

Response to Oxidative Stress Involves a Novel Peroxiredoxin Gene in the Unicellular Cyanobacterium *Synechocystis* sp. PCC 6803

Mari Kobayashi¹, Tomokazu Ishizuka¹, Mitsunori Katayama¹, Minoru Kanehisa², Maitrayee Bhattacharyya-Pakrasi³, Himadri B. Pakrasi³ and Masahiko Ikeuchi^{1,4}

¹ Department of Life Sciences (Biology), The University of Tokyo, Komaba, Meguro, Tokyo, 153-8902 Japan

² Institute for Chemical Research, Kyoto University, Uji, Kyoto, 611-0011 Japan

³ Department of Biology, Washington University, St. Louis, MO 63130-4899, U.S.A.

Exposure to methyl viologen in the presence of light facilitates the production of superoxide that gives severe damage on photosynthetic apparatus as well as many cellular processes in cyanobacteria and plants. The effects of methyl viologen on global gene expression of a unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 were determined by DNA microarray. The ORFs *sll1621*, *slr1738*, *slr0074*, *slr0075*, and *slr0589* were significantly induced by treatment of methyl viologen for 15 min commonly under conditions of normal and high light. One of these genes, *slr1738*, which encodes a ferric uptake repressor (Fur)-type transcriptional regulator, is located divergently next to another induced gene, *sll1621*, in the genome. We deleted *slr1738*, and compared the global gene expression patterns of this mutant to that of wild type under non-stressed conditions. It was found that *sll1621* was derepressed to the greatest extent, while many other genes including *slr0589* but not *slr0074* or *slr0075* were derepressed to lesser extent in the mutant. Genetic disruption of *sll1621*, which encodes a putative type 2 peroxiredoxin, indicates that it is essential for aerobic phototrophic growth in both liquid and solid media in high light and on solid medium even in low light. Slr1738 was prepared as a His-tagged recombinant protein and shown to specifically bind to the intergenic region between *sll1621* and *slr1738*. The binding was enhanced by dithiothreitol and abolished by hydrogen peroxide. We concluded that the Fur homolog, Slr1738, plays a regulatory role in the induction of a potent antioxidant gene, *sll1621*, in response to oxidative stress.

Keywords: Cyanobacterium — DNA microarray — Methyl viologen — Oxidative stress — Peroxiredoxin — Transcriptional regulator.

Abbreviations: DTT, dithiothreitol; Fur, ferric uptake repressor; IPTG, isopropyl- β -D-thiogalactoside; ROS, reactive oxygen species.

Introduction

When aerobic organisms respire to gain energy aerobically, they cannot avoid oxidative stresses due to reactive oxygen spe-

cies (ROS), which are often derived from the recombination of oxygen and electrons on the respiratory chain. Oxygenic photosynthetic organisms suffer even more stress: they generate both oxygen and highly reducing equivalents during active photosynthesis. Oxygen concentrations are expected to be greater in the vicinity of the photosynthetic apparatus than near the respiratory apparatus. Initial reductants of photosynthetic electron transport have lower redox potentials than those in respiration. Thus, we expect that the mechanisms to protect oxygenic photosynthetic organisms from oxidative stresses must be more critical than that in aerobic heterotrophic organisms.

To cope with the oxidative stresses, cyanobacteria and plants appear to have developed a number of antioxidant systems. Recent genome projects of cyanobacteria and plants confirm this and also revealed the presence of genes encoding key components that respond to oxidative stresses, whose roles have been established in other organisms. For example, cyanobacteria and plants have multiple copies of putative thioredoxin genes: nine copies in *Anabaena* (a filamentous cyanobacterium), more than 30 copies for *Arabidopsis* (higher plant), versus six copies for humans and three copies each for *Escherichia coli* and yeast (Kaneko et al. 2001, Arabidopsis Genome Initiative 2000, Goffeau et al. 1996, Spyrou et al. 2001). Genome analysis also revealed the absence of a canonical ascorbate peroxidase in the cyanobacterial genomes, although it seems to be one of the most crucial peroxidases in plant chloroplasts (Asada 2000).

The unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 has been a model organism for gene disruption analysis, DNA microarray analysis and proteomic analysis (Ikeuchi and Tabata 2001, Hihara et al. 2001, Sazuka et al. 1999), since its genome sequence was determined in 1996 (Kaneko et al. 1996). One can assign many putative antioxidant genes that may be involved in protection against oxidative stress based upon gene sequence analysis. They encode superoxide dismutase, catalase-peroxidase, peroxiredoxins (thioredoxin peroxidase), glutathione peroxidase, and thioredoxins. Gene disruption analysis revealed that catalase-peroxidase (*sll1987*) was dispensable for normal growth but is critical for survival when H₂O₂ is added at sublethal concentrations in a growth medium (Tichy and Vermaas 1999). Gene disruption has also

⁴ Corresponding author: Email, mikeuchi@bio.c.u-tokyo.ac.jp; Fax, +81-3-5454-4337.

Table 1 Genes induced by methyl viologen under normal light conditions

Gene : Function ^a	Induction ratio ^b
<i>slr0589</i> : hypothetical protein ^c	2.77±0.09
<i>slr1738</i> : transcription regulator Fur family ^c	2.63±0.24
<i>sll1165</i> : DNA mismatch repair protein	2.62±0.82
<i>sll0865</i> : excinuclease ABC subunit C (uvrC)	2.59±0.29
<i>sll1621</i> : AhpC/TSA family protein ^c	2.57±0.13
<i>slr1474</i> : hypothetical protein	2.37±1.15
<i>slr2107</i> : probable polysaccharide ABC transporter permease protein	2.25±0.41
<i>slr0074</i> : ABC transporter subunit (ycf24) ^c	2.24±0.39
<i>ssr1038</i> : unknown protein	2.13±1.14
<i>slr0075</i> : ABC transporter ATP-binding protein (ycf16) ^c	2.07±1.14
<i>slr1421</i> : unknown protein	2.05±0.44

^a Function description of CyanoBase (<http://www.kazusa.or.jp/cyano/>).

^b Ratio of transcript levels in the treated cells to those in the non-treated cells.

^c Induced genes common to the conditions of normal and high light.

showed that a typical peroxiredoxin encoded by *sll0755* was critical for growth under high light conditions (Yamamoto et al. 1999, Klughammer et al. 1998). No other putative antioxidant genes have been analyzed by gene disruption so far.

In plants, antioxidant genes are either critically regulated upon the onset of an oxidative stress or can be expressed constitutively. Methyl viologen treatments induce expression of genes for superoxide dismutase in *Nicotiana plumbaginifolia* (Tsang et al. 1991) and ascorbate peroxidase in spinach (Yoshimura et al. 2000). DNA microarray analysis also revealed effects of methyl viologen on global gene expression in *Nicotiana tabacum* (Vranova et al. 2002). In cyanobacteria, a few reports suggested induction of superoxide dismutase, catalase or some peroxidases under conditions of oxidative stresses based on activity measurements (Mittler and Tel-Or 1991, Abeliovich et al. 1974). Recently, effects of high light, dark and light, UV, electron transport inhibitors and salt stress have been analyzed by DNA microarray (Gill et al. 2002, Hihara et al. 2001, Kanasaki et al. 2002, Hihara et al. 2003, Huang et al. 2002). It was found that hundreds of genes and ORFs are induced or repressed under stress conditions compared with normal growth conditions. Some of them appear to overlap between different stress conditions while others may be unique to a certain stress. However, little is known about direct mechanisms to recognize ROS or oxidative damages in cyanobacteria or plants.

OxyR and SoxRS systems have been established as ROS sensors to induce various antioxidant components in *E. coli* (Zheng et al. 2001, Pomposiello et al. 2001). OxyR is known as a peroxide sensor to induce *katA* (catalase), *dps* (DNA-binding protein), *oxyS* (small regulatory RNA) and many other genes (Zheng et al. 2001). SoxR and SoxS mediate superoxide-responsive induction of *sodA* (superoxide dismutase), *fpr* (ferredoxin NADPH oxidoreductase), *zwf* (glucose-6-phosphate dehydrogenase) and other genes (Pomposiello et al. 2001).

Although cyanobacteria are Gram-negative bacteria like *E. coli*, no clear homologs of *oxyR* or *soxRS* have been found in any cyanobacterial genomes so far.

In this report, we analyzed the global gene expression of *Synechocystis* sp. PCC 6803 using DNA microarrays in response to methyl viologen under normal and high light conditions. We found that the expression of a novel peroxiredoxin gene, *sll1621*, was increased at the transcript level. Further, we showed by gene disruption studies that *sll1621* was essential for survival under high light conditions. We also showed that the transcriptional regulator (Slr1738) represses the expression of *sll1621* under normal conditions. The Slr1738 protein specifically bound to the upstream region of *sll1621*.

Results

Effects of methyl viologen on the global gene expression were examined by DNA microarray analysis. Expression was compared between the cells incubated with and without 10 μ M methyl viologen for 15 min under conditions of normal light (50 μ E m⁻² s⁻¹) or high light (200 μ E m⁻² s⁻¹). Although long-term incubation (>10 h) with 10 μ M methyl viologen in light was almost lethal to *Synechocystis* cells, we could observe induction of specific genes in response to the treatment for 15 min. Depending upon the induction ratio, expression of 11 genes was greater than 2-fold induced by methyl viologen under normal light conditions and expression of 27 genes was induced under the high light conditions (Table 1, 2). Of these, five genes (*sll1621*, *slr1738*, *slr0074*, *slr0075* and *slr0589*) were found to be commonly induced by methyl viologen under the two light conditions. Induction ratio of *sll1621* was over 20-fold under the high light conditions, while the ratio was less under the normal light conditions (ca. 2.5-fold). The predicted product, Sll1621, was significantly homologous to type 2 peroxiredoxins, which belong to the recently established peroxire-

Table 2 Genes induced by methyl viologen under high light conditions

Gene : Function ^a	Induction ratio ^b
<i>sll1621</i> : AhpC/TSA family protein ^c	26.47±5.48
<i>ssr0692</i> : hypothetical protein	4.47±0.41
<i>slr0513</i> : periplasmic iron-binding protein	4.44±0.87
<i>slr1738</i> : transcription regulator Fur family ^c	4.29±0.19
<i>slr0075</i> : ABC transporter ATP-binding protein (<i>ycf16</i>) ^c	4.25±0.41
<i>slr1634</i> : hypothetical protein	3.73±0.23
<i>sll1620</i> : hypothetical protein	3.66±0.92
<i>slr1295</i> : iron transport system substrate-binding protein (<i>futA1</i>)	3.57±0.78
<i>sll0023</i> : hypothetical protein	3.05±0.47
<i>slr0373</i> : hypothetical protein	3.04±0.15
<i>slr0589</i> : hypothetical protein ^c	2.90±0.18
<i>slr1957</i> : hypothetical protein	2.81±1.05
<i>sll1542</i> : hypothetical protein	2.70±0.76
<i>smr0001</i> : photosystem II PsbT protein (<i>psbT</i>)	2.70±0.59
<i>slr1161</i> : hypothetical protein	2.65±0.66
<i>slr0074</i> : ABC transporter subunit (<i>ycf24</i>) ^c	2.61±0.52
<i>sll0503</i> : hypothetical protein	2.61±1.50
<i>sll1864</i> : probable chloride channel protein	2.51±0.23
<i>slr1816</i> : hypothetical protein	2.48±0.64
<i>sll0525</i> : hypothetical protein	2.47±0.60
<i>sll0217</i> : flavoprotein	2.45±0.48
<i>slr0923</i> : hypothetical protein	2.37±0.35
<i>slr1958</i> : unknown protein	2.37±0.47
<i>slr0651</i> : hypothetical protein	2.28±0.40
<i>sll1740</i> : 50S ribosomal protein L19 (<i>rp119</i>)	2.18±0.56
<i>sll0485</i> : two-component response regulator NarL subfamily	2.17±0.48
<i>slr1932</i> : unknown protein	2.08±0.14

^a Function description of CyanoBase (<http://www.kazusa.or.jp/cyano/>).

^b Ratio of transcript levels in the treated cells to those in the non-treated cells.

^c Induced genes common to the conditions of normal and high light.

doxin superfamily (Dietz et al. 2002, Wood et al. 2003). The peroxiredoxin superfamily is defined as thiol-specific reductases or peroxidases, which do not have redox cofactors in contrast to other peroxidases. In the *Synechocystis* genome, five peroxiredoxin-like genes (*sll1621*, *sll0755*, *slr1198*, *sll0221* and *slr0242*) are detected. These genes except for *sll1621* were not significantly affected by methyl viologen (data not shown). Similar to *sll1621*, a transcriptional regulator gene, *slr1738*, was induced by methyl viologen more pronouncedly under the high light conditions than the normal light. Since *sll1621* and *slr1738* are located next to each other on the chromosome in divergent directions, we reasoned that both genes could be regulated by the same factor. Two contiguous genes, *slr0074* and *slr0075*, were also induced by methyl viologen, suggestive of polycistronic transcription. They are homologous to *sufB* and *sufC* in *E. coli* and *ycf24* and *ycf16* in malarial plastids, respectively, which are involved in as yet unidentified processes in the assembly of various iron-sulfur centers (Takahashi and Tokumoto 2002, Ellis et al. 2001). Interestingly, *sufB* and *sufC*

are also induced by hydrogen peroxide treatment in *E. coli* as a part of *oxyR* regulon (Zheng et al. 2001). Finally, *slr0589* is a hypothetical gene, whose homologs are found only in the genomes of cyanobacteria and *Arabidopsis* but not in other organisms. On the other hand, no significant induction was observed in known antioxidant genes such as superoxide dismutase (*slr1516*), catalase-peroxidase (*sll1987*) or thioredoxins (*sll1980*, *slr0623* and *slr1139*). We also observed specific suppression of many genes in the treatment of methyl viologen especially under high light conditions. They are mostly photosynthesis-related; nitrate transporter operon (*sll1450/sll1451/sll1452*), *ndhD2* (*slr1291*), ferredoxin-nitrite reductase (*slr0898*), ribulose biphosphate carboxylase large subunit (*slr0009*), CO₂-concentrating mechanism operon (*sll1028/sll1029/sll1030/sll1031*), and Photosystem I P700 apoprotein subunits (*slr1834/slr1835*). The complete list of the entire array data is available at web site (<http://www.genome.ad.jp/kegg/expression/>).

We deleted the *slr1738* ORF (Fig. 1) to examine whether it is involved in transcriptional regulation of *sll1621*. We

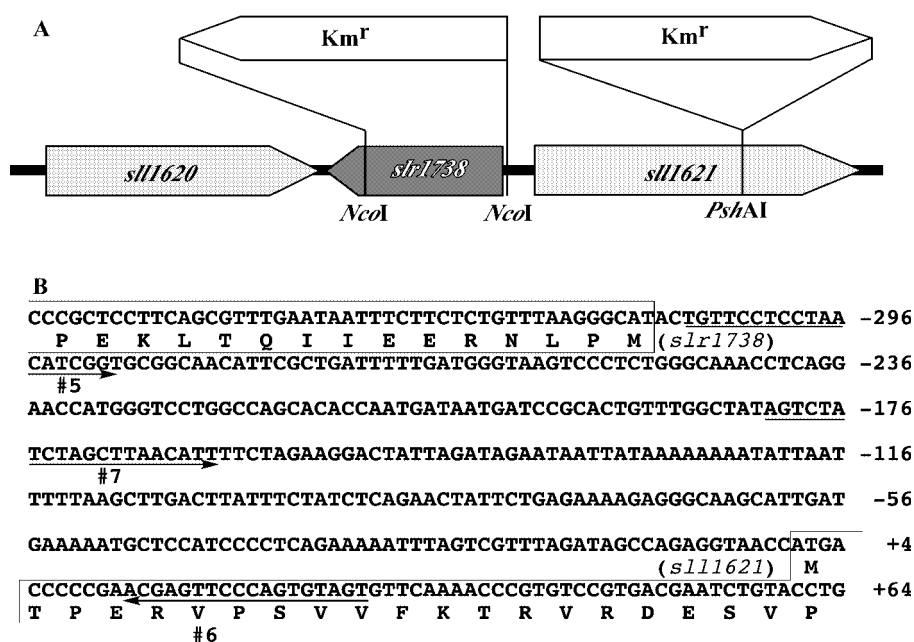


Fig. 1 Schematic arrangement of *sll1620*, *slr1738* and *sll1621* genes (A) and nucleotide sequence of the *slr1738-sll1621* intergenic region (B). Coding sequences of *slr1738* and *sll1621* are boxed. Positions of the primers for probes used in the gel mobility shift assay experiments are shown by arrows.

compared the genome-wide gene expression in this mutant with that in wild type under normal light conditions of $50 \mu\text{E m}^{-2} \text{s}^{-1}$. It was found that at least 26 genes were upregulated by greater than 2.5-fold (Table 3) and over 50 genes were downregulated in the mutant (data not shown). Notably, transcript levels of *sll1621* were approximately 10 times higher in the mutant than in wild type. This strongly suggests that the transcriptional regulator, Slr1738, acts as a repressor for expression of *sll1621*. Since a majority of the coding region of *slr1738* was deleted in the mutant, microarray data of the transcript level of *slr1738* were not considered. No other genes were found to be upregulated commonly under the conditions of methyl viologen treatment and disruption of *slr1738*.

To further confirm the direct involvement of Slr1738 in induction of *sll1621* and *slr1738*, we expressed His-tagged Slr1738 (His-Slr1738) in *E. coli* without IPTG and purified it by Ni-affinity chromatography (Fig. 2A). The recombinant protein was recovered in the soluble fraction in relatively large amounts when *E. coli* was grown without induction, while it was mostly recovered in inclusion bodies when overexpression was induced with IPTG (data not shown). We examined specific binding of His-Slr1738 to the intergenic region of *sll1621* and *slr1738* by gel mobility shift assays. When a DNA fragment from -308 to +29 (position from the putative translation initiation of *sll1621*) was used as a probe (Fig. 1B), specific binding of His-Slr1738 to this fragment was observed as two retarded bands depending on the protein concentration (Fig. 2B, arrows). The smaller fragment from -182 to +29 was also effective for this assay (data not shown). By contrast, no binding to upstream DNA of superoxide dismutase (*slr1516*) or catalase-peroxidase (*sll1987*) was observed even with the same His-Slr1738 preparation (data not shown). Addition of 1 mM

DTT clearly enhanced the binding affinity of the protein to the intergenic region of *sll1621* and *slr1738* (Fig. 2B). By contrast, addition of hydrogen peroxide clearly abolished the binding (Fig. 2C). However, binding affinity and effects of DTT varied depending on preparation of the His-Slr1738 protein. Similar variation in the DNA-binding affinity is already known for some Fur-type regulators. For example, metal composition of *Bacillus* PerR protein varied between preparations and the metals were critical for DNA-binding (Herbig and Helmann 2001). We measured the metal content of Slr1738 by ICP atomic absorption spectroscopy and found that Zn content varied around 0.4–0.6 atom/monomer of Slr1738, while Mn or Fe was not associated at all (Kobayashi, M. and Ikeuchi, M. unpublished results). This suggests to us that the protein may be functional as a dimeric form. More precise evaluation of the DNA-binding affinity should be done with regard to the metal content and sensitivities to redox changes. We also measured the growth of the *slr1738*-disruptant. Regardless of the light conditions, the disruptant showed normal growth that was comparable to the wild type (Fig. 3A).

To gain insights into the physiological role, we disrupted peroxiredoxin-like *sll1621* by insertion of a kanamycin resistance cassette in the *Synechocystis* genome. We could only achieve complete segregation of the disrupted chromosome under anaerobic conditions supplemented with CO_2 (data not shown). When these segregated mutants were grown aerobically on solid medium under low light conditions of $10 \mu\text{E m}^{-2} \text{s}^{-1}$, they showed unusual heterogeneous populations of dead cells, growing cells and cells turning brown after transient growth (Fig. 4). These observations suggest that *sll1621* is required for survival even under the low light conditions. On the other hand, the mutant showed only slight retardation of

Table 3 Upregulated genes in the *slr1738*-disruptant

Gene : Function ^a	Induction ratio ^b
<i>sll1621</i> : AhpC/TSA family protein	10.61±4.55
<i>sll1450</i> : nitrate/nitrite transport system substrate-binding protein (nrtA)	5.77±0.91
<i>sll1688</i> : threonine synthase (thrC)	5.52±0.80
<i>sll0381</i> : hypothetical protein	3.96±1.07
<i>sll0684</i> : phosphate transport ATP-binding protein PstB homolog	3.56±0.41
<i>sll1030</i> : carbon dioxide concentrating mechanism protein CcmL (ccmL)	3.48±0.56
<i>sll0680</i> : phosphate-binding periplasmic protein precursor (PBP)	3.43±1.04
<i>sll1031</i> : carbon dioxide concentrating mechanism protein CcmM (ccmM)	3.35±0.72
<i>sll0616</i> : preprotein translocase SecA subunit (secA)	3.26±1.97
<i>slr0900</i> : molybdopterin biosynthesis MoeA protein (moeA)	3.11±1.21
<i>sll0927</i> : S-adenosylmethionine synthetase	2.99±0.20
<i>slr0898</i> : ferredoxin–nitrite reductase (nirA)	2.92±0.54
<i>slr0364</i> : hypothetical protein	2.85±0.07
<i>sll0681</i> : phosphate transport system permease protein PstC homolog	2.85±1.13
<i>sll1032</i> : carbon dioxide concentrating mechanism protein CcmN(ccmN)	2.82±0.51
<i>sll0385</i> : ATP-binding protein of ABC transporter	2.79±0.38
<i>slr0899</i> : cyanate lyase (cynS)	2.78±0.44
<i>sll0685</i> : hypothetical protein	2.77±0.48
<i>sll1780</i> : putative transposase (ISY203_b)	2.77±1.35
<i>sll1804</i> : 30S ribosomal protein S3 (rps3)	2.75±0.02
<i>sll1397</i> : putative transposase (ISY100_a)	2.69±0.98
<i>sll1071</i> : hypothetical protein	2.68±0.99
<i>slr0628</i> : 30S ribosomal protein S14 (rps14)	2.59±0.20
<i>slr1616</i> : unknown protein	2.57±1.39
<i>sll1771</i> : protein serin-threonin phosphatase (pphA)	2.56±1.03
<i>slr1931</i> : type 4 pilin-like protein (pilA8)	2.52±1.31

^a Function description of CyanoBase (<http://www.kazusa.or.jp/cyano/>).

^b Ratio of transcript levels in the *slr1738*-disruptant to those in the wild type.

growth in liquid medium under the same low light conditions (Fig. 3B). Under conditions of 50 $\mu\text{E m}^{-2} \text{s}^{-1}$, the mutant grew significantly slower than the wild type (Fig. 3B). When grown under the high light conditions of 200 $\mu\text{E m}^{-2} \text{s}^{-1}$, the mutant stopped growing after transient growth (Fig. 3B). We also disrupted other peroxiredoxin-like genes (*sll0755* and *slr1198*), glutathione peroxidase (*slr1992* and *slr1171*) and catalase-peroxidase (*sll1987*). These disruptants completely segregated on agar plates even under aerobic conditions and showed no discernible defect in growth at least under the normal light conditions (data not shown). Thus, we can conclude that *sll1621* plays the critical role in protection against photooxidative stress especially under high light conditions.

Discussion

We have identified by DNA microarray analysis methyl viologen-inducible genes, which could be candidates for ROS-responsive genes. The five genes, *sll1621*, *slr1738*, *slr0074*, *slr0075*, and *slr0589*, were induced by methyl viologen commonly under conditions of normal and high light. Of these,

sll1621 and possibly *slr1738* were shown to be regulated by a Fur-type transcriptional regulator, Slr1738, by DNA microarray analysis of the *slr1738*-disruptant and gel mobility shift assay. We demonstrated that the Slr1738 protein binds specifically to the intergenic region of *sll1621* and *slr1738*. The binding was strictly dependent on the redox conditions of the protein, although we do not know which ROS component is primarily responsible for regulation of the binding affinity. Consistently, we have observed that hydrogen peroxide treatment induced marked induction of *sll1621* under high light conditions as well (Kobayashi, M. and Ikeuchi, M. unpublished results).

The predicted product of *sll1621* is significantly homologous to the known type 2 peroxiredoxin in eukaryotes but more homologous to bacterial peroxiredoxin-like hypothetical proteins. Type 2 peroxiredoxins have been identified as potent antioxidant proteins in yeast, animal and plants (Jeong et al. 1999, Yamashita et al. 1999, Brehelin et al. 2003), which are distantly related to the typical peroxiredoxins (also called type 1). Based on the crystal structure, human peroxiredoxin 5 (a representative of eukaryotic type 2) does not form a dimer or

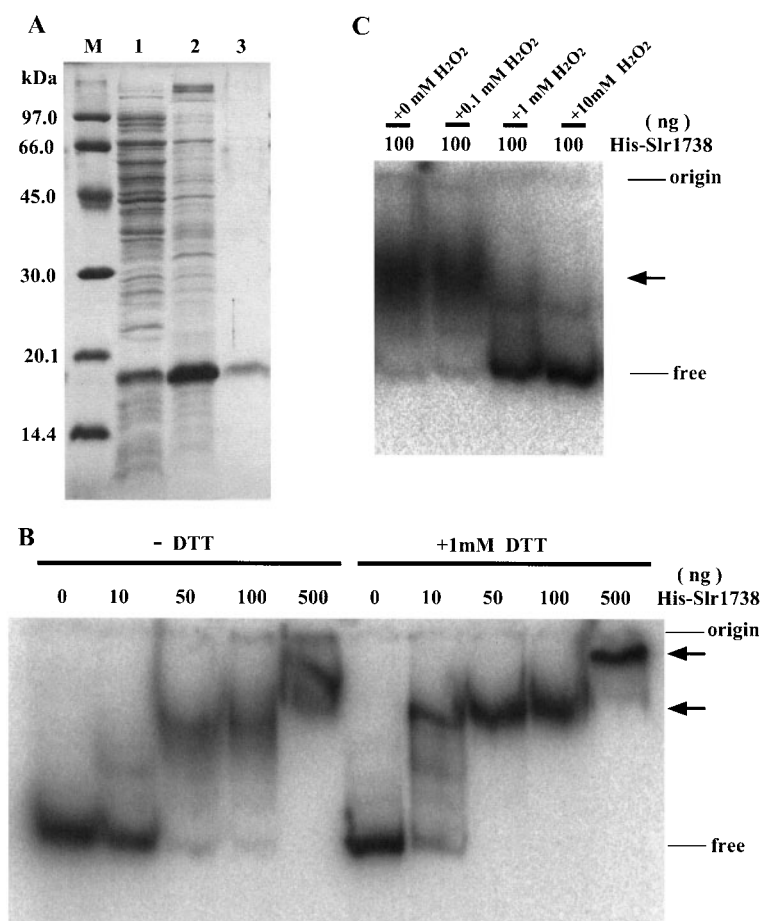


Fig. 2 (A) Preparation of the His-Slr1738 protein from *E. coli* BL21 (DE3). Lanes: M, molecular size markers; 1, the soluble fraction of crude cell extracts; 2, the precipitate of crude cell extracts; 3, His-Slr1738 purified by Ni-chelating affinity chromatography. Proteins were separated by SDS-PAGE (16% acrylamide gel). (B and C) Gel mobility shift assay of *sll1621-slr1738* intergenic sequence with the purified His-Slr1738. The DNA fragment amplified with primers, #5 and #6, was end-labeled with [γ -³²P]ATP and incubated with the His-Slr1738 in the presence or absence of 1 mM DTT or 0.1–10 mM H₂O₂. Arrows show complexes of the DNA fragment with the protein.

multimer like other type of peroxiredoxins but forms intramolecular disulfide bond between N-terminal and C-terminal Cys residues (Cys-47 and Cys-151) during reduction of peroxides (Declercq et al. 2001). Bacterial type 2-like genes including *sll1621* have been simply annotated to encode putative antioxidant proteins without biochemical evidence, since they are significantly related to the eukaryotic type 2 peroxiredoxins especially in the region surrounding the N-terminal Cys residue. By contrast, the C-terminal counterpart Cys residue is not well conserved in the type 2-like bacterial proteins, suggestive of slight difference in the structural configuration. Moreover, physiological role of type 2-like peroxiredoxins has not yet been clarified in bacteria, although eukaryotic type 2 has been reported to play an important role as antioxidant in ROS-generating organelles and an inhibitor of ROS-dependent cell apoptosis (Zhou et al. 2000, Yamashita et al. 1999).

Our data presents the first experimental evidence that the type 2-like peroxiredoxin gene, *sll1621*, is essential for phototrophic growth of *Synechocystis* sp. PCC 6803 under aerobic conditions. Gene disruption analysis clearly demonstrated that *sll1621* is the most important gene for survival under stresses of oxygen and high light among many potential peroxidase genes. It should be noted that the transcript level of *sll1621* was

relatively high even under non-stressed conditions, as judged from the signal to noise ratio of the microarray data. Consistently, the Sll1621 protein was identified as one of the dye-stained spots in a two-dimensional gel electrophoresis followed by N-terminal Edman sequencing in the proteomic analysis in *Synechocystis* (Sazuka et al. 1999). This suggests that Sll1621 protein is present in relatively large amount in cells grown under normal light conditions but further accumulates to greater extent to increase detoxification capacity in response to the ROS-generating methyl viologen treatment. Further studies should be done to confirm the accumulation of the Sll1621 protein under stressed conditions. Recently, Sll1621 protein was isolated from cell extracts by thioredoxin affinity chromatography, where a modified thioredoxin was conjugated to a resin, enabling isolation of adducts between the thioredoxin and thioredoxin-binding proteins (Matsuda et al. 2003, Motohashi et al. 2001). Further, the Sll1621 protein showed peroxidase activity toward H₂O₂ (Matsuda, N. and Hisabori, T. personal communication).

Sll1621 protein is a divergent homolog of the type 2 peroxiredoxins as mentioned above. In the literature, various peroxiredoxins have been shown to play a key role in protection against oxidative stress in aerobes and in the redox-

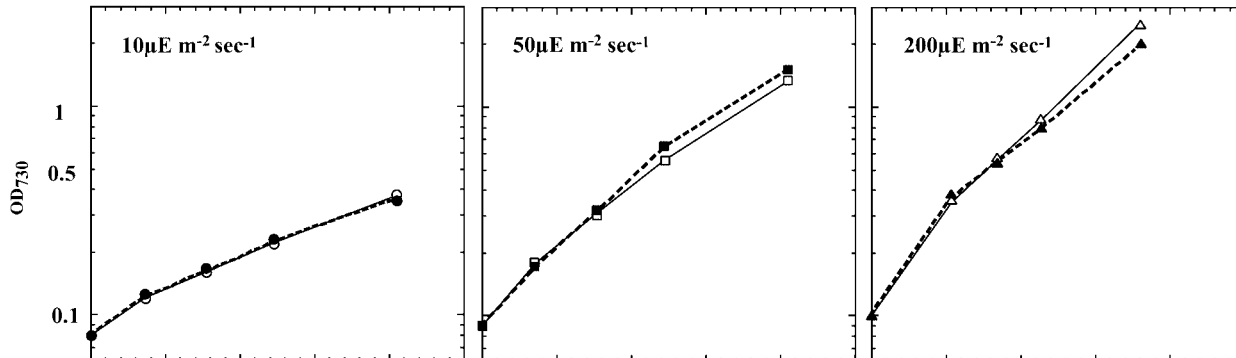
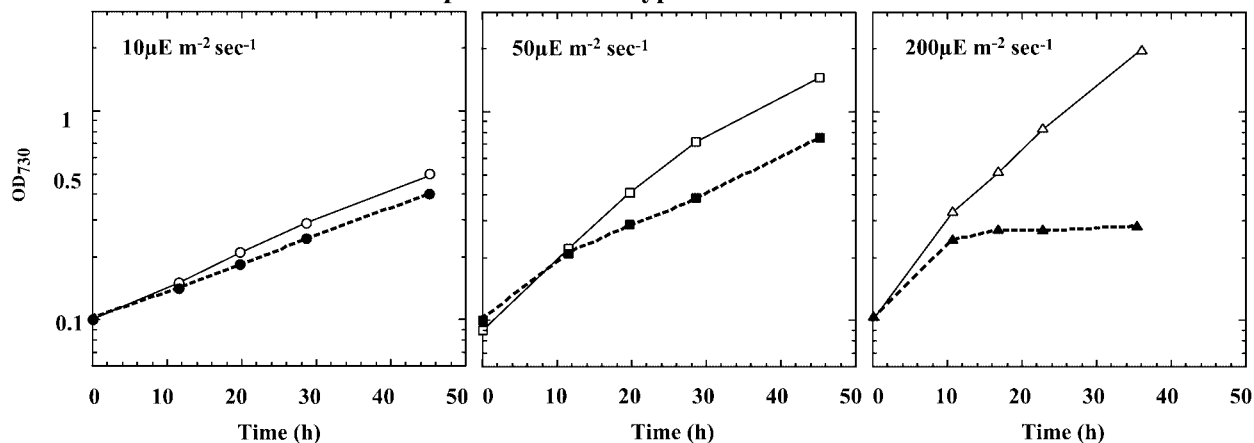
A : Growth curves of *slr1738*-disruptant and wild type**B : Growth curves of *sll1621*-disruptant and wild type**

Fig. 3 Growth curves of wild type and *slr1738*-disruptant (A) and *sll1621*-disruptant (B) under the photoautotrophic conditions at 10 $\mu\text{E m}^{-2} \text{s}^{-1}$ (circle), 50 $\mu\text{E m}^{-2} \text{s}^{-1}$ (square) and 200 $\mu\text{E m}^{-2} \text{s}^{-1}$ (triangle). Open symbol, wild type; closed symbol, mutants.

regulation of cellular signaling and differentiation in higher animals (Hofmann et al. 2002). Biochemically, peroxiredoxins are featured as low efficiency peroxidase activity with promiscuous substrate specificities: hydrogen peroxide, alkyl hydroperoxide, and peroxyxynitrite as peroxides and glutathione, thioredoxin, glutaredoxin, trypanedoxin as thiol reductants (Bryk et al. 2000). Such variation of peroxiredoxins may account for the multiple genes in *Synechocystis* in contrast to single genes for superoxide dismutase (*slr1516*) and peroxidase-catalase (*sll1987*). Namely, at least five peroxiredoxins are encoded in the *Synechocystis* genome, Sll0755 (type 1, subtype 2-Cys), Slr1198 (type 1, subtype 1-Cys), Sll0221 and Slr0242 (peroxiredoxin Q or bacterioferritin comigratory protein) and Sll1621 (type 2). Detailed analysis of the enzymatic properties of Sll1621 and other peroxiredoxins would lead to thorough understanding of the photooxidative stresses and protection in cyanobacteria.

In our microarray analysis, we detected the five genes as well as many other genes, whose transcript levels were upregulated in the methyl viologen treatment under conditions of low or high light. They are the likely targets of yet unidentified

transcriptional regulators in response to stresses due to specific or general ROS or changes in specific or general redox components. Those stresses could be shared with other treatments such as high light, UV, high salts, or low temperature. When the results were compared with the high light experiments (Hihara et al. 2001, Huang et al. 2002), only *sll1621* was also upregulated (2- to 3.9-fold), while the other four genes were not significantly affected. Instead, a number of genes induced

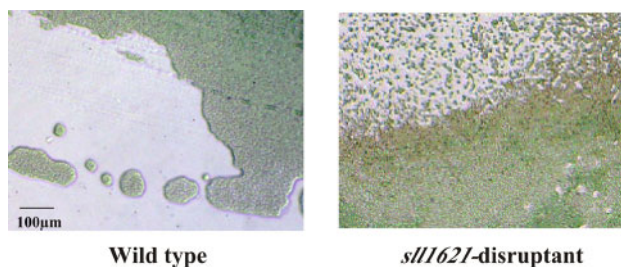


Fig. 4 Colony morphology of wild type and *sll1621*-disruptant on BG11 plates. Cells were grown photoautotrophically at light intensity of 10 $\mu\text{E m}^{-2} \text{s}^{-1}$ under aerobic conditions for 4 d.

by high light were not induced by the treatment of methyl viologen. For example, superoxide dismutase and glutathione peroxidase (*slr1992*) were induced throughout the high light illumination. In addition to these genes, UV-B illumination induced expression of a number of genes including other anti-oxidant-related genes such as glutathione peroxidase (*slr1171*), glutaredoxin (*ssr2061*), thioredoxin M (*slr0233*), and Fur-type regulator (*slr1738*) (Huang et al. 2002). High salt (0.5 M NaCl) or high osmotic conditions (0.5 M sorbitol) strongly induced superoxide dismutase in addition to moderate induction of *sll1621* and *slr1738* (Kanesaki et al. 2002, Suzuki et al. 2001). This suggests that many antioxidant genes may be distinctively induced via several regulatory systems in response to oxidative stresses, despite that the methyl viologen seems to induce specifically *sll1621* and *slr1738* via the Slr1738 regulator protein.

Slr1738 belongs to a family of Fur-like proteins. In the *Synechocystis* genome, there are three such genes (*sll0567*, *sll1937*, and *slr1738*) (Kaneko et al. 1996). First, *sll0567* is a homolog of *fur* that plays a key role for regulation of ferrous ion uptake genes in some Gram-positive and many Gram-negative bacteria including cyanobacteria (Escolar et al. 1999, Ghassemian and Straus 1996, Hernandez et al. 2002). Fur represses transcription by binding to the target genes when Fe^{2+} is bound to the protein (Escolar et al. 1999). Second, *Sll1937* may be a repressor for Zn^{2+} uptake genes like Zur protein in *E. coli* (Patzner and Hantke 1998) and *Bacillus subtilis* (Gaballa and Helmann 1998). Although *Sll1937* is not clear ortholog of Zur, the *sll1937*-inactivated mutant of *Synechocystis* showed constitutive expression of *znuCAB* operon encoding Zn^{2+} -uptake transporter (Pakrasi et al. 2002). Finally, *slr1738* is slightly homologous to diverged *perR* families in *B. subtilis* and *Staphylococcus aureus* (Bsat et al. 1998, Herbig and Helmann 2001, Horsburgh et al. 2001). The *Bacillus* PerR protein has been extensively studied and it was now established that it is a peroxide-responsive Fur-type metal-binding transcriptional regulator that represses certain antioxidant-related genes such as *kata* (catalase) and *ahpCF* (alkyl hydroperoxide reductase), etc. under non-stressed conditions (Bsat et al. 1998). AhpC is a member of typical peroxiredoxins (type 1), although it forms a complex with AhpF to detoxify hydrogen peroxide with the aid of reduced NADPH (Storz et al. 1989). Although the detailed mechanism has not yet been elucidated for peroxide-induced changes even in the PerR protein, H_2O_2 treatment was shown to abolish its DNA-binding of PerR, while DTT recovered the binding activity (Herbig and Helmann 2001). Our data that DTT enhanced the DNA-binding affinity of Slr1738 appear to be consistent with the effects of DTT on the *Bacillus* PerR. However, in *B. subtilis* no specific responses in gene expression have been observed against the methyl viologen treatment by DNA microarray analysis (Mongkolsuk and Helmann 2002). This suggests that recognition mechanism of Slr1738 may be slightly different from the *Bacillus* PerR protein.

To define the Slr1738 regulon, it is essential to know its target sequence. By gel mobility shift assay, we showed that Slr1738 protein binds to the DNA fragment in an intergenic region between *sll1621* and *slr1738* (Fig. 2). This suggests that the same binding site is responsible for bidirectional transcriptional regulation of *sll1621* and *slr1738* in response to ROS stresses. Bacterial Fur, Zur and PerR proteins recognize related target sequences of 15–21 bp (Fuangthong and Helmann 2003). They are featured as AT-rich inverted repeat of 7-1-7 or 10-1-10. Since amino acid sequence of the putative DNA binding region of Slr1738 is slightly homologous to the bacterial Fur-like proteins, we may expect a specific box for binding of Slr1738. Gel mobility shift assay with several oligonucleotide probes is currently being undertaken to determine the target sequence. It would allow us to search the other target genes of Slr1738 in the genome, which might have escaped the DNA microarray analysis due to less dramatic response to the treatment of methyl viologen.

Materials and Methods

Growth

The glucose-tolerant strain of *Synechocystis* sp. PCC 6803 (Williams 1988) was used for microarray analysis of methyl viologen and the *slr1738*-disruptant. A motile strain of *Synechocystis* sp. PCC 6803 (Yoshihara et al. 2000) was used for analysis of the *sll1621*-disruptant. The wild-type and mutant cells were grown in BG11 medium buffered with *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid-KOH, pH 7.0 at 31°C at a light intensity of $40 \mu\text{E m}^{-2} \text{s}^{-1}$. Solid medium was supplemented with 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate and used for screening of mutants. To maintain gene-disrupted mutants, $20 \mu\text{g ml}^{-1}$ kanamycin was added, while antibiotics were not included for characterization of the mutant phenotype. For complete segregation of the *sll1621*-disruptant, cells were grown on plates under anaerobic conditions supplemented with approximately 5% CO_2 at $10 \mu\text{E m}^{-2} \text{s}^{-1}$ (AnaeroPack Kenki, Mitsubishi Gas Chemical, Tokyo, Japan). *E. coli* strain JM109 was used for cloning and subcloning of plasmids, while BL21(DE3) was used for expression of His-Slr1738 with pET28a (Novagen, Madison, WI, U.S.A.).

Gene disruption

A DNA fragment of 1,839 bp containing *sll1621* and *slr1738* was amplified by PCR using primers, #1 5'-GGCACAGTCACTTGGCAA-3' and #2 5'-TTTGTGCGATGACCTCGAA-3' and cloned into pT7Blue (Novagen). A kanamycin-resistant cassette was inserted into *sll1621* at *PshAI* in the direction of *sll1621*. Alternatively, the almost entire coding region of *slr1738* was deleted with *NcoI* and replaced with the kanamycin-resistant cassette. Mutants were generated by transformation of *Synechocystis* cells with this DNA and selected on BG11 plates containing $20 \mu\text{g ml}^{-1}$ kanamycin. Complete segregation was confirmed by PCR with the same primers as mentioned above (not shown).

DNA microarray analysis

Total RNA was isolated by a hot phenol method as described by Muramatsu and Hihara (2003). DNA microarray analysis was performed basically according to Hihara et al. (2001) except for using M-MLV reverse transcriptase (Takara Bio, Otsu, Japan). Prehybridization was omitted. CyanoChip Version 1.5 was obtained from Takara Bio. Fluorescence images were obtained on a ScanArray 4000 (GSI Lumonics, Watertown, MA, U.S.A.) using auto-balance/auto-range

feature and the data were analyzed using ArrayVision software (Version 6, Imaging Research, Ontario, Canada). The fluorescence intensity of each spot in both Cy3 and Cy5 images was quantified, and fluorescence levels of the local background were subtracted. Cy3 and Cy5 images were normalized by adjusting the total signal intensities of two images ("global normalization"). After normalization, the ratio of transcript level of each gene of a sample to the reference was calculated. Since these included not so accurate data due to low expression level, the data of signal to noise ratio less than 2 in both Cy3 and Cy5 images were removed from further analysis. Finally, all ratios were represented as averages of two independent experiments including four sets of data. The raw data are available on Internet (<http://www.genome.ad.jp/kegg/expression>).

Expression and purification of His-Slr1738

A transcriptional regulator gene, *slr1738*, was amplified by primers, #3 5'-CCATATGCCCTTAAACAGAGAAG-3' and #4 5'-CTC-CATTACCAAGGAGAT-3', with Pfu polymerase and cloned into pPCRscript (Stratagene, La Jolla, CA, U.S.A.) according to manufacturer's instruction. The coding region was excised with *NdeI* and *BglII* and subcloned into pET28a for expression of a fusion protein with N-terminal His-tag. The nucleotide sequence was confirmed by DNA sequencing using the BigDye terminator method (Applied Biosystems, Foster City, CA, U.S.A.). Soluble His-Slr1738 was expressed in *E. coli* strain BL21 (DE3) in a culture medium for overnight without induction. Proteins were extracted by breakage of cells in a medium of 20 mM HEPES-NaOH (pH 7.5), 500 mM NaCl and 10% (v/v) glycerol with sonication (model 200M, Kubota Co., Tokyo, Japan) and centrifuged at 100,000×g for 30 min at 4°C. His-Slr1738 was purified by Ni-affinity column chromatography using a HiTrap chelating column (Amersham Biosciences, Piscataway, NJ, U.S.A.). The column equilibrated with 20 mM HEPES-NaOH (pH 7.5), 500 mM NaCl, 10% (v/v) glycerol and 5 mM imidazole was loaded with the soluble fraction and eluted with a linear gradient of imidazole from 5 mM to 600 mM. Fractions containing nearly homogenous His-Slr1738 were pooled and dialyzed against 20 mM Tris-HCl (pH 8.0) overnight at 4°C. Protein composition was examined by SDS-PAGE with 16% (w/v) polyacrylamide gel followed by staining with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Gel mobility shift assay

The DNA probes were amplified with primers, #5 5'-TGTTCTCCTAACATCGG-3' and #6 5'-ACTACTGTTGGAACTCGT-3', and #6 and #7 5'-AGTCTATCTAGCTTAACATT-3' (Fig. 1). The amplified DNA fragments were gel-purified and end-labeled with Klenow fragment and [γ - 32 P]ATP (Amersham Biosciences). After purification with a spin column (NAP5, Amersham Biosciences), the labeled probe was added to His-Slr1738 in a total volume of 20 μ l of the binding buffer (20 mM Tris-HCl (pH 7.5), 80 mM NaCl, 50 ng of poly(dA-dT), 5% (v/v) glycerol and 50 μ g ml $^{-1}$ bovine serum albumin with or without 1 mM DTT) for 30 min at room temperature. The mixtures were loaded onto 6% (w/v) polyacrylamide gel with 40 mM Tris-acetate (pH 7.5). After electrophoresis, gels were dried and autoradiographed (BAS2500, Fujifilm, Tokyo, Japan).

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