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Running head: Oxidative stress and group 2 sigma factors

Corresponding author: T. Tyystjärvi

Department of Biochemistry / Molecular Plant Biology, University of Turku, FI-20014 Turku, Finland

Tel. +358-400-546259, fax +358-02-333-5549, E-mail: taityy@utu.fi

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Group 2 Sigma Factors Are Central Regulators of Oxidative Stress Acclimation in Cyanobacteria

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Kaisa Hakkila[1], Dimitar Valev[1], Taras Antal[2], Esa Tyystjärvi[1] and Taina Tyystjärvi[1]*

[1]Department of Biochemistry/Molecular Plant Biology, University of Turku, FI-20014 Turku, Finland

[2]Biological Faculty, Moscow State University, Vorobyevi Gory 119992, Moscow, Russia

*Corresponding author: E-mail taityy@utu.fi

Abbreviations:

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Abstract

Regulatory σ factors of the RNA polymerase (RNAP) adjust gene expression according to environmental cues when the cyanobacterium *Synechocystis* sp. PCC 6803 acclimates to suboptimal conditions. Here we show central roles of the non-essential group 2 σ factors in oxidative stress responses. Cells missing all group 2 σ factors fail to acclimate to chemically induced singlet oxygen, superoxide or H_2O_2 stresses and lose pigments in high light. The SigB and SigD are the major σ factors in oxidative stress whereas SigC and SigE play only minor roles. The SigD factor is upregulated in high light, singlet oxygen and H_2O_2 stresses, and overproduction of the SigD factor in the ΔsigBCE strain leads to superior growth of ΔsigBCE cells in those stress conditions. Superoxide does not induce the production of the SigD factor but instead SigB and SigC factors are moderately induced. The SigB factor alone in ΔsigCDE can support almost as fast growth in superoxide stress as full complement of σ factors in the control strain but an overdose of the stationary-phase-related SigC factor causes growth arrest of ΔsigBDE in superoxide stress. Drastic decrease of the functional RNA polymerase limits the transcription capacity of the cells in H_2O_2 stress, which explains why cyanobacteria are sensitive to H_2O_2. Formation of RNAP-SigB and RNAP-SigD holoenzymes is highly enhanced in H_2O_2 stress and cells containing only SigB (ΔsigCDE) or SigD (ΔsigBCE) show superior growth in H_2O_2 stress.

Introduction

Oxygenic photosynthesis in cyanobacteria leads to inevitable production of reactive oxygen species (ROS) that harm the cell by oxidizing proteins, lipids and pigments but might also function in cell signaling. Different species of ROS are produced and scavenged by particular cellular processes (for reviews see Latifi et al. 2009, Mattila et al. 2015). The major source of singlet oxygen is a reaction of triplet chlorophyll and oxygen in photosystem II (PSII), and in bright light singlet oxygen production may lower the oxygen evolving activity of PSII (Rehman et al. 2013). Carotenoids, α-tocopherol and plastoquinone act as singlet oxygen quenchers and scavengers in cyanobacteria (Inoue et al. 2011, Hakkila et al. 2014). The importance of carotenoids is obvious as the carotenoid knock out strain of the model cyanobacterium *Synechocystis* sp. PCC 6803 is not viable in the light (Sozer et al. 2010).

Superoxide, in turn, is mainly produced via one electron reduction of O_2 by photosystem I (PSI) in Mehler's reaction (Fujii et al. 1990). The probability of Mehler's reaction in cyanobacteria is lowered by a Mehler-like reaction in which flavodiiron proteins safely transfer electrons from PSI to O_2 converting O_2 to H_2O without ROS production (Helman et al. 2003). Superoxide is further processed to H_2O_2 by a superoxide dismutase encoded by a single essential gene in *Synechocystis* (Ke et al. 2014). H_2O_2 is detoxified by peroxidases and catalases; *Synechocystis* genome encodes five peroxiredoxins, two glutathione peroxidases and a catalase-peroxidase KatG (Cui et al. 2012). Detoxification of H_2O_2 is important because in the presence of Fe^{2+}, the Fenton reaction converts H_2O_2 to the highly reactive hydrogen radical.

Unlike in chloroplasts of higher plants, the ascorbate concentration of cyanobacteria is low and they miss ascorbate peroxidase. The tripeptide glutathione is abundant and important in protection against oxidative stress in cyanobacteria (Cameron and Pakrasi 2010). In addition, some defense mechanisms balance cellular functions preventing ROS formation. The orange carotenoid protein associates with the phycobilisome antenna and dissipates excess energy as heat (Kirilovsky 2010), and state transitions balance energy distribution between the photosystems (van Thor et al. 1998). Adjustment of gene expression according to environmental changes is a central mechanism behind the acclimation processes. In cyanobacteria, transcription initiation plays a major role in gene regulation, and the regulatory σ factors of the RNA polymerase (RNAP) are mainly responsible for overall changes of the transcription pattern. The RNAP core recruits one of the σ factors to form a transcription initiation competent RNAP holoenzyme that recognizes a particular set of genes.

The model cyanobacterium *Synechocystis* sp. PCC 6803 encodes nine different σ factors. SigA is the essential primary σ factor that is mainly responsible for expression of housekeeping genes during normal growth (Imamura et al. 2003b). Group 2 σ factors are non-essential in standard conditions, and all single, double, triple and quadruple inactivation

strains of group 2 factors grow well in standard growth conditions (Imamura et al. 2003b, Pollari et al. 2008, Pollari et al. 2011, Koskinen et al. 2016). RNAP holoenzymes with group 2 σ factors are present in low quantity in standard conditions but form in high quantity in different stress conditions like heat, high light or high salt (Koskinen et al. 2016). SigD has shown to be important in high light (Imamura et al. 2003a, Imamura et al. 2003b, Pollari et al. 2008, Pollari et al. 2009). In high salt, recruitment of SigB, SigC and SigE increases (Koskinen et al. 2016) and accordingly, ΔsigB, ΔsigC and ΔsigE strains show slow growth and other defects in high salt (Pollari et al. 2008, Nikkinen et al. 2012, Tyystjärvi et al. 2013). For heat stress acclimation, SigB and SigC are the most crucial σ factors (Singh et al. 2006, Tuominen et al. 2006, Tuominen et al. 2008, Gunnelius et al. 2010) whereas simultaneous deletion of SigB and SigD interferes with acclimation to low nitrogen (Antal et al. 2016). Possibility for enhanced ROS production has been connected to all these stress conditions.

We studied the roles of different group 2 σ factors in oxidative stress by comparing the performance of a quadruple mutant strain without any group 2 σ factor and triple inactivation strains with only one functional group 2 σ factor with the control strain in high-light and after chemically induced singlet oxygen, superoxide or H_2O_2 stresses. In addition, σ factor content and recruitment of σ factors by the RNAP core were studied in superoxide and H_2O_2 stresses. According to the results, group 2 σ factors are necessary for acclimation and protection against oxidative stress, and SigB and SigD playing the major roles.

Results

The σ factor content of triple and quadruple inactivation strains of group 2 σ factors of Synechocystis

The roles of group 2 σ factors in oxidative stress were studied using the group 2 σ factor inactivation strains of *Synechocystis* sp. PCC 6803. Each inactivation strain contains either only one functional group 2 σ factor (strains ΔsigBCD, ΔsigBCE ΔsigBDE ΔsigCDE; strains are described in Pollari et al. 2011) or no group 2 σ factors at all (strain ΔsigBCDE, Koskinen et al. 2016). In the standard growth conditions, inactivation of the other group 2 σ factors doubled the SigB content of ΔsigCDE and SigD content of ΔsigBCE, the SigE content of ΔsigBCD increased 1.5 fold whereas the SigC content of ΔsigBDE remained similar as in the control strain (CS) (Fig.1A).

Roles of group 2 σ factors in singlet oxygen stress

The transcriptomes of the triple and quadruple mutant strains were compared by DNA microarray analysis. RNAs were isolated from cells grown in the standard conditions, and the transcriptome of each mutant strain was compared to that of

CS. The whole transcriptome data have been collected to Supplemental Dataset 1, and the original data have been deposited to GEO; strains ΔsigBCD, ΔsigBCE and ΔsigBDE accession GSE117478, strains CS and ΔsigCDE accession GSE50060 and ΔsigBCDE accession GSE69981.

Expression data of genes known to play roles in oxidative stress responses of *Synechocystis* have been collected in Table 1. Carotenoids and tocopherols provide protection against singlet oxygen. In the quadruple mutant, many α-tocopherol synthesis genes were downregulated (Table 1) and accordingly, low α-tocopherol content was measured in the ΔsigBCDE strain (Koskinen et al. 2016). Some carotenoid synthesis genes were upregulated in ΔsigBCDE (Table 1) and the total carotenoid content of ΔsigBCDE was slightly upregulated compared to CS (Fig. 2A). Carotenoid synthesis genes were expressed similarly in all triple inactivation strains as in CS (Table 1) but the carotenoid content was high in ΔsigCDE and low in ΔsigBCD, ΔsigBCE and ΔsigBDE (Fig. 2A)) pointing to a central role for post-transcriptional regulation of carotenoid synthesis in *Synechocystis*.

The major source of singlet oxygen in cyanobacteria is PSII, and therefore adjustment of PSII function might affect the production of singlet oxygen. Upregulation of the flavodiiron operon *flv4-sll0218-flv2* has been shown to protect PSII against light damage (Hakkila et al. 2013) and deletion strains of the *flv4-sll0218-flv2* operon are light sensitive (Zhang et al. 2012). Flv2 protein content was high in all mutant strains (Fig. 2B). In another mechanism known to protect PSII, the orange carotenoid protein (OCP) dissipates excitation energy collected by the phycobilisome antenna (Kirilovsky and Kerfeld. 2012, Wilson et al. 2006) and might also directly quench singlet oxygen (Sedoud et al. 2014). The *ocp* transcript was upregulated in ΔsigBCD but not in the other mutant strains (Table 1). However, OCP protein content was high in ΔsigBCD and ΔsigBCDE (Fig. 2B) and accordingly, non-photochemical quenching (NPQ) was highest in those strains (Fig. 2C). The OCP protein and NPQ were also enhanced in ΔsigBDE and ΔsigCDE, and only in ΔsigBCE the OCP content and NPQ were similar as in CS (Figs. 2B and 2C).

To directly induce singlet oxygen stress, the cell culture was supplemented with 5 μM methylene blue and cells were grown in otherwise standard conditions. The quadruple mutant was not able to grow and ΔsigBCE (SigD active) grew better than CS (Fig. 2D). ΔsigCDE (SigB active) grew almost as well as CS whereas the OD_{730} values of ΔsigBCD and ΔsigBDE were only half of that measured in CS after three days of growth (Fig. 2D). These results indicate that SigD has a central role in protection against singlet oxygen stress, SigB is important, and SigC and SigE are also involved.

Group 2 σ factors and high light induced oxidative stress

To further test the roles of group 2 σ factors, oxidative stress was induced with high light treatment. Cells were grown under constant illumination, PPFD 350 μmol $m^{-2}s^{-1}$, for three days in otherwise standard conditions. Strain ΔsigBCE grew faster than the other strains, and ΔsigBCDE, ΔsigBCD and ΔsigBDE grew more slowly than CS (Fig. 3A). However, growth differences were only moderate compared to the color changes of the cultures (Fig. 3B). After three days, ΔsigBCDE and ΔsigBCD had lost the majority of their pigments (Fig. 3C). ΔsigBCDE retained more carotenoids than ΔsigBCD, which explains the more yellowish appearance of ΔsigBCDE cells than ΔsigBCD cells. The ΔsigBCE strain remained greener than the other strains due to a high Chl *a* and phycobilin to carotenoid ratio whereas a high carotenoid content explains the yellowish-green color of ΔsigCDE (Figs. 3B and 3C).

The ROS-dependence of the high light effects was tested by growing CS and ΔsigBCDE at PPFD 500 μmol $m^{-2}s^{-1}$ in microaerobic conditions obtained by bubbling the growth medium with N_2 gas containing an atmospheric concentration of CO_2 (Fig. 3D). ΔsigBCDE grew more slowly than CS, just like in ambient air, indicating that group 2 σ factors also regulate high light acclimation processes that are not directly related to ROS. Loss of pigments in ΔsigBCDE was less prominent in microaerobic conditions than in ambient air (Inset of Fig. 3D and Fig. 3B).

Growth of σ factor mutants in chemically induced superoxide or hydrogen peroxidase stresses

PSI function might lead to superoxide production in Mehler's reaction. In *Synechocystis*, the flavodiiron protein dimer Flv1/Flv3 transfers electrons from PSI to O_2, forming H_2O (Helman et al. 2003) and thereby reducing superoxide production by Mehler's reaction. However, the Flv3 protein content was low in all mutant strains in standard conditions, which might lower the efficiency of the Mehler-like reaction (Fig. 4A). To test mutant strains in superoxide stress, the production of superoxide was chemically induced with methyl viologen. The quadruple mutant did not grow in superoxide stress, and the growth of ΔsigBDE was completely arrested after 12 h (Fig. 4A). Obviously the SigB factor is the most important one for superoxide stress responses, as ΔsigCDE grew almost as well as CS while the growth rates of ΔsigBCD and ΔsigBCE were retarded after 1.5 days (Fig. 4B).

Cellular effects of superoxide and H_2O_2 are related because superoxide dismutases convert superoxide to H_2O_2. In *Synechocystis*, the *sodB* gene encodes the superoxide dismutase FeSOD. In standard conditions, the *sodB* gene was up-regulated at the transcript level in ΔsigBCD but not in the other strains (Table 1). To directly test the sensitivity of the mutant strains to added H_2O_2, the BG-11 growth medium was supplemented with 0.2 mM H_2O_2. The quadruple mutant was

unable to grow in H_2O_2 stress while strains containing only SigB (ΔsigCDE) or SigD (ΔsigBCE) grew better than CS. H_2O_2 is detoxified by multiple cellular processes including the detoxifying enzymes, peroxidases and catalase (Latifi et al. 2009). The mutant strains might have some differences in these processes as the catalase peroxidase KatG was circa 1.5-fold more abundant in ΔsigBCDE, ΔsigBCD, ΔsigBDE, and 15% less abundant in ΔsigCDE and ΔsigBCE than in CS (Fig. 4A). The expression of the *gpx2* gene, encoding glutathione peroxidase, was similar in all strains whereas ΔsigBCDE, ΔsigBCD and ΔsigBDE had reduced amounts of transcripts of another glutathione peroxidase, *gps1* (Table 1).

RNAP responses differ in superoxide and H_2O_2 stresses

According to the growth experiments, group 2 σ factors showed variable importance in superoxide and H_2O_2 stresses. To get a more comprehensive picture, we next followed changes in the amounts of different σ factors and their recruitment by RNAP core upon induction of superoxide or H_2O_2 stress. The RNAP-His strain of *Synechocystis* was used in these experiments. In the RNAP-His strain, a His-tag was added to the γ subunit of the RNAP core to allow collection of intact RNAP complexes (Koskinen et al. 2016).

To induce superoxide stress, the RNAP-His strain was illuminated in the presence of 7.5 μM methyl viologen for 0, 15 min or 1, 2 or 6 h. After the illumination, soluble proteins were isolated and the σ factor and RNAP core contents were measured with specific antibodies. Both SigA content (Fig. 5A) and RNAP core protein contents (Fig. 5B) slowly decreased by circa 20% during the methyl viologen treatment. To directly measure the RNAP-SigA holoenzyme content, the RNAPs were pulled down and the SigA content per RNAP core was calculated and compared to that obtained without the treatments. Initially the RNAP-SigA holoenzyme content more than doubled in superoxide stress but then returned back to the same level as in standard growth conditions (Fig. 5C). The RNAP-SigB and RNAP-SigC holoenzymes slightly increased upon methyl viologen treatment while RNAP-SigD content was below the detection limit (Fig. 5C).

Previous studies on short term effects of H_2O_2 on transcription and translation have used 1.5 to 10 mM H_2O_2 (Li et al. 2004, Singh et al. 2004, Kojima et al. 2009), and based on those experiments we decided to use 4 mM H_2O_2 in our experiments. Addition of H_2O_2 rapidly reduced the SigA content of cells to one-third of that measured in standard conditions (Fig. 6A). On the contrary, the SigB content of the cells increased in H_2O_2 stress and an increase was detected also in SigD content but only after 6 h of exposure to H_2O_2 (Fig. 6A). SigC or SigE proteins did not show any clear changes (Fig. 6A).

When RNAP complexes were pulled down after H_2O_2 treatments of the RNAP-His strain, the amounts of the RNAP core subunits α and β drastically decreased in H_2O_2 stress, but the amount of the His-tag-containing γ subunit

decreased only moderately (Fig. 6B). However, in the soluble protein fraction, the amounts of α and β subunits decreased only moderately (Fig. 6C). This circa 20% decrease of RNAP core subunits was a specific response, as the overall protein level remained constant during the 6-h H_2O_2 treatment (Fig. 6D). These results suggest that the H_2O_2 treatment caused disassembly of the RNAP complex, as only the γ subunit, not the whole complex, was efficiently collected by the pull-down technique. Although the RNAP core is partially disassembled, the σ factor content of the RNAP holoenzyme can be still analyzed with pull down technique as only the intact RNAP core can bind a σ factor. Formation of holoenzyme requires interactions between β, β', and γ subunits of the RNAP core and the σ factor (Pollari et al. 2008) and separate core subunits do not bind σ factors.

The amounts of all σ factors increased upon H_2O_2 exposure in pull down samples indicating that a higher proportion of RNAP was in the RNAP holoenzyme form in H_2O_2 stress than in the control conditions (Fig. 6E). Since the amount of intact RNAP complexes after 1 h exposure to H_2O_2 was only one-fourth of that measured in standard growth conditions but the relative amount of RNAP-SigA holoenzyme was doubled, the H_2O_2 treatment can be estimated to have reduced the number of transcription initiation competent RNAP-SigA complexes to one-half of that present in standard conditions.

In accordance with an increased SigB content of cells (Fig. 6A), SigB became highly abundant in RNAP holoenzymes after 1h exposure to H_2O_2 (Fig. 6E). Although the SigD protein content of the cells only slowly and moderately increased upon H_2O_2 treatment (Fig. 6A), recruitment of SigD by RNAP core prominently increased after 1h of H_2O_2 treatment and remained high until the end of the experiment (Fig. 6E). Thus, the importance of SigB and SigD factors in H_2O_2 stress is clear; the abundancy and recruitment of SigB and SigD proteins increases in H_2O_2 stress (Fig. 6A and 6E) and strains containing only SigB or only SigD grew better in H_2O_2 stress than CS (Fig. 4C). Although the SigC and SigE contents of cells remained unchanged by H_2O_2 exposure, the content of RNAP holoenzyme with SigC or SigE increased upon H_2O_2 treatment but less prominently than those with SigB or SigD (Fig. 6E). Overall, the ratio of group 2 σ factors to the primary σ factors in RNAP holoenzyme increased upon H_2O_2 stress.

Discussion

The SigD factor plays major roles in singlet oxygen and high light stresses

The main source of singlet oxygen in cyanobacteria is assumed to be a reaction between the triplet state of PSII reaction center chlorophyll, originating from recombination reactions, and O_2 (for a review, see Fischer et al. 2013). SigD seems to

be important for cells to cope with singlet oxygen stress. The ΔsigBCE strain containing only SigD (Fig. 1) shows superior growth in singlet oxygen stress (Fig. 2D). The ΔsigBCE cells grow faster than CS also in high light (Fig. 2A) retaining higher phycocyanobilin and chlorophyll contents than CS (Fig. 2C). The *sigD* gene is upregulated in high light (Imamura et al. 2003b, Tuominen et al. 2003), which leads to increased formation of RNAP-SigD holoenzymes (Koskinen et al. 2016). Strains without SigD, in turn, grow poorly already in moderate light (Pollari et al. 2008) and if both SigD and SigB are missing, the repair of photodamaged PSII is compromised as cells are not able to efficiently upregulate the expression of the *psbA* genes encoding the D1 protein (Pollari et al. 2009).

The ΔsigCDE strain, containing only SigB, is characterized by a high carotenoid content (Figs. 2A and 3C) and grows quite well in singlet oxygen and high light stresses (Fig. 2D and 3A). Carotenoids quench singlet oxygen within the membrane (Fischer et al. 2013) and play an important role in the light. High carotenoid and low singlet oxygen contents of ΔsigCDE protect cells against the damaging reaction of photoinhibition (Hakkila et al. 2013) whereas low β-carotene content of a carotenoid synthesis mutant strain leads to high singlet oxygen content and rapid damage of PSII (Vajravel et al. 2016). In addition to the damaging reaction of photoinhibition, the PSII repair cycle is vulnerable to singlet oxygen (Nishiyama et al. 2004), as translation elongation factors EF-G (Kojima et al. 2007) and EF-Tu (Jimbo et al. 2018) are vulnerable to oxidative stress. Thus, the rapid loss of PSII activity in ΔsigBCD, ΔsigBCE and ΔsigBDE (Pollari et al. 2011) might be due to PSII repair problems, as the carotenoid contents of these strains are low (Fig. 1A).

The ΔsigBCD strain loses carotenoids faster and chlorophylls and phycocyanin similarly as the quadruple mutant in high light (Figs. 3B and 3C), suggesting that the expression of SigE-activated genes might have a negative impact in high light. SigE activates sugar catabolic reactions in darkness (Osanai et al. 2005, Osanai et al. 2011) and in some stress conditions like nitrogen starvation (Osanai et al. 2006). The activity of the SigE factor is controlled mostly post-translationally. In the light, the H subunit of the Mg chelatase acts as an anti-σ factor for SigE in addition to its well-known function in chlorophyll synthesis (Osanai et al. 2009). Due to regulation by the anti-σ factor, formation of RNAP-SigE holoenzyme is rapid in darkness, slow in normal growth light conditions and goes further down in high light (Koskinen et al. 2016), allowing expression of sugar catabolic genes mainly in darkness. In ΔsigBCD, the high SigE content, the missing competition by the other group 2 σ factors, and probably a low content of the anti-σ factor (*chlH* is down-regulated at transcriptional level in high light, (Hakkila et al. 2013) obviously mix up normal regulation.

The group 2 σ factors only moderately respond to superoxide stress

The σ factor mutant strains respond differently to externally induced superoxide and H_2O_2 stresses (Figs. 4-6), suggesting that superoxide and H_2O_2 exert specific effects. The fact that H_2O_2 was administered externally whereas methyl viologen treatment induces superoxide production inside the cells, might provide an alternative explanation. However, H_2O_2 is known to diffuse through some aquaporins (Bienert and Chaumont. 2014, Tamma et al. 2018) suggesting that this simple explanation might not be correct.

The overall responses of σ factors and the RNA polymerase are only moderate in superoxide stress (Figs. 5), suggesting that superoxide does not function as an efficient signaling molecule. The growth experiments indicated that the the SigB factor is important (Fig. 4B), and some increase of the SigB protein was detected upon methyl viologen treatment of cells but the cellular content of the RNAP-SigB holoenzyme remained constant (Fig. 5). Further studies are required to see whether the formation of the RNAP-SigB holoenzyme was accelerated upon methyl viologen treatments, but so slow that our treatments were not long enough to detect it, or if low RNAP-SigB holoenzyme can guarantee expression of genes required for acclimation to superoxide stress. Particularly notable is that SigD induction is completely missing and overproduction of SigD in ΔsigBCE does not provide superior protection towards superoxide stress, contrary to H_2O_2, high light and singlet oxygen stresses.

When SigC is the only group 2 σ factor, cells do not grow in superoxide stress. The SigC factor has been suggested to regulate genes involved in the stationary phase (Asayama et al. 2004) and a high RNAP-SigC holoenzyme content due to missing regulatory 6S RNA (Heilmann et al. 2017) or missing competition by other group 2 σ factors in ΔsigBDE strain (Antal et al. 2016) lengthens the lag period before cells resume active growth when nitrogen depleted cells are supplemented with nitrate. In the RNAP-His strain that contains all σ factors, contents of the RNAP-SigA, RNAP-SigB and RNAP-SigC holoenzymes are slightly up after 6 h of induction of superoxide stress (Fig. 5). In ΔsigBDE cells, when other group 2 σ factors are missing, formation of the RNAP-SigC might be more frequent than in the RNAP-His strain and lead to halted growth of ΔsigBDE.

Transcription machinery largely disassembles in H_2O_2 stress

Unlike superoxide stress, H_2O_2 added to the growth medium completely blocked growth of all strains for the first 12 h (Fig 4C) and reduced the RNAP content of cells, suggesting that the transcription capacity was lowered. Furthermore, the ratio of RNAP holoenzyme to RNAP core complex increases in H_2O_2, suggesting that the transcription initiation process takes a long time in H_2O_2 stress. Binding of RNAP holoenzyme to the promoter region forms a closed promoter complex that is

first converted to an open promoter complex and then after synthesis of first circa 9 nucleotides, RNAP conformation changes, the σ subunit is released and RNAP proceeds further to the elongation phase and leaves the promoter region (Browning and Busby 2016). Any of these transcription initiation steps might be delayed in H_2O_2 stress, either due to oxidative damage to the RNAP components or DNA, or due to an effect of H_2O_2 on yet unknown regulatory factor(s).

External H_2O_2 stress has been shown to reduce translation activity in *Synechocystis* (Nishiyama et al. 2001) and enhanced translation rate was detected in *Synechococcus elongatus* PCC 7942 cells overexpressing simultaneously SOD and catalase in high light (Sae-Tang et al. 2016). The production of transcription and translation machineries was recently shown to be co-regulated (Koskinen et al. 2018) and it is thus possible that also the amount of the translation machinery is reduced. Reduced gene expression and protein synthesis capacity in H_2O_2 stress impairs acclimation and might be one of the reasons why cyanobacteria are so sensitive to H_2O_2 that it can be used to eliminate toxic cyanobacterial blooms from lakes (Matthijs et al. 2012).

When SigB (ΔsigCDE) or SigD (ΔsigBCE) is the only remaining group 2 σ factor, the strain shows superior growth in external H_2O_2 stress (Fig. 4C). Recently H_2O_2 was suggested to function as a signaling molecule in cold stress (Fedurayev et al. 2018) and interestingly, strains that contain only SigB or SigD acclimate better to cold stress than those containing only SigC or SigE (Pollari et al. 2011). Similarly, strains containing SigB or SigD manage better in nitrogen deficiency than those containing only SigC or SigE (Antal et al. 2016). Both cold and nutrient deficiency cause an imbalance between harvested energy, photosynthetic electron flow and CO_2 assimilation, thus possibly inducing oxidative stress.

According to our results, construction of a strain that overproduces either SigB or SigD or both of them might be useful in biotechnology, especially if the product of a cyanofactory is potentially toxic to cells or if production conditions create a risk of oxidative stress. It has already been shown that a strain overexpressing SigB tolerates higher butanol concentrations than the control strain and this resistance was connected to the reduced ROS content of the mutant cells (Kaczmarzyk et al. 2014).

Conclusions

In standard growth conditions when the oxidative stress level is low, the group 2 σ factor less ΔsigBCDE strain grows well and the formation of RNAP holoenzymes with group 2 σ factors occurs but not in high quantities (Fig. 7A). In high light conditions, especially the formation of the RNAP-SigD holoenzyme is induced and the overproduction of SigD in the

ΔsigBCE strain leads to superior growth in high light (Fig. 7B) and in singlet oxygen stress. Contrary to that, formation of extra RNAP-SigE holoenzymes in high light is harmful as ΔsigBCD has an even more bleached phenotype than ΔsigBCDE (Fig. 7B). Group 2 σ factors are essential for acclimation to chemically induced superoxide stress, SigB being the most important one as it alone can support almost similar growth as all σ factors in the control strain (Fig. 7C). In addition to SigB, also the SigC factor is moderately upregulated in superoxide stress but an overdose of the stationary-phase-related SigC ceases the growth (Fig. 7C). In H_2O_2 stress, drastic decrease of RNA polymerase content of cells limits the transcription capacity of the cells, formation of RNAP-SigB and RNAP-SigD holoenzymes is strongly enhanced and overexpression of either SigB or SigD improves growth.

Materials and methods

Strains and growth conditions

The glucose tolerant *Synechocystis* sp. PCC 6803 (Williams 1988) was used as a control strain (CS). The construction of the group 2 σ factor triple inactivation mutants, ΔsigBCD, ΔsigBDE, ΔsigBCE and ΔsigCDE (Pollari et al. 2011), and the quadruple mutant, ΔsigBCDE (Koskinen et al. 2016) have been described earlier. The 30-ml cell cultures (OD_{730} was set to 0.1 in the beginning) were grown in 100-ml flasks in BG-11 medium supplemented with 20 mM HEPES-NaOH, pH 7.5, under continuous illumination with the photosynthetic photon flux density (PPFD) of 40 µmol $m^{-2}s^{-1}$ at 32°C in ambient CO_2, and the cultures were shaken at 90 rpm. The plates for triple inactivation strains were supplemented with kanamycin (50 µg ml^{-1}), chloramphenicol (10 µg ml^{-1}), streptomycin (20 µg ml^{-1}) and spectinomycin (10 µg ml^{-1}), and for the quadruple mutant nourseothricin (10 µg ml^{-1}) was added in addition. For the experiments, all strains were grown without antibiotics in liquid BG-11 medium.

For bright light experiments, the cells were grown in liquid cultures as described above, except that the PPFD was 350 µmol $m^{-2}s^{-1}$. To test external oxidative stress, singlet oxygen production was induced with 5 µM methylene blue, superoxide production with 4 µM methyl viologen, or the growth medium was supplemented with 0.2 mM H_2O_2 and cells were grown in standard growth conditions. Three independent biological replicates were measured.

For microaerobic conditions, 80-ml of cell cultures, starting with OD_{730} =0.1, were grown in Multi-Cultivator MC1000 (PSI Instruments). Cultures were continuously bubbled with a gas mixture (0.04% CO_2 and 99.6% N_2) with a flow rate of 75±2 mL/min, adjusted by measuring the gas inlet of each tube with a gas flow meter (Agilent technologies). Light intensity was set to PPFD of 500 µmol $m^{-2}s^{-1}$ using US-SQS/L submersible light sensor (Heinz Walz) and LI-250A light

meter (Li-COR). The temperature was 32°C. Three independent biological replicates were measured. Each cultivation tube was equipped with a Firesting TROXSP5 TRACE Oxygen Sensor Spotfor O_2 concentration detection with FireStingO2 optical oxygen meter (Pyro Science). The oxygen concentrations were 0.140±0.054% and 0.114±0.014% for CS and ΔsigBCDE, respectively.

In vivo absorbance measurements

The absorbance spectra were measured with OLIS CLARiTY 17 UV/VIS/NIR spectrophotometer (On Line Instrument Systems, Inc). Cells were grown for three days in the standard or high light conditions, and the cultures were diluted to OD_{730} = 1 before measurement.

Non-photochemical quenching

Cells (30 ml, OD_{730}=1) were concentrated to OD_{730}=2.5 and kept is standard growth conditions for 2 h. Then non-photochemical quenching was measured with PAM-2000 fluorometer using blue actinic light (500 nm shortpass filter) with the PPFD of 300 µmol photons m^{-2} s^{-1} as described earlier (Pollari et al. 2009).

DNA microarray analysis

The DNA microarray analysis was done in standard growth conditions as described earlier (Hakkila et al. 2013, Hakkila et al. 2014, Koskinen et al. 2016) using an 8×15 K custom designed cyanobacterium *Synechocystis* sp. PCC 6803 array (Agilent) (Eisenhut et al. 2007). At least three biological replicates were analyzed. DNA microarray data has been deposited to GEO in accession GSE117478 (ΔsigBCD, ΔsigBCE, ΔsigBDE), GSE50060 (CS and ΔsigCDE) and GSE69981 (ΔsigBCDE).

Analyses of protein content by western blotting

Cells (OD_{730} was 1, 30 ml) were harvested and then total proteins (Pollari et al. 2011) or soluble proteins were isolated. To isolate soluble proteins, isolated total proteins were centrifuged twice at 18 000 × g for 15 min and membrane pellets were discarded. Protein concentration was determined using a DC protein assay kit (Bio Rad).

Proteins were solubilized for 10 min at 75°C with Next gel® sample loading buffer (Amresco), separated with 10 % NEXT GEL® SDS-PAGE (Amresco), and transferred to Immobilon-P membrane (Millipore) with Trans-Blot® (BioRad)

according to the manufacturer's instructions. Proteins were detected using specific antibodies. The custom antibodies against the α and β subunits of the RNA polymerase (Gunnelius et al. 2014) and the σ factor antibodies against SigA, SigB, SigC, SigD and SigE (Gunnelius et al. 2010, Gunnelius et al. 2014, Koskinen et al. 2016) has been described earlier. Flv2 and Flv3 antibodies were generous gifts from Prof. Eva-Mari Aro and OCP from Prof. Diana Kirilovsky, and the KatG antibody (AS08374) was purchased from Agrisera. Primary antibodies were detected with the goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate (Zymed) and the CDP-star Chemiluminescence Reagent (PerkinElmer), and quantified with a FluorChem image analyzer (Alpha Innotech Corp). At least three independent biological replicates were analyzed.

Analyses of RNA polymerase holoenzymes

A His-tag was added to the γ subunit of the RNA polymerase core (Koskinen et al. 2016). Cells (OD_{730} was 1; 30 ml) were treated with 7.5 μM methylviologen, or 4 mM H_2O_2 in standard growth conditions for 0 min, 15 min, 1h, 2h, or 6 h. Cells were rapidly cooled to 4°C using pre-frozen centrifuge tubes, and harvested by centrifugation at 4500 × g for 6 min at 4°C. The cell pellet was resuspended into 1-ml of B/W buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 0.01% TWEEN-20, Protease Inhibitor Cocktail (Roche)) and cells were collected by centrifugation at 10 000 × g for 1 min. The supernatant was removed, the pellet volume of acid-washed 150-212 μm glass beads (Sigma), 370 μl of B/W buffer, 5 μl of RNase and DNase were added and the cells were broken by vortexing 8 x 1min at 4°C. Unbroken cells, cell debris and membranes were removed by centrifugations at 10 000 × g for 5 min and 18 000 × g for 15 min, respectively.

The concentration of soluble proteins was measured and 900 ng of proteins in 350 μl of W/B buffer was used for His-tag purification using DynebeadsTM His-tag Isolation and Pulldown (ThermoFisher) system. Samples were mixed with 50 μl of Dynabeads at room temperature for 5 min, the beads were collected with a magnet and washed 4 times with 600 μl of W/B buffer. RNAP complexes were released with 75 μl of Elution buffer (300 mM Imidazole, 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 0.01% TWEEN-20). To detect the σ factors, 7 μl (SigA), 15 μl (SigB, SigD, SigE) or 5 μl (SigC) of eluate was solubilized with Next gel® sample loading buffer, and proteins were separated on SDS-PAGE and analyzed with western blotting as described earlier (Koskinen et al. 2016). All membranes were re-probed with the α subunit of the RNA polymerase to calculate the final results as the relative amount of the particular σ factor per the amount of RNA polymerase core.

Supplementary data

Supplementary Dataset 1 contains microarray data for all genes studied.

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Disclosures

Conflicts of interest: No conflicts of interest declared

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Figure legends

Table 1. Expression of selected ROS protection related genes in *Synechocystis* sp. PCC 6803 group 2 σ factor mutants in standard growth conditions.

Gene		ΔsigBCDE		ΔsigCDE		ΔsigBDE		ΔsigBCE		ΔsigBCD	
		FC	p-value	FC	p-value	FC	p-value	FC	p-value	FC	p-value
α-tocopherol synthesis											
slr0090		-0.97	0.0058	-0.29	0.4350	-0.42	0.1855	-0.35	0.3570	-0.25	0.4390
slr1736	*HPT1*	-0.24	0.4392	-0.76	0.0205	-0.74	0.0295	-0.05	0.8900	-0.14	0.6141
slr1737		-0.90	0.0001	-0.68	0.0046	-0.81	0.0028	-0.10	0.6366	-0.36	0.0352
sll0418		-0.40	0.0202	0.16	0.4543	-0.64	0.0106	0.04	0.8900	-0.82	0.0012
slr0089	*VTE4*	-1.71	0.0000	-0.59	0.0140	-1.78	0.0001	-0.02	0.9363	-0.71	0.0030
Carotenoid synthesis											
slr0739	*crtE*	0.35	0.1048	0.25	0.3389	0.35	0.0821	0.42	0.1307	0.56	0.0194
slr1255	*crtB*	0.95	0.0011	0.24	0.4131	0.59	0.0205	0.21	0.5439	0.33	0.2007
slr1293	*crtD*	0.76	0.0014	-0.09	0.7684	0.67	0.0150	-0.15	0.5701	0.08	0.7117
slr1254	*crtP*	0.79	0.0071	0.01	0.9915	0.57	0.0541	0.03	0.9478	0.73	0.0190
slr0940	*crtQ*	0.37	0.1316	-0.35	0.2770	0.39	0.1419	0.16	0.7008	0.55	0.0634
sll0033	*crtH*	-0.40	0.0275	-0.20	0.3485	-0.32	0.1084	0.52	0.0681	-0.25	0.3020
slr0088	*crtO*	0.26	0.0930	0.16	0.4028	0.35	0.0783	-0.27	0.2376	-0.14	0.4204
sll1468	*crtR*	0.13	0.4344	-0.01	0.9709	0.37	0.1337	0.37	0.1983	0.40	0.0376
Flavodiiron proteins											
sll0217	*flv4*	-0.10	0.8509	0.59	0.4557	0.83	0.1355	1.98	0.0402	0.08	0.9177
sll0218		-0.36	0.5430	0.73	0.4435	0.68	0.2982	2.03	0.0479	0.00	0.9980
sll0219	*flv2*	-0.40	0.5496	0.75	0.4940	0.42	0.5712	2.10	0.0494	-0.45	0.6239
sll1521	*flv1*	-0.47	0.0252	-0.22	0.4079	0.01	0.9675	-0.12	0.7344	0.18	0.4175
sll0550	*flv3*	-1.36	0.0004	-1.13	0.0073	-0.62	0.0371	-0.45	0.2722	-0.76	0.0182
Orange carotenoid protein											
slr1963	*ocp*	-0.04	0.8781	0.09	0.8061	0.08	0.7876	0.18	0.6578	1.78	0.0000
slr1964	*frp*	-0.01	0.9814	0.02	0.9540	-0.16	0.4635	0.79	0.0226	1.17	0.0014
Antioxidant enzymes											
slr1516	*sodB*	0.16	0.2606	0.21	0.2736	0.14	0.3640	-0.17	0.4969	0.92	0.0012
sll1987	*katG*	-0.60	0.0017	-0.32	0.0773	-0.01	0.9448	-0.22	0.2855	0.27	0.0968
slr1171	*gpx1*	-1.59	0.0000	-0.14	0.5314	-1.56	0.0001	0.19	0.4348	-1.40	0.0000
slr1992	*gpx*	0.02	0.9287	0.11	0.7011	0.24	0.4155	-0.01	0.9771	0.44	0.0481
sll0755	*2-cys prx*	0.34	0.0714	0.45	0.0248	0.31	0.0605	-0.31	0.1934	-0.15	0.3243
slr1198	*1-cys prx*	-0.75	0.0050	0.29	0.1568	-0.45	0.0389	-0.28	0.2236	0.02	0.9414
sll0221	*prxQ-B2*	-1.26	0.0002	-0.17	0.6182	-0.85	0.0043	0.14	0.6667	-0.81	0.0060
slr0242	*prxQ-B1*	-0.57	0.0331	0.03	0.9420	-0.59	0.0589	0.19	0.7339	-0.43	0.1607
sll1621	*type II prx*	1.11	0.0005	-0.01	0.9614	1.07	0.0007	0.62	0.0633	0.60	0.0197

The transcript level of each mutant strain is compared to that of the control strain. The fold changes (FC), expressed as $\log_2$ values, and the P values were calculated from microarray data obtained from at least three biological replicates.

Fig. 1. The amounts of primary and group 2 σ factors in the control and σ factor mutant strains. The soluble protein fraction was isolated from cells grown in standard conditions and the amounts of SigA (A) and group 2 σ factors (B) were measured with Western blotting. Three independent biological replicates were measured, representative western blots are shown and the values indicate the amount of each σ factor in the mutant strains compared to those measured in the control strain.

Fig. 2. Photoprotective mechanisms in σ factor mutants. (A) *In vivo* absorption spectra of CS, ΔsigBCDE, ΔsigCDE, ΔsigBDE, ΔsigBCE and ΔsigBCD in standard growth conditions. The red peak of Chl *a* (red arrow), the phycobilin peak (orange arrow) and the carotenoid peak (blue arrow) are indicated. (B) The amounts of the flavodiiron protein Flv2 and the orange carotenoid protein OCP. Proteins were isolated from cells grown in standard conditions, and 50 μg (Flv2) or 10 μg (OCP) of proteins were solubilized, separated by SDS-PAGE and proteins were detected with western blotting using specific polyclonal antibodies against Flv2 or OCP. The dashed line indicates that the last sample in the row was moved to the first place to keep the order of the samples same in the whole figure. (C) Non-photochemical quenching in mutant strains was measured with PAM-2000. (D) OD_{730} was set to 0.1 and cells were grown in standard conditions in BG-11 medium supplemented with 5 μM methylene blue to induce singlet oxygen production.

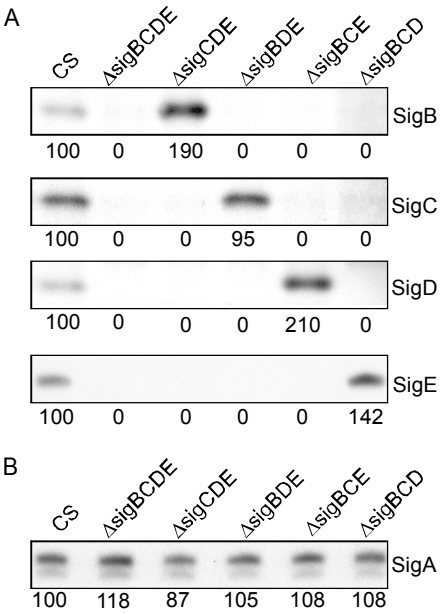
Fig. 3. Growth and pigment properties of the σ factor mutant strains in high light. (A) Cells were grown under constant illumination, PPFD 350 μmol $m^{-2}s^{-1}$, at 32 °C in ambient air. (B) Cultures grown three days at high light. (C) Absorption spectra of cells grown for three days in high light. (D) Growth of CS and ΔsigBCDE at PPFD 500 μmol $m^{-2}s^{-1}$ in microaerobic conditions.

Fig. 4. Abundance of Flv3 and KatG proteins in mutant strains, and their growth in superoxide and H_2O_2 stresses. (A) Proteins were isolated from cells grown in standard conditions, and 50 μg (Flv3) or 15 μg (KatG) of proteins were solubilized, separated by SDS-PAGE and proteins were detected with western blotting using specific polyclonal antibodies against Flv3 or KatG. The dashed line indicates that the last sample in the row was moved to the first place to keep the order of the samples same in the whole figure. (B) OD_{730} was set to 0.1 and cells were grown in standard conditions in BG-11 medium supplemented with 4 μM methyl viologen. (C) OD_{730} was set 0.1 and cells were grown in standard conditions in BG-11 medium supplemented with 0.2 mM H_2O_2.

Fig. 5. The σ factors and RNAP holoenzyme in superoxide stress. (A) Cells of the RNAP-His strain were exposed to 7.5 μM methyl viologen for 15 min, 1h, 2h or 6 h, soluble proteins were isolated, and the amounts of SigA and group 2 σ factors were measured with western blotting and compared to those measured in the standard conditions. (B) The amounts of RNAP core proteins α and β were measured with western blotting after exposure of RNAP-His strain to methyl viologen. (C) The RNAP complexes well pulled down after 15 min, 1 h, 2 h or 6 h treatment with 7.5 μM methyl viologen and the amount of each σ factor in RNAP was measured with western blotting and compared to the amount of the same σ factor in the standard conditions.

Fig. 6. The σ factors and RNAP holoenzyme in hydrogen peroxide stress. (A) Cells of the RNAP-His strain were exposed to 4 mM H_2O_2 for 15 min, 1 h, 2 h or 6 h, soluble proteins were isolated, and the amounts of SigA and group 2 σ factors were measured with western blotting and compared to those measured in the standard conditions. (B) The RNAP complexes were pulled down after exposure of RNAP-His strain to H_2O_2 for 15 min, 1h, 2h or 6 h., and the amounts of RNAP core proteins α, β and γ were measured with western blotting. (C) The amount of RNAP core subunits α and β after exposure of RNAP-His cells to H_2O_2 for 15 min, 1 h, 2 h or 6 h. (D) Relative amounts of soluble proteins in RNAP-His cells after 15 min, 1 h, 2 h or 6 h exposure to H_2O_2. (E) The RNAP complexes well pulled down after 15min, 1 h, 2 h or 6 h treatment with H_2O_2 and amounts of different σ factors were measured with western blotting and compared to the amount of the same σ factor in the standard conditions.

Fig. 7. Roles of group 2 σ factors in regulating oxidative stress responses. (A) Standard growth conditions (B) High light stress (C) Superoxide stress (D) H_2O_2 stress. The color of culture indicates growth of cells in stress conditions. The size of each σ factor indicates changes compared to the growth conditions, and for the mutant cultures, the σ factors present in each strain are shown. Part of growth conditions and high light results are taken from (Koskinen et al. 2016).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ΔsigCDE
ΔsigBDE
ΔsigBCE
ΔsigBCD
SigB
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SigC
100 0 0 95 0 0
SigD
100 0 0 0 210 0
SigE
100 0 0 0 0 142
B
CS
ΔsigBCDE
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ΔsigBDE
ΔsigBCE
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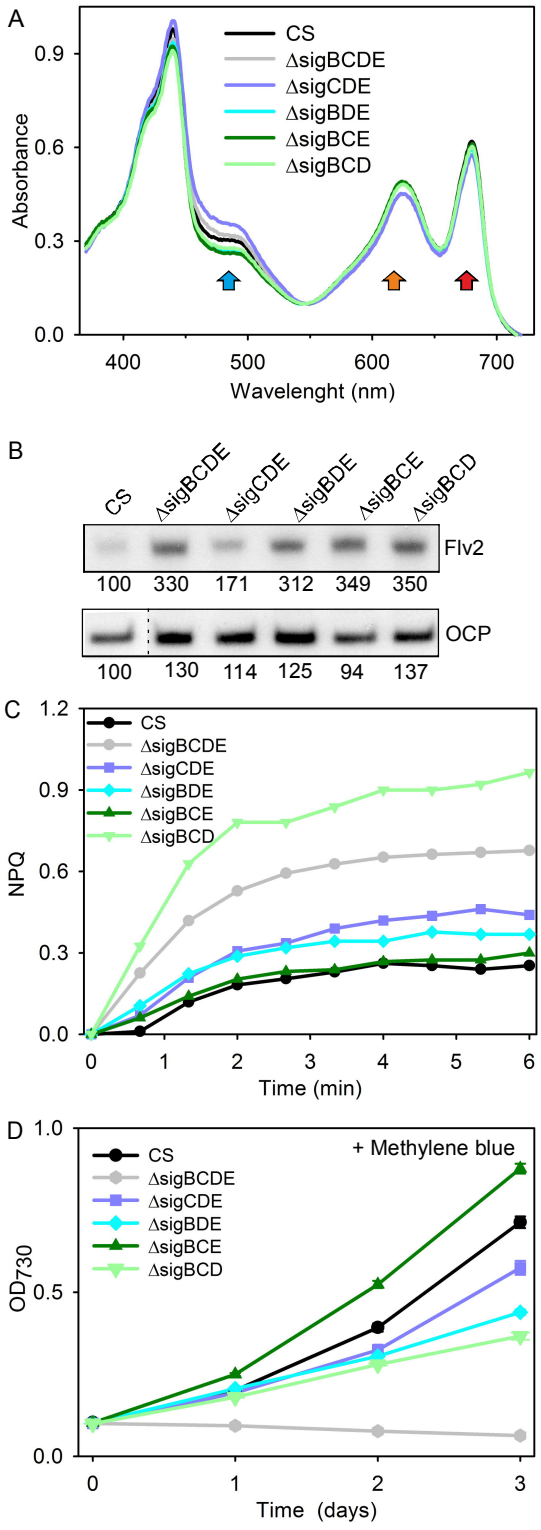
SigA
100 118 87 105 108 108

Figure 1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CS
ΔsigBCDE
ΔsigCDE
ΔsigBDE
ΔsigBCE
ΔsigBCD
Absorbance
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0.3
0.6
0.9
400
500
600
700
Wavelenght (nm)
B
CS
ΔsigBCDE
ΔsigCDE
ΔsigBDE
ΔsigBCE
ΔsigBCD
Flv2
100 330 171 312 349 350
OCP
100 130 114 125 94 137
C
CS
ΔsigBCDE
ΔsigCDE
ΔsigBDE
ΔsigBCE
ΔsigBCD
NPQ
0.0
0.3
0.6
0.9
1.2
0
1
2
3
4
5
6
Time (min)
D
+ Methylene blue
CS
ΔsigBCDE
ΔsigCDE
ΔsigBDE
ΔsigBCE
ΔsigBCD
OD730
0.0
0.5
1.0
0
1
2
3
Time (days)

Figure 2

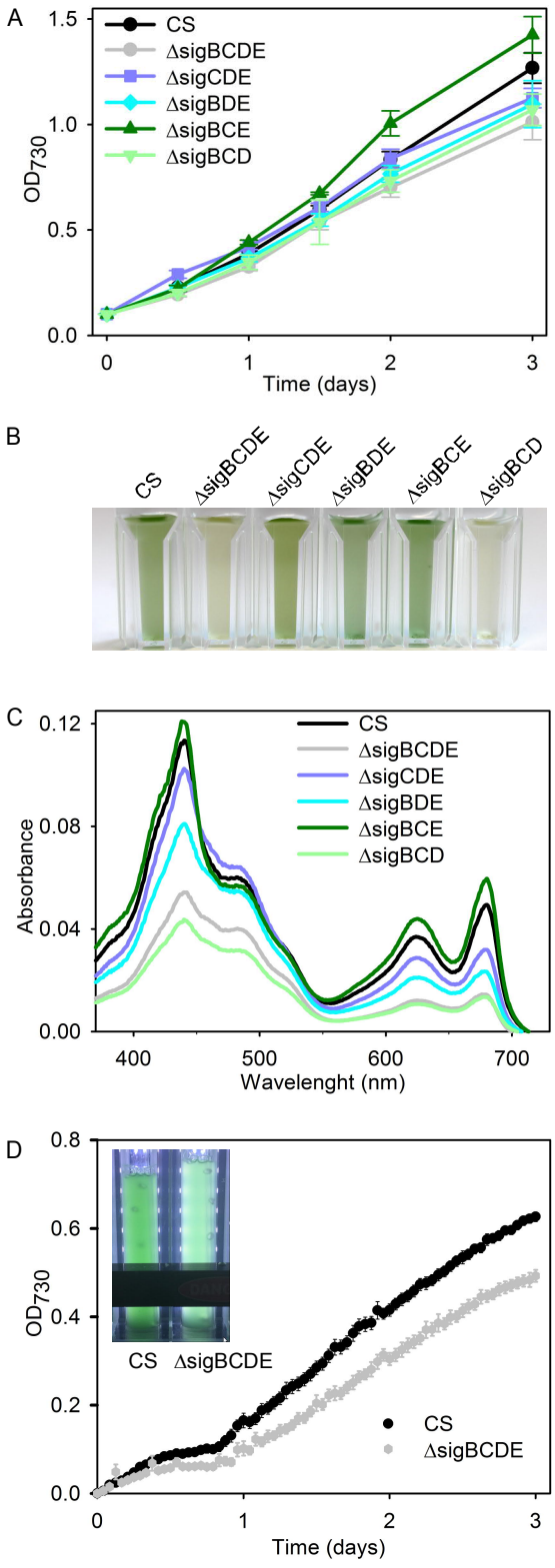
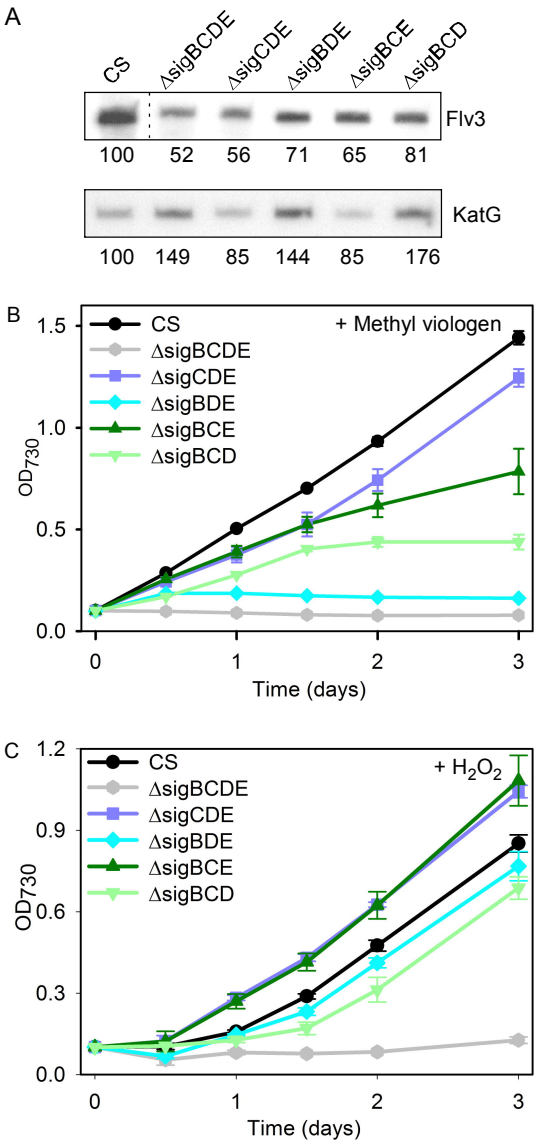

A
CS
ΔsigBCDE
ΔsigCDE
ΔsigBDE
ΔsigBCE
ΔsigBCD
OD730
Time (days)
B
CS
ΔsigBCDE
ΔsigCDE
ΔsigBDE
ΔsigBCE
ΔsigBCD
C
Absorbance
Wavelenght (nm)
D
OD730
CS ΔsigBCDE
Time (days)

Figure 3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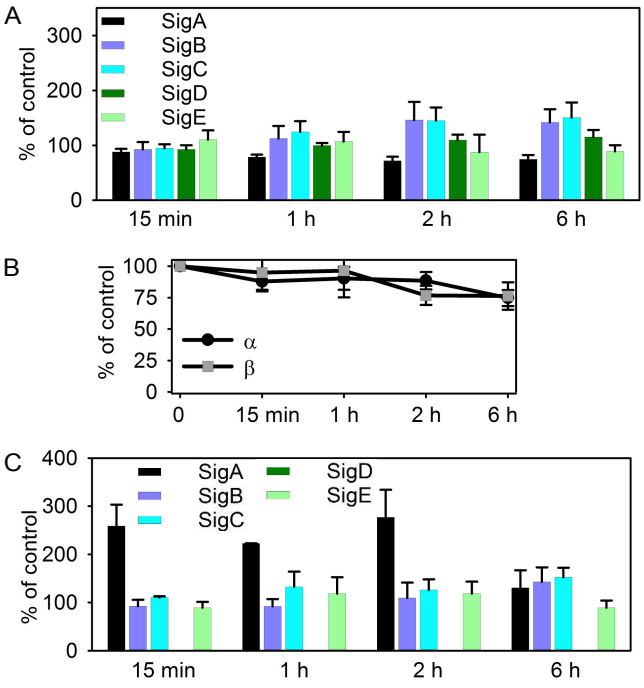
CS
ΔsigBCDE
ΔsigCDE
ΔsigBDE
ΔsigBCE
ΔsigBCD
Flv3
100 52 56 71 65 81
KatG
100 149 85 144 85 176
B
+ Methyl viologen
CS
ΔsigBCDE
ΔsigCDE
ΔsigBDE
ΔsigBCE
ΔsigBCD
OD730
Time (days)
C
+ H2O2
CS
ΔsigBCDE
ΔsigCDE
ΔsigBDE
ΔsigBCE
ΔsigBCD
OD730
Time (days)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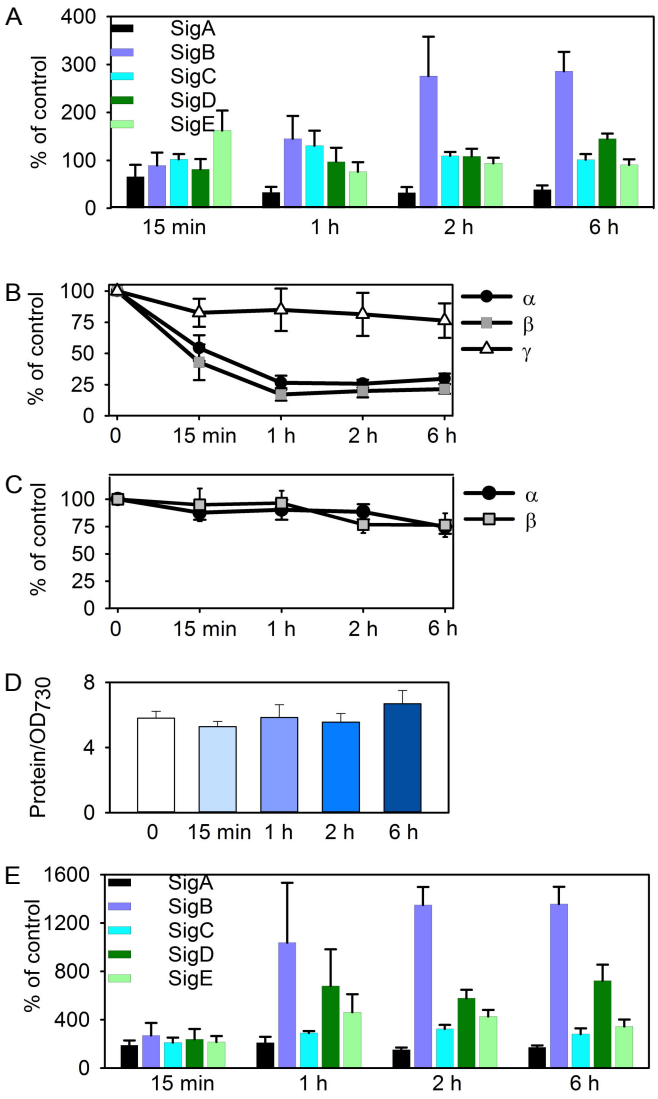
% of control
300
200
100
0
SigA
SigB
SigC
SigD
SigE
15 min
1 h
2 h
6 h
B
% of control
100
75
50
25
0
α
β
0
15 min
1 h
2 h
6 h
C
% of control
400
300
200
100
0
SigA
SigB
SigC
SigD
SigE
15 min
1 h
2 h
6 h

Figure 5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400
300
200
100
0
% of control
SigA
SigB
SigC
SigD
SigE
15 min
1 h
2 h
6 h
B
100
75
50
25
0
% of control
α
β
γ
0
15 min
1 h
2 h
6 h
C
100
75
50
25
0
% of control
α
β
0
15 min
1 h
2 h
6 h
D
8
4
0
Protein/OD730
0
15 min
1 h
2 h
6 h
E
1600
1200
800
400
0
% of control
SigA
SigB
SigC
SigD
SigE
15 min
1 h
2 h
6 h

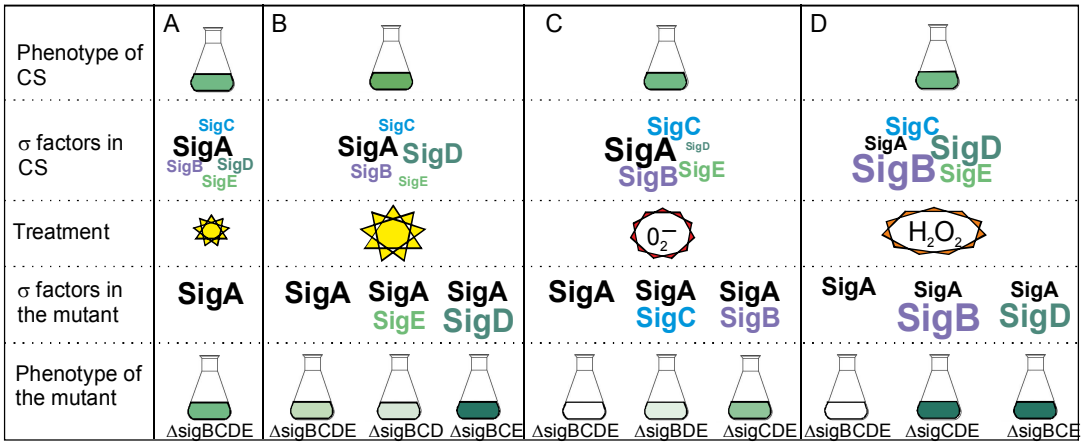
Phenotype of CS
σ factors in CS
Treatment
σ factors in the mutant
Phenotype of the mutant
A
B
C
D
SigC
SigA
SigB
SigD
SigE
O_2^-
H_2O_2
ΔsigBCDE
ΔsigBCDE
ΔsigBCD
ΔsigBCE
ΔsigBCDE
ΔsigBDE
ΔsigCDE
ΔsigBCDE
ΔsigCDE
ΔsigBCE

Figure 7.