Methylglyoxal detoxification by an aldo-keto reductase in the cyanobacterium *Synechococcus* sp. PCC 7002

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Received 26 January 2006

Revised 26 March 2006

Accepted 29 March 2006

Aldo-keto reductases (AKRs) are a superfamily of enzymes that reduce aldehydes and ketones, and have a broad range of substrates. An AKR gene, *sakR1*, was identified in the cyanobacterium *Synechococcus* sp. PCC 7002. A mutant strain with *sakR1* inactivated was sensitive to glycerol, a carbon source that can support heterotrophic growth of *Synechococcus* sp. PCC 7002. It was found that the *sakR1* null mutant accumulated more toxic methylglyoxal than the wild-type when glycerol was added to growth medium, suggesting that SakR1 is involved in the detoxification of methylglyoxal, a highly toxic metabolite that can damage cellular macromolecules. Enzymic analysis of recombinant SakR1 protein showed that it can efficiently reduce methylglyoxal with NADPH. Based on immunoblotting, SakR1 was not upregulated at an increased cellular methylglyoxal concentration. A pH-dependent enzyme-activity profile suggested that SakR1 activity could be regulated by cellular pH in *Synechococcus* sp. PCC 7002. The broad substrate specificity of SakR1 implies that SakR1 could play other roles in cellular metabolism.

INTRODUCTION

Cyanobacteria are a diverse group of prokaryotes that carry out oxygenic photosynthesis and grow photoautotrophically. Some cyanobacteria can also grow heterotrophically in the presence of organic substrates (Rippka *et al.*, 1979). The substrates that support heterotrophic growth of cyanobacteria include the sugars fructose and glucose. However, in the cyanobacterium *Synechococcus* sp. PCC 7002, glycerol is the only substrate that supports heterotrophic growth. A high concentration of exogenous glycerol is inhibitory to cyanobacterial growth, and it has been observed that glycerol-dependent growth of *Synechococcus* sp. PCC 7002 is only established after a period of adaptation to glycerol.

Although the pathway of glycerol metabolism has not been studied in cyanobacteria in detail, it is expected that it would be the same as that of other bacteria such as *Escherichia coli*, since, based on cyanobacterial genome sequences, cyanobacterial cells have all the genes to encode the enzymes required for glycerol metabolism (www.kazusa.or.jp/cyanobase; J. Zhao and D. A. Bryant, unpublished data). The inhibitory effect of glycerol to cyanobacterial growth could largely be due to toxic products of glycerol metabolism. One highly toxic product of cellular glycerol metabolism is methylglyoxal (Freedberg *et al.*, 1971), which is an electrophile and reacts with cellular macromolecules such as

proteins and DNA (Ferguson *et al.*, 1998). One of the pathways leading to methylglyoxal production in most cells is the formation of methylglyoxal from dihydroxyacetone phosphate (DHAP) enzymically or non-enzymically (Ferguson *et al.*, 1998; Kalapos, 1999). In most cells, detoxification of methylglyoxal is through glyoxalase I-II systems (Inoue & Kimmura, 1995; Kalapos, 1999). Another important mechanism is reduction of methylglyoxal by aldo-keto reductases (AKRs), which catalyse the formation of acetol from methylglyoxal (Kalapos, 1999).

AKRs are a large superfamily of related proteins that carry out NADPH-dependent reduction of various aldehydes and ketones (Jez *et al.*, 1997, 2001; Ellis, 2002). A common feature of the superfamily is that they share a (α/β)₈-barrel motif found in triose phosphate isomerase. The AKR superfamily consists of 14 families, based on their structures and sequences (Jez *et al.*, 2001). Although a detailed mechanism has been revealed for some of the AKRs, the physiological function of most putative members of this superfamily is still unclear, partly due to their broad substrate specificity and partly because of the difficulty of genetic analysis, as many organisms have multiple genes that encode AKRs. In bacteria, a small number of AKRs have been characterized (Ellis, 2002). For example, YghZ of *E. coli* has been shown to reduce methylglyoxal and enhance methylglyoxal resistance when the gene is overproduced (Grant *et al.*, 2003). Ko *et al.* (2005) recently showed that AKRs play an important role *in vivo* in methylglyoxal detoxification in *E. coli*.

Abbreviations: AKR, aldo-keto reductase; DHAP, dihydroxyacetone phosphate.

In the study of glycerol metabolism in the cyanobacterium *Synechococcus* sp. PCC 7002, we noticed that a transposon-generated mutant with an insertion in an ORF encoding a putative AKR was sensitive to glycerol. Here we show that this gene encodes an AKR and is required for methylglyoxal detoxification in *Synechococcus* sp. PCC 7002.

METHODS

Bacterial strains and growth conditions. *Synechococcus* sp. PCC 7002 was grown in A⁺ medium (Stevens *et al.*, 1973), either in liquid form or on 1.5% agar plates. When grown in liquid medium, *Synechococcus* sp. PCC 7002 was bubbled with air plus 1% CO₂ and illuminated with cool white fluorescent light at a light intensity of 100 µM photons m⁻² s⁻¹. When needed, kanamycin (100 µg ml⁻¹) or streptomycin (100 µg ml⁻¹) was added for growth of mutant strains of *Synechococcus* sp. PCC 7002. Growth measurement for *Synechococcus* sp. PCC 7002 in liquid medium was by monitoring OD₇₃₀. Growth measurement for *Synechococcus* sp. PCC 7002 on solid medium was based on colony size after 3 weeks incubation on agar plates. The plates with colonies were first recorded by a digital camera, and colony sizes were measured with CS-Tian software (Tian-neng). For photoheterotrophic growth, various amounts of glycerol from a 30% (w/v) stock were added to the growth medium, while the growth conditions were the same as those of photoautotrophic growth. *E. coli* was grown in LB medium at 37 °C. The strain DH5α was used for all routine cloning purposes, and strain BL21(DE3) was used for overproduction of recombinant protein.

Gene inactivation and overexpression. Total genomic DNA was isolated from *Synechococcus* sp. PCC 7002 in exponential growth with a kit from Omega DNA. Inactivation of the *Synechococcus* sp. PCC 7002 *sakR1* gene was performed as follows. A fragment containing *sakR1* was amplified from genomic DNA by PCR using primers 5'-AGCCGAGGAACTGTGCCATAGCAAC-3' and 5'-AAGGATCCGCTCCGGAGAAAACGCCGG-3'. The amplified fragment was cloned into pGEM-T (Promega). The resulting plasmid pSAKR1 was used as template for inverse PCR using primers 5'-AATTTGGCCCCGATCACCAG-3' and 5'-CAGAATTCCACGTTCCCGCTG-3'. The amplified fragment was digested with *HincII* and ligated with a DNA fragment containing the *npt* gene encoding resistance to kanamycin. The orientation of the *npt* gene was the same as that of *sakR1* so that it would not interfere with the transcription of *fesM* that is located downstream of *sakR1* (Xu *et al.*, 2005). The resulting plasmid was digested with *XhoI* and transformed into *Synechococcus* sp. PCC 7002, as previously described (Zhao *et al.*, 2001). Confirmation that the mutant was completely segregated was obtained by Southern hybridization, using a 0.6 kb fragment obtained by PCR with the primers 5'-CAGAATTCCACGTTCCCGCTG-3' and 5'-TTGGATCCCTGTTGGGGAGGGAGGGT-TTG-3', followed by random primer labelling with radioactive nucleotides. For complementation of the *sakR1* null mutant, a gene cartridge encoding streptomycin resistance (*Sm*) was first used to replace the *npt* gene on the shuttle plasmid pAQE19 that is a derivative of pAQE1 of *Synechococcus* sp. PCC 7002 (Buzby *et al.*, 1985), generating a new shuttle vector pAQE-Sm. A 1.5 kb fragment containing *sakR1* and its promoter region from pSAKR1 was inserted into pAQE-Sm at a *HindIII* site. The resulting plasmid pAQE-Sm-sakR1 was then transformed into the mutant by selection with streptomycin and kanamycin. The complemented strain was named AKm-c. For immunoblotting, anti-SakR1 antibodies were raised in a rabbit using recombinant SakR1 as antigen. Recombinant SakR1 and cellular extracts from *Synechococcus* sp. PCC 7002 wild-type and the AKm1 mutant were separated by SDS-PAGE, followed by blotting onto a nitrocellulose membrane. The proteins cross-reacting with

anti-SakR1 antibodies were detected with alkaline phosphatase conjugated to secondary antibodies, as described previously (Zhou *et al.*, 1998).

For overproduction of the SakR1 protein, *sakR1* was amplified from genomic DNA using the primers 5'-AACATATGACCCGCCACA-AAAACG-3' and 5'-TTCCTCGAGCTTACCAGTTCATAGGAG-3'. The amplified fragment was digested with *NdeI* and *XhoI* and cloned into pET15b (Novagen). The resulting plasmid pET-sakR1 was transformed into *E. coli* strain BL21(DE3). Expression of *sakR1* as a fusion gene was induced by IPTG at 0.5 mM for 12 h at 22 °C. The cells were collected by centrifugation and broken with a French press at 168 MPa. The cell extracts were centrifuged at 10 000 g for 5 min and the pellet was discarded. The supernatant was loaded onto a nickel-chelating Sepharose column (Amersham Biosciences), and the column was washed several times with phosphate/NaCl buffer (20 mM NaKPO₄, pH 7.4, 0.5 M NaCl), followed by elution in the same buffer containing various concentrations of imidazole from 50 to 500 mM. The His-tagged SakR1 was eluted at an imidazole concentration of 150 mM. The fractions containing the fusion protein were pooled and dialysed against phosphate buffer (20 mM NaKPO₄, pH 7.4). The His tag was then removed by digestion with thrombin. The correct N-terminal sequence of the recombinant SakR1 was confirmed by protein sequencing.

Enzymic analyses of SakR1. The aldehyde- and ketone-reducing activity of SakR1 was measured by monitoring the substrate-dependent absorption change of NADPH at 340 nm ($\epsilon = 6270 \text{ M}^{-1} \text{ cm}^{-1}$). Unless otherwise specified, the reactions were carried out at 25 °C in phosphate buffer (100 mM NaKPO₄, pH 6.0; initial NADPH concentration 0.2 mM). Values of K_m and k_{cat} were determined by the initial rates of enzymic reactions at different concentrations of substrate using the curve-fit software Dynafit (Biokin). Substrates for measurement of SakR1 activities were purchased from Sigma-Aldrich, and were used at the concentrations indicated in Table 1. For measurement of SakR1 activities in reducing methylglyoxal at different pHs, the following buffer systems were used. In the range

Table 1. Specific activities of recombinant SakR1

Substrate	Concentration (mM)	Specific activity (nmol min ⁻¹ mg ⁻¹)
Methylglyoxal	1	1278 ± 87
2-Nitrobenzaldehyde	1	1206 ± 101
4-Nitrobenzaldehyde	1	1091 ± 67
3-Nitrobenzaldehyde	1	1072 ± 93
Benzaldehyde	1	651 ± 66
Isopentaldehyde	1	641 ± 35
DL-Glyceraldehyde	1	507 ± 26
Diacetyl	1	373 ± 12
Succinic semialdehyde	1	225 ± 26
Ninhydrin	1	211 ± 15
Dihydroxyacetone	1	48 ± 15
Crotonaldehyde	1	47 ± 8
Isatin	1	47 ± 5
Acetaldehyde	1	38 ± 7
D-Xylose	50	660 ± 22
L-Arabinose	50	364 ± 32
D-Galactose	50	240 ± 31
D-Glucose	50	109 ± 6

pH 4.0–5.5, a sodium acetate/acetic acid system (100 mM) was used; in the range pH 6.0–7.0, a phosphate buffer system (100 mM) was used; in the range pH 7.5–9.0, a Tris/HCl system (100 mM) was used.

Analysis of cellular methylglyoxal. Cellular methylglyoxal concentration was determined as follows. A *Synechococcus* sp. PCC 7002 culture was grown until the OD₇₃₀ reached 1.5. The cells were harvested by centrifugation and resuspended in fresh A⁺ medium at an OD₇₃₀ of 1.5. The culture was then grown for 1 h at 37 °C at a light intensity of 100 µM photons m⁻² s⁻¹ before glycerol was added to a final concentration of 6 mM. Portions of the culture were collected and used for the measurement of methylglyoxal at the times indicated. The concentration of cellular methylglyoxal was determined according to Cordeiro & Freire (1996). To 20 ml of the culture mentioned above, 2 ml perchloric acid stock solution (5 M) was added and the solution was put in an ice/water bath for 10 min. The samples were centrifuged at 4 °C for 10 min at 12 000 g and immediately analysed by the methylquinoxaline method (Cordeiro & Freire, 1996). Final analyses were performed by HPLC using a C18 column from Vydac (4.6 nm × 250 nm, 5 mm). The mobile phase was 40 % (v/v) ammonium formate (25 mM, pH 3.4) and 60 % (v/v) methanol at a flow rate of 0.8 ml min⁻¹. Derivatives of methylglyoxal were monitored spectrophotometrically at 320 nm.

Other methods. Chlorophyll concentration was determined according to MacKinney (1941). Protein concentration was determined according to Peterson (1974). SDS-PAGE was performed according to Laemmli (1970). Sequence comparison and alignment were performed using CLUSTAL W (Thompson *et al.*, 1994).

RESULTS

Cloning and inactivation of *sakR1* encoding an AKR from *Synechococcus* sp. PCC 7002

During the study of glycerol metabolism in *Synechococcus* sp. PCC 7002, we isolated a transposon-generated mutant that, compared to the wild-type, showed sensitivity to glycerol at millimolar concentrations. Characterization of the mutant showed that the transposon was inserted into an ORF that encoded a putative AKR, and the gene was named *sakR1*. Fig. 1 shows a sequence alignment of the deduced SakR1 protein with those of several other closely related AKRs. The *sakR1* gene has two potential translation initiation sites. Based on the immunoblotting result (see Fig. 5B) and a prediction of codon usage preference in this organism (data not shown), we assigned the first ATG as the translation start site. The deduced SakR1 from *Synechococcus* sp. PCC 7002 has a high similarity to AKRs from other cyanobacteria and a moderately high similarity (57 %) to YhdN from *Bacillus subtilis* (Kunst *et al.*, 1997). It is shown in Fig. 1 that four key catalytic residues of SakR1, Asp60, Tyr65, Lys90 and His123, are conserved in *Synechococcus* sp. PCC 7002, as in AKRs from other species. Based on the sequences shown in Fig. 1, SakR1 from *Synechococcus* sp. PCC 7002 belongs to family 11 of the AKR superfamily and has been assigned the name AKR11B3 according to the AKR nomenclature system (Jez *et al.*, 1997, 2001; www.med.upenn.edu/akr). The enzyme activity of the *sakR1* gene product was confirmed using a recombinant protein (see below).

To further study *sakR1*, we reconstructed a mutant by partial deletion of the gene through a double recombination event. As shown in Fig. 2, the *npt* gene encoding kanamycin resistance replaced part of the coding sequence of *sakR1*, and the mutant was completely segregated, as confirmed by Southern hybridization. The mutant was named AKm1.

Characterization of AKm1

It has previously been observed that only a small portion of *Synechococcus* sp. PCC 7002 cells plated onto medium containing glycerol survives (J. Zhao and D. Xu, unpublished results). As mentioned above, the transposon-generated mutant of *sakR1* was more sensitive to glycerol. The strain AKm1 had the same phenotype as the transposon-generated mutant when plated onto glycerol-containing plates (Fig. 3). When the glycerol concentration reached 6 mM, approximately one in 300 cells formed colonies in the wild-type, while only one in 100 000 AKm1 cells formed colonies, showing that glycerol was more inhibitory to the growth of AKm1 than to that of the wild-type. Complementation of AKm1 strains with a wild-type *sakR1* gene on a shuttle vector restored the wild-type phenotype. The growth rates of the wild-type and AKm1 in A⁺ media were measured, and we observed that there was a longer lag time before exponential growth when AKm1 cells were transferred to new liquid medium in the absence of glycerol (Fig. 4A). However, once the mutant cells reached the exponential growth phase, their growth rate was similar to that of the wild-type. In contrast to the growth in liquid medium, AKm1 cells showed a much slower growth rate on solid medium. The mean size of AKm1 colonies was 55 % that of wild-type colonies after 3 weeks growth on solid medium (Fig. 4B).

To understand why AKm1 was sensitive to glycerol, we measured the cellular concentration of methylglyoxal, which in most organisms is a toxic intermediate of glycerol metabolism. As demonstrated in Fig. 5(A), when 6 mM glycerol was present in the growth medium of the wild-type, cellular methylglyoxal increased rapidly from 0.1 to 0.6 µM within 1 h. The methylglyoxal level then gradually declined to the initial level within 24 h. In AKm1, a similar but faster initial increase of methylglyoxal was observed after the addition of 6 mM glycerol. Unlike the wild-type, however, the cellular methylglyoxal level in AKm1 increased until it reached its highest level (2.5 µM) 24 h after the addition of glycerol. These results show that AKm1 is unable to efficiently detoxify methylglyoxal. Although intracellular methylglyoxal levels reached a maximum 1 h after the addition of glycerol and declined during the following 12 h in the wild-type, as demonstrated in Fig. 5(A), SakR1 protein levels did not increase during that period (Fig. 5B). Immunoblotting also showed that a protein cross-reacting with antibodies against recombinant SakR1 was absent in AKm1, confirming that the mutant was fully segregated. The molecular mass of the native protein detected by immunoblotting was very close to that of recombinant SakR1, supporting our assignment of the first ATG as the initiation codon shown in Fig. 1.

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The toxic effect of exogenous methylglyoxal to the growth of *Synechococcus* sp. PCC 7002 was studied and the results are shown in Fig. 6. The wild-type and AKm1 responded to exogenous methylglyoxal differently. The methylglyoxal concentrations in the growth medium that reduced growth rates by 50 % were 1.1 and 2.0 mM for AKm1 and the wild-type, respectively (Fig. 5B). At a concentration of 3 mM, exogenous methylglyoxal completely inhibited the growth of both strains.

To determine whether SakR1 had the ability to reduce methylglyoxal, we overproduced His-tagged SakR1 protein in *E. coli* (Fig. 7A). The His tag was later removed with thrombin. N-terminal sequencing of the recombinant SakR1 revealed that it had the sequence SGMTRQKNE-LMKTRQ, as predicted from the expression construction. The activities of the recombinant SakR1 were measured

Fig. 2. Physical map of *sakR1* of *Synechococcus* sp. PCC 7002 and characterization of AKm1 (*sakR1* mutant) by Southern blotting. (A) Schematic drawing of a physical map of *sakR1* and insertional mutagenesis of the gene. An *npt* cartridge was inserted into the *sakR1* gene, which was partially deleted by inverse PCR. H, restriction enzyme *Hind*III. (B) Southern blotting confirming that AKm1 is completely segregated. Total DNA from the wild-type (WT) and AKm1 was digested with *Hind*III and separated by electrophoresis before being transferred onto a nitrocellulose membrane. The molecular masses of the bands that hybridized with the labelled probe are indicated on the left.

with various substrates, and the results in Table 1 show that SakR1 was able to reduce a variety of aldehydes and ketones. These activities are comparable to those of *E. coli* YghZ (Grant *et al.*, 2003) and rat AKR7A1 (Ellis *et al.*, 1993). At a methylglyoxal concentration of 1 mM, SakR1 had a high methylglyoxal-reducing activity, while it had low reducing activities for sugars such as xylose and glucose. We also determined apparent kinetic constants of SakR1 with selected substrates (see Table 2). The affinity of SakR1 for methylglyoxal was quite high ($K_m = 80 \mu\text{M}$), suggesting that it could reduce methylglyoxal *in vivo* under physiological conditions. The highest ratio of k_{cat}/K_m was obtained with methylglyoxal as substrate, and the lowest ratios were obtained with sugar substrates such as galactose and glucose. The specificity (k_{cat}/K_m) of SakR1 to methylglyoxal was 5.9×10^5 , suggesting that an efficient reduction of methylglyoxal could be catalysed by SakR1. At concentrations of 50 mM, the k_{cat}/K_m ratios obtained with the sugars tested in

Fig. 3. c.f.u. of *Synechococcus* sp. PCC 7002 strains in the presence of various concentrations of glycerol. Cells in the late-exponential growth phase were diluted with A^+ medium and plated on solid medium containing different concentrations of glycerol. Colonies were counted after 2 weeks growth. The values for all points were normalized by comparing them with those obtained in the absence of glycerol. ●, wild-type; ■, AKm1; ▲, AKm-c, which is a strain of AKm1 containing a wild-type *sakR1* gene on a shuttle plasmid. Error bars show SD.

Table 2 were low, suggesting that these sugars were unlikely to be the substrates of SakR1 *in vivo*. The pH-dependent enzymic activity of SakR1 was also determined (Fig. 7B). The highest activity was obtained at pH 6; however, the enzymic activity declined rapidly when pH values were higher than 7.5 or lower than 5.

DISCUSSION

Among cyanobacteria that can grow heterotrophically with organic substrates, *Synechococcus* sp. PCC 7002 is the only species known to utilize glycerol efficiently (Rippka *et al.*, 1979). Glycerol metabolism in many organisms leads to an increase of cellular methylglyoxal, which is a toxic by-product of carbon metabolism (Freedberg *et al.*, 1971; Burke & Tempest, 1990; Russell & Cook, 1995). Methylglyoxal production has been found in nearly all cells. The major route of methylglyoxal production related to glycerol metabolism is the methylglyoxal synthase pathway (Hopper & Cooper, 1971). In *Synechococcus* sp. PCC 7002 and most other cyanobacteria, genes encoding methylglyoxal synthases are missing, based on BLAST searches of cyanobacterial genome sequences. It is speculated that the production of methylglyoxal from glycerol in *Synechococcus* sp. PCC 7002 (Fig. 5) is by a non-enzymic reaction from DHAP (Phillips & Thornalley, 1993). The highly toxic methylglyoxal is removed promptly from cells by glyoxalase detoxification pathways and through AKRs that convert methylglyoxal to acetol (Ferguson *et al.*, 1998; Kalapos, 1999; Booth *et al.*, 2003).

Fig. 4. Growth of *Synechococcus* sp. PCC 7002 wild-type and AKm1 in A⁺ liquid medium (A) and on agar plates (B). Cells from a culture in A⁺ medium were transferred to fresh liquid or solid A⁺ media with appropriate dilutions. (A) The growth of the wild-type (●) and AKm1 (■) at 35 °C was measured by monitoring OD₇₃₀. Semi-log plots of the data in the first 24 h of growth of both strains are shown in the inset. (B) The growth of the wild-type (solid bars) and AKm1 (open bars) on solid A⁺ medium at 33 °C was measured by colony size using a digital recorder and analysed by CS-Tian software, as described in Methods. Data are the means of three separate measurements; error bars show SD. Colony size is expressed as unit area, and one unit area is equal to 0.064 mm².

There are several lines of evidence that show that SakR1 is responsible for methylglyoxal detoxification. The strain AKm1 is more sensitive to glycerol (Fig. 3), suggesting that the gene product is involved in the protection of cells from toxic metabolites of glycerol. In *E. coli*, an uncontrolled carbon metabolism results in an increase in cellular methylglyoxal (Kim *et al.*, 2004), and AKRs are required for its detoxification (Ko *et al.*, 2005). The addition of glycerol to the growth medium induces a drastic increase in cellular methylglyoxal concentration in AKm1 (Fig. 5), while the glycerol-induced increase of the cellular methylglyoxal concentration in the wild-type is only transient and the methylglyoxal levels return to normal within 1 h. Both the wild-type and the AKm1 mutant were quite resistant to

Fig. 5. Production of intracellular methylglyoxal after addition of glycerol to the growth medium, and immunoblotting of SakR1. (A) Intracellular concentration of methylglyoxal (MG) as a function of time, after the addition of glycerol to the growth medium. To cultures of *Synechococcus* sp. PCC 7002 wild-type (●) and AKm1 (■), glycerol was added to a final concentration of 6 mM, and their growth was continued. Cells were collected by centrifugation at the times indicated and their intracellular methylglyoxal concentration was determined. Error bars show SD. (B) Immunoblotting of native SakR1 with anti-SakR1 antibodies in *Synechococcus* sp. PCC 7002. Lane 1, recombinant SakR1; lane 2, total cell extract from AKm1; lanes 3–6, total cellular extracts from the wild-type after exposure to glycerol for 0, 1, 3 and 12 h. Molecular mass (in kDa) is shown on the left.

exogenous methylglyoxal. The growth of both strains was normal at methylglyoxal concentrations in the growth medium of less than 1 mM (Fig. 6), indicating that *Synechococcus* sp. PCC 7002 has an effective mechanism to protect its cells from exogenous methylglyoxal. However, at higher concentrations of methylglyoxal in the growth medium, the growth of AKm1 was more severely inhibited than that of the wild-type. These results provide evidence that SakR1 is involved in the detoxification of methylglyoxal in *Synechococcus* sp. PCC 7002. This suggestion is in agreement with other reports that AKRs are involved in methylglyoxal reduction in other organisms (Wermuth *et al.*, 1977;

Fig. 6. Inhibition of growth by exogenous methylglyoxal. The growth of the wild-type (●) and AKm1 (■) in the presence of exogenous methylglyoxal at the concentrations indicated was measured. The cultures were inoculated at an initial optical density of 0.05 and were grown for 28 h before final optical densities were measured. Each point represents the mean of five individual measurements; error bars show SD.

O'Connor *et al.*, 1999; Hinshelwood *et al.*, 2002; Ko *et al.*, 2005). Compared with other AKRs (Ellis, 2002), recombinant SakR1 has a high affinity for methylglyoxal and its catalytic efficiency in reducing methylglyoxal is moderately high, supporting the proposal that it plays an important role in methylglyoxal detoxification *in vivo*.

One interesting observation is that AKm1 has a slower rate of growth on solid medium than the wild-type, while it has a similar growth rate to that of the wild-type in liquid medium. We speculate that there is some methylglyoxal production in *Synechococcus* sp. PCC 7002 even in the absence of exogenous glycerol, and that excretion of methylglyoxal, which is also a detoxification mechanism in other bacteria (Tempest & Neijssel 1984; Baskaran *et al.*, 1989; Russel, 1993), is not as efficient on solid medium as in liquid medium.

The transient increase of methylglyoxal in the wild-type after addition of glycerol to the growth medium (Fig. 5) suggests that the detoxification system is regulated. Because the amount of SakR1 remains unchanged by the increased methylglyoxal concentration (Fig. 5B), we speculate that there exists a mechanism of regulating enzyme activity. The profile of enzyme activities at different pH values (Fig. 7B) shows that SakR1 activity increases nearly fivefold from pH 8 to 7. This suggests that cells could increase SakR1 activity by lowering cytosolic pH when cellular methylglyoxal concentration increases, a strategy adopted by many organisms (Ferguson *et al.*, 1998).

The function of many putative AKRs in various organisms is unclear. This is largely due to their broad substrate

Fig. 7. Overproduction of recombinant SakR1 and its pH-dependent activity in the reduction of methylglyoxal. (A) SDS-PAGE analysis of recombinant SakR1 produced in *E. coli*. Lane 1, molecular mass standards (kDa); lane 2, total cellular extracts of *E. coli* overproducing His-tagged SakR1; lane 3, His-tagged SakR1 affinity-purified by an Ni-chelating column; lane 4, purified recombinant SakR1 with His tag removed by thrombin. (B) pH-dependent activity of recombinant SakR1 in the reduction of methylglyoxal with NADPH. Sodium acetate/acetic acid (100 mM), phosphate (NaKPO₄) (100 mM) and Tris/HCl (100 mM) were used for the pH ranges 4.0–5.5, 6.0–7.0 and 7.5–9.0, respectively. Enzyme activities are expressed as a percentage of that at pH 6.0 (100%).

specificity and to the difficulty in performing genetic analysis, because many organisms have multiple genes encoding putative AKRs that could be functionally redundant. For example, *E. coli* has six AKR genes (Blattner *et al.*, 1997), while yeast has 14 (Goffeau *et al.*, 1996). Our survey shows that the number of AKR genes in cyanobacteria varies greatly. *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 6301 and *Nostoc punctiforme* have four, two and 21 potential AKR genes, respectively. There are four potential AKR genes in *Synechococcus* sp. PCC 7002, based on its genomic

Table 2. Apparent enzymic kinetic constants of recombinant SakR1

Substrate concentrations and enzymic reaction conditions are the same as those in Table 1.

Substrate	k_{cat} (min^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1} \text{min}^{-1}$)
Methylglyoxal	50.8 ± 1.7	0.08 ± 0.011	5.9×10^5
2-Nitrobenzaldehyde	78.6 ± 3.6	0.31 ± 0.03	2.6×10^5
3-Nitrobenzaldehyde	66.9 ± 1.6	0.91 ± 0.57	2.2×10^5
4-Nitrobenzaldehyde	74.6 ± 5.9	0.85 ± 0.12	8.8×10^4
DL-Glyceraldehyde	31.1 ± 3.2	0.56 ± 0.12	5.5×10^4
Benzaldehyde	62.7 ± 15.5	1.69 ± 0.70	3.7×10^4
Isopentaldehyde	38.9 ± 3.8	1.33 ± 0.32	2.9×10^4
D-Xylose	72.9 ± 6.0	104 ± 14	7.0×10^2
L-Arabinose	76.4 ± 3.2	234 ± 13	3.3×10^2
D-Galactose	40.8 ± 15.3	160 ± 24	2.6×10^2
D-Glucose	36.9 ± 14.5	445 ± 102	8.0×10

sequence. Besides recombinant SakR1, we have obtained a soluble recombinant protein encoded by another potential AKR gene. However, we did not detect any enzyme activity with this recombinant protein using NADPH and the substrates listed in Table 1 (Xu and Zhao, unpublished results). The function of the other three putative AKRs in *Synechococcus* sp. PCC 7002 is therefore at present unknown.

The detoxification of methylglyoxal by SakR1 in *Synechococcus* sp. PCC 7002 suggests that the AKRs of other cyanobacteria could have a similar function in the removal of methylglyoxal. The broad substrate range of SakR1 also suggests that it is involved in other biochemical reactions. Further study is required to understand the roles that AKRs play in *Synechococcus* sp. PCC 7002 and other cyanobacteria.

ACKNOWLEDGEMENTS

We would like to thank Dr Trevor M. Penning of the Department of Pharmacology, University of Pennsylvania, for analysis and assignment of the *sakR1* gene. This research was supported by the Ministry of Science and Technology of China (2004AA626021) and by the Natural Science Foundation of China (30230040).

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