Synechocystis* (Houot *et al.*, 2007; Kanesaki *et al.*, 2007). The transient inactivation of the GS caused by the addition of $H_2O_2$ could have the physiological function of attenuating the assimilation of ammonium while the intracellular concentrations of $H_2O_2$ remain above the threshold of toxicity and therefore represent a situation of oxidative stress.

Taking into account the fact that the global regulator NtcA mediates GS regulation, our attention was focused on evaluating whether the effects on N metabolism caused by peroxide treatment were in some way channelled by this regulator. The data obtained in this work, together with previously reported data (Houot *et al.*, 2007; Kanesaki *et al.*, 2007), clearly show that $H_2O_2$ affects the entire NtcA regulon. The effects of $H_2O_2$ on this regulon are dose-dependent and similar to those caused by the decrease in the concentrations of 2-OG, the natural effector of NtcA. Two possibilities could explain these results: the peroxide could interfere in the interaction of 2-OG with NtcA or it

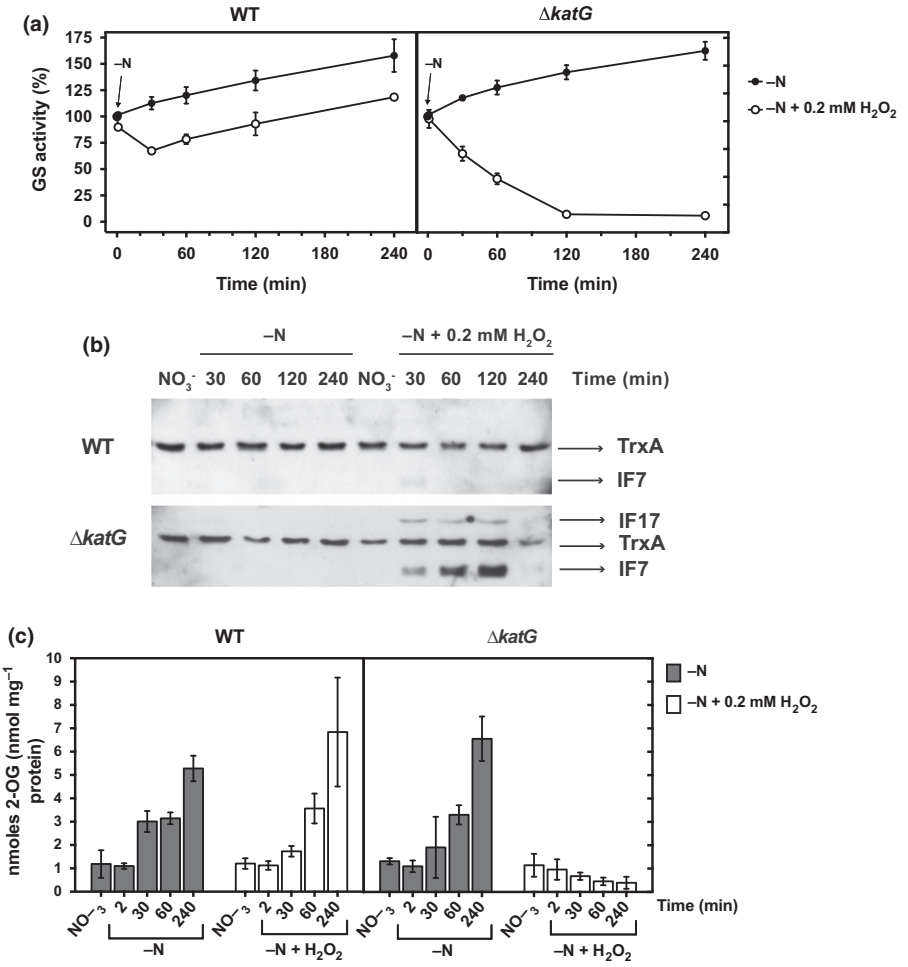


**Fig. 8** Effect of hydrogen peroxide ($H_2O_2$) on 2-oxoglutarate (2-OG) intracellular concentrations in wild-type (WT) and Δ*katG* strains under nitrogen starvation. (a) Kinetics of glutamine synthetase (GS) activity after addition of 0.2 mM $H_2O_2$, at the same time as nitrogen removal, to WT and Δ*katG* cells. Immediately after the elimination of nitrogen, 0.2 mM $H_2O_2$ was added. A control culture of each strain was maintained without $H_2O_2$ addition (–N). The GS activity values are relativized to the value at time '0' before the treatments. The error bars correspond to the SD of three independent experiments. (b) Western blot of IFs after addition of 0.2 mM $H_2O_2$ at the same time as nitrogen elimination. From the same cultures used to determine GS activity, samples were taken before (0) and after $H_2O_2$ addition at the indicated times. Total proteins were isolated and resolved on sodium dodecyl sulphate-polyacrylamide gel electrophoresis, blotted and incubated with anti-IF7 and anti-IF17. As a control for protein loading, membranes were also incubated with anti-TrxA antibodies. (c) Quantification of intracellular concentrations of 2-OG at the indicated times in WT and Δ*katG* cells following the marked treatments. Values are calculated as nmol $mg^{-1}$ total protein. The error bars correspond to the SD of three independent experiments.

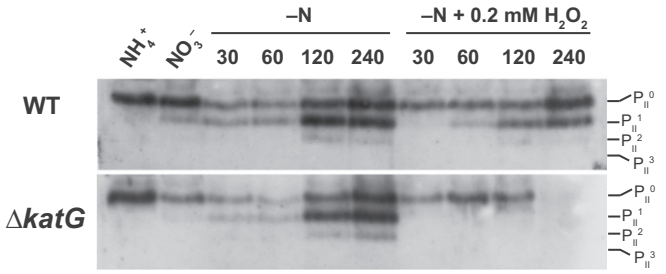


**Fig. 9** Effect of hydrogen peroxide ($H_2O_2$) on PII phosphorylation in wild-type (WT) and Δ*katG* strains under nitrogen starvation. The degree of phosphorylation of the PII trimeric protein in the different conditions was analysed by Western blot. Cells cultivated under standard conditions were washed to remove the nitrogen source and incubated in the presence of 0.2 mM $H_2O_2$ during 4 h. Cell samples were taken (equalized by $OD_{750\ nm}$) and cell extracts were obtained. Twenty micrograms of total protein were analysed on native-polyacrylamide gel electrophoresis, blotted and incubated with anti-PII. Cultures of each strain under standard conditions ($NO_3^-$) or with ammonium added ($NH_4^+$) were analysed as controls. $P_{II}^0$ denotes the nonphosphorylated form of PII. $P_{II}^1$, $P_{II}^2$ and $P_{II}^3$ indicate the phosphorylation of one, two or three subunits, respectively.

could act by altering the concentrations of 2-OG. It could be that the $H_2O_2$ molecule, with two hydroxyl groups, presents a certain structural analogy with 2-OG. In this sense, it could interfere with the binding of 2-OG to NtcA, which is well characterized (Zhao *et al.*, 2010). However, EMSA assays in which 2-OG was added before incubation with $H_2O_2$ or vice versa (results not shown) allowed us to rule out the possibility that the interference of $H_2O_2$ in the transcriptional regulation mediated by NtcA is a result of a displacement of the effector 2-OG from its interaction site. Therefore, the results suggest that the concentrations of 2-OG are altered by its reaction with peroxide.

The emerging role of 2-OG as an antioxidant agent has been studied in different organisms. The antioxidant properties are attributed to the nonenzymatic interaction of 2-OG with $H_2O_2$ that leads to the oxidative decarboxylation of 2-OG and gives rise to the formation of succinate, carbon dioxide and water (Mailloux *et al.*, 2009; Bayliak *et al.*, 2015). Other ketoacids such as pyruvate react with $H_2O_2$ and can eliminate this compound from culture media (Winterbourn, 2013). In *Saccharomyces cerevisiae*, it has been described that growth in the presence of 2-OG protects cells against the stress caused by $H_2O_2$ and other oxidants; however, this protection disappears in mutants lacking some antioxidant enzymes such as catalases or superoxide dismutases. Taking this into account, the effect is partially attributed to a role of 2-OG in the induction of defence systems against oxidative stress (Bayliak *et al.*, 2017). By contrast, in the case of *Synechocystis*, the protective effect of 2-OG is also observed in the Δ*katG* mutant, lacking the main enzyme involved in the defence against $H_2O_2$ (Fig. 6). In this sense, our results suggest a role of 2-OG in the detoxification of $H_2O_2$ mediated by direct interaction with this compound.

One of the parameters indicative of ROS homeostasis that increases in *Saccharomyces* in the presence of 2-OG is glutathione reductase activity (GR), the enzyme responsible for regenerating oxidized glutathione (Bayliak *et al.*, 2017). However, *Synechocystis* lacks this enzyme (Narainsamy *et al.*, 2013), so the possible mechanism involved in the reduction of oxidized glutathione is unknown, despite the fact that an essential role of reduced glutathione in the protection against ROS has been shown in this cyanobacterium (Cameron & Pakrasi, 2010). In *Anabaena sp.* PCC 7120 (*Anabaena*), it was described that the gene coding for GR (*gor*) is differentially transcribed as a function of the N source, and the GR activity also depends on the N conditions (Jiang *et al.*, 1995). More recently, transcriptomic data have confirmed these observations (Mitschke *et al.*, 2011). The authors suggested that it was difficult to predict the role of N control on a gene related to ROS homeostasis. In view of our results in *Synechocystis*, it could be hypothesized that a regulated GR activity would be favourable for *Anabaena* as an antioxidant element. It is worth noting that this organism lacks the KatG enzyme (Bernroitner *et al.*, 2009).

In summary, our results clearly indicate that the modulation of 2-OG concentration by $H_2O_2$ is responsible for the response of N metabolism to this oxidizing compound, suggesting the possible role of 2-OG as an antioxidant agent in *Synechocystis*, although more work would be required to clearly demonstrate this duty. We can conclude, therefore, that 2-OG is the key element in the connection between oxidative stress and N metabolism. Several studies revealed that 2-OG is a master regulator metabolite in different organisms, including bacteria, plants and humans (Huergo & Dixon, 2015; Zhang *et al.*, 2018). In this sense, our work highlights a new function of this signalling molecule in cyanobacteria.

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## Author contributions

RR-R performed the experiments and analysed the data. MIM-P and FJF conceived and designed the experiments, supervised the work and analysed the data. All the authors contributed to writing and revising the article.

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Construction and analysis of the Δ*katG Synechocystis* strain.

**Fig. S2** Construction and analysis of the KGTP *Synechocystis* strain.

**Fig. S3** Effect of $H_2O_2$ on NtcA binding to the *gifB* promoter region.

**Fig. S4** Northern blots of NtcA-regulated genes in wild-type (a) and Δ*katG* (b) strains under standard growth conditions ($NO_3$) or after nitrogen starvation and addition of hydrogen peroxide (0.2–0.3 mM).

**Methods S1** Oxidation test of ferrous ion in the presence of xylenol orange (FOX).

**Methods S2** Enzymatic determination of 2-OG.

**Table S1** List and sequence of oligonucleotides used in this work.

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