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REVIEW PAPER

Occurrence, phylogeny, structure, and function of catalases and peroxidases in cyanobacteria

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Abstract

Cyanobacteria have evolved $\sim 3\times10^9$ years ago from ancient phototrophic microorganisms that already lived on our planet Earth. By opening the era of an aerobic, oxygen-containing biosphere, they are the true pacemakers of geological and biological evolution. Cyanobacteria must have been among the first organisms to elaborate mechanisms for the detoxification of partially reduced oxygen species including (hydrogen) peroxide. Since there is still an suprising lack of knowledge on the type, role, and mechanism(s) of peroxide-degrading enzymes in these bacteria, all 44 fully or partially sequenced genomes for haem and non-haem catalases and peroxidases have been critically analysed based on well known structure–function relationships of the corresponding oxidoreductases. It is demonstrated that H_2O_2-dismutating enzymes are mainly represented by bifunctional (haem) catalase–peroxidases and (binuclear) manganese catalases, with the latter being almost exclusively found in diazotrophic species. Several strains even lack a gene that encodes an enzyme with catalase activity. Two groups of peroxidases are found. Genes encoding putative (primordial) haem peroxidases (with homology to corresponding mammalian enzymes) and vanadium-containing iodoperoxidases are found only in a few species, whereas genes encoding peroxiredoxins (1-Cys, 2-Cys, type II, and Q-type) are ubiquitous in cyanobacteria. In addition, $\sim$70% contain NADPH-dependent glutathione peroxidase-like proteins. The occurrence and phylogeny of these enzymes is discussed, as well as the present knowledge of their physiological role(s).

Key words: Catalase–peroxidase, *Cyanobacterium*, glutathione peroxidase, manganese catalase, oxidative stress, peroxide detoxification, peroxiredoxin, vanadium peroxidase.

Introduction

It is generally accepted by the scientific community that our aerobic biosphere ultimately dates back to the advent of cyanobacteria. Primordial blue-green algae (cyanobacteria) have evolved $\sim 3\times10^9$ years ago from ancient phototrophic organisms that already lived on our planet Earth (Barghoorn, 1971; Rasmussen *et al.*, 2008). They had succeeded in linking photosynthetic electron flow from water as the (energy-requiring!) photoreductant through an oxygen-evolving complex of a newly elaborated photosystem II (PSII), which is thought to have originated from a uniform primordial photosystem by gene duplication. The resulting tandem operation of two photosystems (namely a high-potential water-oxidizing PSII and a low-potential ferredoxin-reducing PSI) is now known as oxygenic or plant-type photosynthesis. This most decisive evolutionary step marked a turning point in evolution on our Earth, opening up the era of an aerobic,

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Abbreviations: KatG, catalase–peroxidase; MnCat, manganese catalase; PxDo, peroxidockerin; LPO, lactoperoxidase; DiHCCP, dihaem cytochrome *c* peroxidase; Prx, peroxiredoxin; GPx, glutathione peroxidase; LGT, lateral gene transfer; ORF, open reading frame; GSH, glutathione; GSSG, oxidized glutathione.

oxygen-containing biosphere and atmosphere. The impact of this free dioxygen on the further evolution of life on Earth can hardly be overemphasized, and cyanobacteria thus introduce themselves as true pacemakers of (terrestrial) evolution, in both a geological and a biological sense.

Clearly, the first evolution of molecular O_2 must have been a deadly threat to all extant organisms in those anoxic days and it is logical to assume that cyanobacteria must have been among the first organisms to elaborate mechanisms for the detoxification of partially reduced, reactive oxygen species (ROS) originating from one- (i.e. $O_2^{\cdot-}$ or $HO_2^{\cdot}$) and two-electron reduction of O_2 (i.e. H_2O_2) (Regelsberger *et al.*, 2002).

Here, an analysis is presented of all 44 fully or partially sequenced cyanobacterial genomes (Table 1, August 2008) for peroxidases and catalases, which are ubiquitous oxidoreductases capable of the reductive heterolytic cleavage of the peroxidic bond, predominantly in hydrogen peroxide (H-O-O-H), but also in organic peroxides (R-O-O-H). Peroxidases reduce peroxides by means of two one-electron donors (Reaction 1) or one two-electron donor (Reaction 2). One-electron donors (AH) can be aromatic (e.g. phenols) or aliphatic [e.g. glutathione (GSH)] molecules, small inorganic anions (e.g. NO_2^-), or even metal cations (e.g. Mn^{2+}), and the corresponding oxidation product (e.g. a radical, $^{\cdot}A$) eventually dimerizes [e.g. forming glutathione disulphide (GSSG)]. Two-electron donors (X^-) can be halides (chloride, bromide, iodide, or thiocyanate), with the corresponding oxidation product (HOX) being hypohalous acid or hypothiocyanate. In this respect, peroxidases that catalyse Reaction 2 are often denominated haloperoxidases (irrespective of the protein family that catalyses this reaction).

$$\mathrm{HOOH\,(ROOH)} + 2\,\mathrm{AH} \rightarrow 2\,{}^{\cdot}\mathrm{A} + 2\,\mathrm{H_2O\,(H_2O + ROH)} \qquad \text{Reaction 1}$$

$$\mathrm{HOOH} + \mathrm{X^-} + \mathrm{H^+} \rightarrow \mathrm{HOX} + \mathrm{H_2O} \qquad \text{Reaction 2}$$

Catalases have the unique catalytic capacity to dismutate hydrogen peroxide (H_2O_2; Reaction 3) by their striking ability to evolve molecular oxygen (O_2) by oxidation of H_2O_2.

$$\mathrm{H_2O_2} + \mathrm{H_2O_2} \rightarrow \mathrm{O_2} + 2\mathrm{H_2O} \qquad \text{Reaction 3}$$

Several gene families evolved in the ancestral genomes capable of reduction of H_2O_2 or organic peroxides. Among them are haem and non-haem oxidoreductases. Haem-containing enzymes can be divided in three main (super)-families, namely (i) typical or 'monofunctional' catalases found in all domains of life (Zamocky *et al.*, 2008*a*); (ii) the haem peroxidase superfamily with members in plants, fungi, protists, and (archae)bacteria (Welinder, 1992; Passardi *et al.*, 2007); and (iii) the peroxidase–cyclooxygenase superfamily also distributed in all domains of life (Zamocky *et al.*, 2008*b*). In addition, some other minor groups of haem-containing peroxidases are found, including the novel dye-decolorizing peroxidase family found in archaea, bacteria, and fungi (Zubieta *et al.*, 2007) or bacterial dihaem peroxidases (Echalier *et al.*, 2006).

Non-haem peroxide-utilizing enzymes are manganase catalases (Zamocky *et al.*, 2008a), vanadium peroxidases (Littlechild, 1999), and ubiquitous thiol peroxidases (peroxiredoxins and glutathione peroxidases), which catalyse the reduction of peroxides by catalytic cysteine residues and thiol-containing proteins as reductants (Rouhier and Jacquot, 2005).

Based on similarity searches, multiple sequence alignments, and critical sequence analysis based on well known structure–function relationships, the occurrence, phylogeny, and physiological relevance of these oxidoreductases in phototrophic oxygenic cyanobacteria are described. This is discussed with respect to the knowledge of corresponding enzymes in higher plants, where photosynthesis is compartmentalized within the chloroplast that presumably derived from an endosymbiotic cyanobacterium.

Results and discussion

H_2O_2 is well known to have an inhibitory effect on the phototrophic growth of cyanobacteria (Samuilov *et al.*, 1999). Nevertheless, and despite their unique role in evolution, our understanding of the enzymatic mechanisms of H_2O_2 degradation in cyanobacteria as well as the physiological role(s) of the various reported enzymes is scarce. Still often only enzymatic activities are described without precisely referring to the type of oxidoreductase that is actually responsible for the observed enzymatic conversions.

In the 1980s and even still in the beginning of the 1990s only crude extracts of cyanobacteria were tested for peroxide-degrading enzymes. Catalase activity (Tel-Or *et al.*, 1985), ascorbate peroxidase activity (Miyake *et al.*, 1991), and glutathione peroxidase activity (Tözum and Gallon, 1979) have been reported. Originally, cyanobacteria were divided into two groups, those that have and those that lack ascorbate peroxidase (Miyake *et al.*, 1991). This was based on the observation that the first group scavenges H_2O_2 with a peroxidase using a photoreductant as electron donor. Thus, it was thought that—similarly to chloroplasts—the oxidation products of ascorbate peroxidase are reduced by dehydroascorbate reductase (Hossain and Asada, 1984) and monodehydroascorbate reductase (Hossain and Asada, 1985), with glutathione and NADPH as electron donors (the production of both depending on photosynthetic acitivity). However, the concentration of ascorbate in the cyanobacterial cytosol (20–100 μM) is ~250-fold lower than in the stroma of chloroplasts (Tel-Or *et al.*, 1985), and analysis of cyanobacterial genomes (Passardi *et al.*, 2007) has demonstrated unequivocally that cyanobacteria do not contain ascorbate peroxidases.

Bifunctional catalase–peroxidases (KatGs) were the first H_2O_2-dismutating cyanobacterial enzymes which were characterized by both biochemical and genetic methods. KatGs were originally isolated from the unicellular species *Synechococcus* PCC7942 (Mutsuda *et al.*, 1996; Perelman *et al.*,

Table 1. List of abbreviations and access codes of all cyanobacterial genomes or catalase and peroxidase genes of all mentioned sequences

Abbreviations	Sequenced cyanobacteria	Accession numbers
Aca.mar	*Acaryochloris marina* MBIC11017	YP_001516080
Ana.var	*Anabaena variabilis* ATCC29413	YP_3214520.1
Cro.wat	*Crocosphaera watsonii* WH8501	ZP_00514853
Cya51142	*Cyanothece* sp. ATCC51142	ZP_001804678
Cya0110	*Cyanothece* sp. CCY0110	ZP_01731923
Cya7424	*Cyanothece* sp. PCC7424	ZP_02974490
Cya8801	*Cyanothece* sp. PCC8801	ZP_02940003
Glo.vio	*Gloeobacter violaceus* PCC7421	NP_925728
Lyn8106	*Lyngbya* sp. PCC8106	ZP_01623400
Mic.aer	*Microcystis aeruginosa* NIES-843	YP_001655793
Nod.spu	*Nodularia spumigena* CCY9414	ZP_01628632
Nos.pun	*Nostoc punctiforme* PCC73102	YP_001868279
Nos7120	*Nostoc* (*Anabaena*) sp. PCC7120	NP_485408
Pro9601	*Prochlorococcus marinus* str. AS9601	YP_001009553
Pro9211	*Prochlorococcus marinus* str. MIT9211	YP_001551018
Pro9215	*Prochlorococcus marinus* str. MIT9215	YP_0014844393
Pro9301	*Prochlorococcus marinus* str. MIT9301	YP_001091387
Pro9303	*Prochlorococcus marinus* str. MIT9303	YP_001016925
Pro9312	*Prochlorococcus marinus* str. MIT9312	YP_397563
Pro9313	*Prochlorococcus marinus* str. MIT9313	NP_894962
Pro9515	*Prochlorococcus marinus* str. MIT9515	NC_008817
ProNATL1A	*Prochlorococcus marinus* str. NATL1A	YP_001015351
ProNATL2A	*Prochlorococcus marinus* str. NATL2A	YP_291889
Pro1375	*Prochlorococcus marinus subsp. marinus* str. CCMP1375	NP_875535
Pro1986	*Prochlorococcus marinus subsp. marinus* str. CCMP1986	NP_893174
Syn6301	*Synechococcus elongatus* PCC6301	YP_173174
Syn7942	*Synechococcus elongatus* PCC7942	YP_400495
Syn107	*Synechococcus* sp. BL107	ZP_01468329
Syn9311	*Synechococcus* sp. CC9311	YP_730179
Syn9605	*Synechococcus* sp. CC9605	YP_382244
Syn9902	*Synechococcus* sp. CC9902	YP_376729
SynJA23	*Synechococcus* sp. JA-2-3B'a(2-13)	YP_476915
SynJA33	*Synechococcus* sp. JA-3-3Ab	YP_47007
Syn7002	*Synechococcus* sp. PCC7002	YP_001733641
Syn307	*Synechococcus* sp. RCC307	YP_001226989
Syn9916	*Synechococcus* sp. RS9916	ZP_01470366
Syn9917	*Synechococcus* sp. RS9917	ZP_01080660
Syn5701	*Synechococcus* sp. WH5701	ZP_01084959
Syn7803	*Synechococcus* sp. WH7803	YP_001225319
Syn7805	*Synechococcus* sp. WH7805	ZP_01124489
Syn8102	*Synechococcus* sp. WH8102	NP_896813
Syc6803	*Synechocystis* sp. PCC6803	NP_441492
The.elo	*Thermosynechococcus elongatus* BP-1	NP_683219
Tri.ery	*Trichodesmium erythraeum* IMS101	YP_721847
	Non-cyanobacterial sequences	
Arch.ful.KatG	*Archaeoglobus fulgidus* DSM 4304	NP_071058
BcaMnCat	*Bacteroides caccae* ATCC 43185	ZP_01960863
BcapMnCat	*Bacteroides capillosus* ATCC 29799	ZP_02036380
BceMnCat	*Bacillus cereus* E33L	YP_084426
BcenMnCat	*Burkholderia cenocepacia* AU 1054	YP_621456
BclMnCat	*Bacillus clausii* KSM-K16	YP_175570
BhaKatG	*Bacillus halodurans* C-125	NP_241772
BhaMnCat	*Bacillus halodurans* C-125	NP_241935
BmaPxDo1	*Blastopirellula marina* DSM 3645	ZP_01093887
B.t._LPO	*Bos taurus*	NP_776358
Bur.pse. KatG1	*Burkholderia pseudomallei* K96243	YP_109459
DdPxDo1	*Dictyostelium discoideum* AX4	XP_642775
E. coli HPI	*Escherichia coli* str. K12 substr. MG1655	NP_418377
E. coli KatP	*Escherichia coli* O157:H7 EDL933	YP_325577
GtMnCat	*Geobacillus thermodenitrificans* NG80-2	YP_001127084
HalmaKatG1	*Haloarcula marismortui* ATCC 43049	YP_135827
HalsaKatG	*Halobacterium sp.* NRC-1	NP_395796
Leg.pn. KatG	*Legionella pneumophila subsp. pneumophila* str. Philadelphia 1	YP_094248
LplMnCat1	*Lactobacillus plantarum*	P60355
Ma. sp. KatG	*Marinomonas* sp. MWYL1	YP_001340448
MagKatG1	*Magnaporthe grisea* 70-15	XP_361863
MflaMnCat	*Methylobacillus flagellatus* KT	YP_545545
Mic.mar. KatG	*Microscilla marina* ATCC 23134	ZP_01693331
Msi KatG	*Methylocella silvestris* BL2	ZP_02946304
MsiMnCat1	*Methylocella silvestris* BL2	ZP_02947034
MspMnCat	*Mesorhizobium sp.* BNC1	YP_674434
Myc.tub. KatG	*Mycobacterium tuberculosis* H37Rv	NP_216424
NcrKatG1	*Neurospora crassa* OR74A	XP_959745
NmoPxDo1	*Nitrococcus mobilis* Nb-231	ZP_01126301
OsiPxDo1	*Oryza sativa* Indica group	CT836978
PaerMnCat1	*Pseudomonas aeruginosa* PAO1	NP_250875
PfMnCat1	*Pseudomonas fluorescens* Pf-5	YP_261802
PlmaPxDo1	*Planctomyces maris* DSM 8797	ZP_01857045
Pse.put. KatG	*Pseudomonas putida* KT2440	NP_745804
PYcaMnCat	*Pyrobaculum calidifontis* JCM 11548	YP_001055621
RbaPxDo1	*Rhodopirellula baltica* SH 1	NP_869799
RbaPxDo2	*Rhodopirellula baltica* SH 1	NP_864006
ReMnCat	*Ralstonia eutropha* JMP134	YP_298605
RpPxDo2	*Rhodopseudomonas palustris* BisA53	YP_783237
RxMnCat	*Rubrobacter xylanophilus* DSM 9941	YP_643463
Sal.typ. HPI	*Salmonella typhimurium*	CAA37187
SarPxDo1	*Salinispora arenicola* CNS-205	YP_001537753
SerMnCat	*Saccharopolyspora erythraea* NRRL 2338	YP_001105249
TtMnCat	*Thermus thermophilus* HB27	YP_005841
Yer.pes. KatG	*Yersinia pestis* CO92	NP_406785

2003), *Synechococcus* PCC6301 (Engleder *et al.*, 2000), and *Synechocystis* PCC6803 (Jakopitsch *et al.*, 1999; Tichy and Vermaas, 1999). Later on, the occurrence of a gene in *Nostoc punctiforme* that encodes a typical (monofunctional) catalase was reported, and also genes for putative manganese catalases (MnCats) were ascribed to *N. punctiforme* and *Nostoc* sp. PCC7120 (Regelsberger *et al.*, 2002). Furthermore, it has been demonstrated that (multiple) genes for peroxiredoxins (Prxs) (Yamamoto *et al.*, 1999; Perelman *et al.*, 2003) and glutathione peroxidases (GPxs) (Gaber *et al.*, 2001) exist in cyanobacteria and that functional enzymes are expressed. Apparently, these enzymes (and not ascorbate peroxidase) were responsible for the 'photoreductant peroxidase' described earlier in the literature.

Now, the availability of 44 partially or fully sequenced genomes, together with improved knowledge on structure–function relationships, for the first time allows a more

systematic and comprehensive analysis of peroxide-degrading enzymes in cyanobacteria, thereby setting out the framework for future studies on expression and functional role(s).

Catalases

Several gene families evolved in the ancestral genomes capable of H_2O_2 dismutation according to Reaction 3. The most abundant are haem-containing enzymes that are spread among Bacteria, Archaea, and Eukarya. They are divided into two main groups, typical or 'monofunctional' catalases (EC 1.11.1.6) and KatGs. Both types of haem enzymes exhibit high catalase activities, but have significant differences, including absence of any sequence similarity and very different active site, tertiary, and quaternary structures. Enzymatic classification of bifunctional KatGs is not clear because, besides their catalase activity (EC 1.11.1.6, hydrogen peroxide, hydrogen peroxide oxidoreductase), they exhibit a peroxidase activity similar to that of conventional peroxidases (EC 1.11.1.7, hydrogen peroxide, donor oxidoreductase). Non-haem MnCats constitute a third (minor) group of enzymes with catalase activity. MnCats (EC 1.11.1.6), initially referred to as pseudocatalases, are present only in bacteria.

Typical (monofunctional) catalase: Though being the largest group of H_2O_2-dismutating enzymes that segregated rather early in the evolution into three main clades through at least two gene-duplicating events (Zamocky *et al.*, 2008) and even occuring in anaerobic bacteria, it was interesting to see that genes for typical catalases are very unusual in cyanobacteria (Table 2). At the moment, the only complete and non-fused gene in which all essential amino acids of typical catalases are conserved (i.e. proximal haem ligand tyrosine and the conserved distal residues histidine, asparagine, and serine) (Zamocky *et al.*, 2008) is found in *N. punctiforme* PCC73102. The genome of this species in addition shows two open reading frames (ORFs) that encode MnCats (see below). *Nostoc punctiforme* catalase belongs to clade 3 of small-subunit catalases that contain haem *b* at the active site and use NADPH as a second redox-active cofactor. A recently performed phylogenetic analysis (Zamocky *et al.*, 2008) suggests a lateral gene transfer from an ancestral proteobacterium to *N. punctiforme*. Incomplete (C-terminal truncated) catalase genes are found in *Synechococcus elongatus* PCC7942 and *Cyanothece* sp. ATCC 51142. The putative proteins would contain all essential amino acids but lack the C-terminal α-helical domain that is known to be essential in folding of typical catalases (Chelikani *et al.*, 2004). Another protein of this family in *Nostoc* sp. PCC7120 is part of a fusion protein between a typical catalase-related domain and a putative lipoxygenase domain, with significant similarity to the well-investigated fusion protein of allene oxide synthase from *Plexaura homomalla* and related corals (Oldham *et al.*, 2005).

The physiological role of the typical catalase in *N. punctiforme* is unknown. From the published kinetic parameters of this protein family [k_{cat} within 54 000–833 000 s^{-1}, K_M within 38–600 mM and activities that are essentially pH independent from pH 5 to pH 10) (Chelikani *et al.*, 2003)], it is reasonble to assume that it has a protective role against environmental H_2O_2 generated in the ecosystem similar to the proposed role of bifunctional KatGs (see below).

Bifunctional catalase–peroxidase: In contrast to typical catalases, ~30% of all known cyanobacterial genomes contain one gene (*katG*) encoding a bifunctional KatG. KatG represents the only peroxidase of both haem peroxidase superfamilies with a reasonable high catalase activity (Reaction 3), besides a usual peroxidase activity (Reaction 1). Together with ascorbate peroxidases, present in chloroplastic organisms, and cytochrome *c* peroxidase, found mainly in mitochondrial organisms, KatGs constitute class I of the plant, fungal, protist, and bacterial haem peroxidase superfamily (Welinder, 1992; Passardi *et al.*, 2007). The predominant form of haem *b*-containing KatGs in solution is a dimer or tetramer (Welinder, 1992; Chelikani *et al.*, 2004), and each subunit is composed of two distinct sequence-related N- and C-terminal domains, which led to the proposal that the large gene size of KatG had arisen through a gene duplication and fusion event of a primordial peroxidase gene after which the C-terminal domain likely lost its functionality (Welinder, 1991).

The evolution of KatGs was analysed recently (Passardi *et al.*, 2007). The most important output of this investigation is that *katG* genes are distributed in ~40% of bacterial genomes, and sometimes even closely related species differ in possessing *katG* genes of different origin or even do not possess any *katG* genes. It has to be mentioned that KatGs are also found in eukaryotes, including some algae, protists, and fungi. Phylogenetic analysis (Fig. 1) reveals the presence of a distinct and well-segregated clade of cyanobacterial KatGs, suggesting early segregation in evolution. In addition, at least two independent lateral gene transfers occurred during the evolution of this protein family between various bacterial taxa and ancestors of (i) a group formed by *S. elongatus* PCC6301, *Synechococcus* PCC7002, and *Synechocystis* PCC6803; (ii) *Gloeobacter violaceus*; and (iii) most probably *Acaryochloris marina* (Fig. 1). *Cyanothece* sp. CCY0110 is the only diazotrophic cyanobacterium with a *katG* gene; all other species that contain this haem protein are unable to fix nitrogen (Table 2). No paralogues are found within one cyanobacterial species.

The four available crystal structures of the KatGs, i.e. those for *Haloarcula marismortui* (1ITK) (Yamada *et al.*, 2002), *Burkholderia pseudomallei* (1MWV) (Carpena *et al.*, 2003), *Mycobacterium tuberculosis* (1SJ2) (Bertrand *et al.*, 2004), and of the cyanobacterium *Synechococcus* PCC7942 (1UB2) (Wada *et al.*, 2002), revealed that KatGs have proximal and distal conserved amino acids at almost identical positions to those in other class I peroxidases (Smulevich *et al.*, 2006). In particular, both the triads histidine/tryptophan/aspartate (His259, Trp341, and Asp402; *Synechocystis* PCC6803 numbering) and histidine/arginine/tryptophan (His123, Arg119, and Trp122) are conserved (Figs 2, 3A). Moreover, the distal histidine is

Table 2. ORFs and genes of cyanobacterial catalases and peroxidases in 44 completely or partially (*) sequenced strains (including genome size)

Nitrogen-fixing cyanobacteria are shaded in light grey, and heterocyst-forming species in dark grey. Classification of cyanobacteria: I, Pleurocapsales; II, Chroococcales; III, Oscillatoriales; IV, Nostocales; V, unclassified. The percentage quantifies differences within the species of the *Prochlorococcus* and the *Synechococcus* group.

Cyanobacteria	Genome size (Mb)	Catalases			Peroxidases							
		(Monofunctional) haem catalase	(Class 1) Catalase–peroxidase	(Binuclear) manganese catalase	Peroxi-dockerin-related peroxidase	Dihaem peroxi-dase	Vanadium peroxidase	Peroxiredoxin				Glutathione peroxidase-related peroxidase
								1-cys Prx	2-cys Prx	Type II Prx	PrxQ	
12×*Prochlorococcus marinus*[a] (I)	1.6–2.7								1	15% 1 85% 0	85% 2 15% 1	1
Gloeobacter violaceus PCC7421 (II)	4.6		1	1				1	1		4	1
Microcystis aeruginosa NIES-843 (II)	5.8							1	1	1	3	
Synechococcus elongatus PCC6301 (II)	2.7		1					1	1		4	1
Synechococcus elongatus PCC7942 (II)	2.7	1[c]	1					1	1		4	1
14×*Synechococcus sp.*[b] (II)	2.2–3.0		60% 1 40% 0				5% 1 95% 0	40% 1 60% 0	1	15% 1 85% 0	5% 4 45% 3 45% 2 5% 1	5% 2 80% 1 15% 0
Synechocystis sp. PCC 6803 (II)	3.6		1					1	1	1	2	2
Thermosynechococcus el. BP-1 (II)	2.6							1	1		3	
Acaryochloris marina (V)	6.5		1		1	(2?)	2 (1)	2	1	1	4	
Crocosphaera watsonii WH8501* (II)	6.2				1		1	1	1	1	2	1
Cyanothece sp. ATCC51142* (II)	4.9	1[c]			1		(1)	1	1	1	2	
Cyanothece sp. CCY0110* (II)	5.9		1	1	1			1	1	1	3	
Cyanothece sp. PCC7424* (II)	5.9			3				1	1	1	4	
Cyanothece sp. PCC8801* (II)	5.9							1	1	1	3	1
Lyngbya sp. PCC8106*(III)	7.0			1	1			1	1	1	3	
Trichodesmium erythraeum IMS101 (III)	7.7							1	1	1	3	
Anabaena variabilis ATCC29413 (IV)	6.3			1		(1?)		1	1	1	4	
Nodularia spumigena CCY9414* (IV)	5.3			1			1	1	1	1	4	
Nostoc punctiforme PCC73102* (IV)	9.0	1		2				1	1	1	4	1
Nostoc (*Anabaena*) *sp.* PCC7120 (IV)	6.4	1[c]		2				1	1	1	4	

[a] The *Prochlorococcus marinus* genus includes the following strains: Pro9601, Pro9211, Pro9215, Pro9301, Pro9303, Pro9312, Pro9313, Pro9515, ProNATL1A, ProNATL2A, Pro1375, and Pro1986.

[b] The following strains are included in the *Synechococcus* group: Syn107*, Syn9311, Syn9605, Syn9902, SynJA23, SynJA33, Syn7002, Syn307, Syn9916*, Syn9917*, Syn5701*, Syn7803, and Syn7805*.

[c] Pseudo-gene (incomplete or fusion gene).

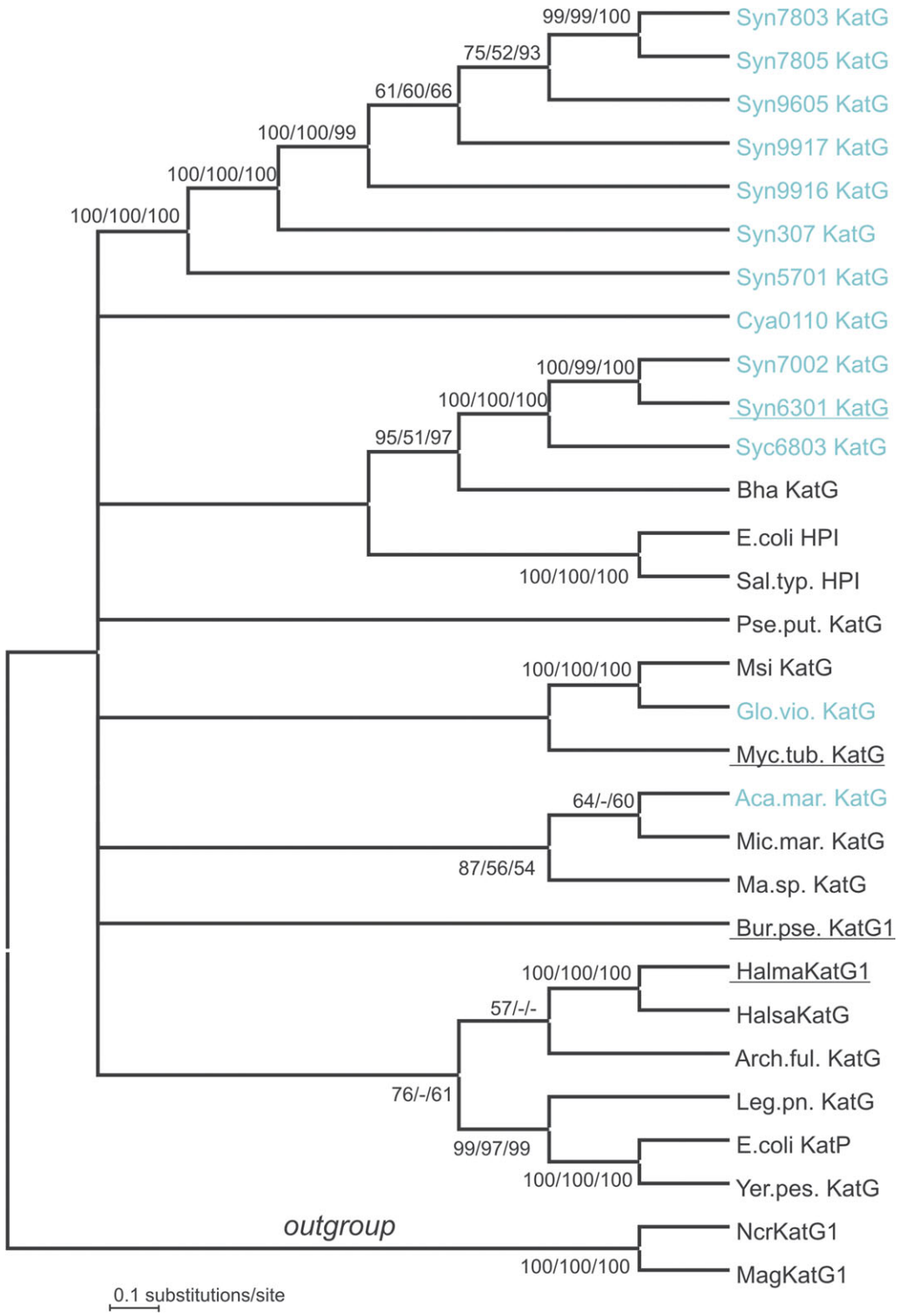

Fig. 1. Reconstructed phylogenetic tree of 30 catalase–peroxidases (KatGs) rooted with an outgroup (i.e. fungal KatGs). Presented is the tree obtained by the MEGA4 package (Tamura *et al.*, 2007). The Neighbor–Joining method was applied with 1000 bootstrap replications and the Jones–Taylor–Thornton model of amino acid substitutions. Further, complete deletion of gaps, a homogenous pattern among lineages, and uniform rates among sites were used. Nearly identical trees were also obtained with the maximum parsimony method (within the MEGA4 package, 1000 bootstraps) and with the maximum likelihood method (from Phylip package 3.68 http://evolution.gs.washington.edu/phylip.html 100 bootstraps). The reconstructed consensus tree is presented using the Tree Explorer included in the MEGA package. Numbers on the branches represent bootstrap values as obtained from NJ/MP/ML, respectively (only values >50% are presented; lower values are denoted '-'). Cyanobacterial KatGs are highlighted in cyan. For abbreviations, gene assignments, and accession numbers of cyanobacterial KatGs, see Table 1. Sequences with known 3D structures are underlined. The scale bar indicates the frequency of substitutions per site.

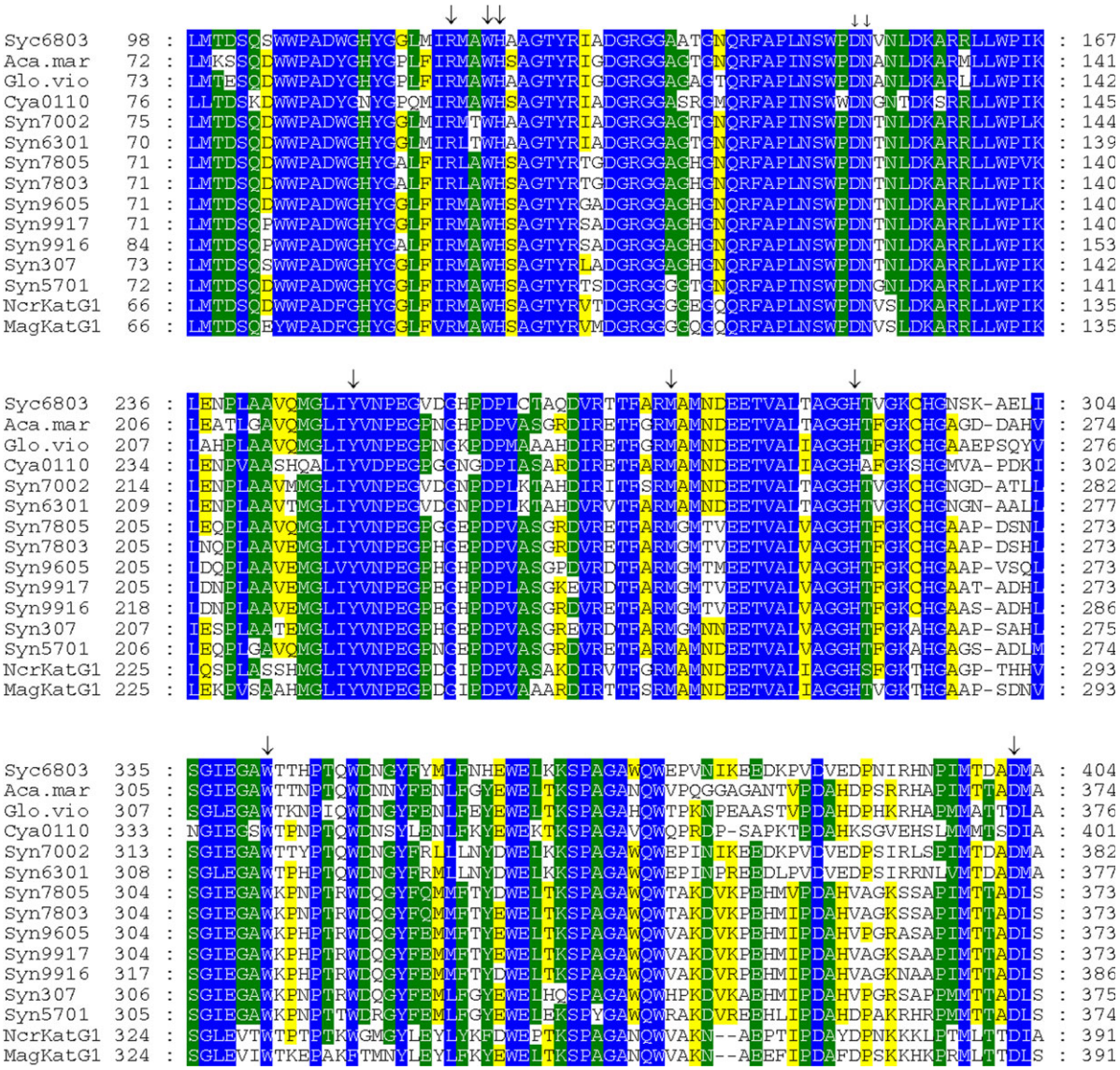

Fig. 2. Selected parts of the multiple sequence alignment of all cyanobacterial catalase–peroxidases (KatGs) sequenced so far. Amino acid sequences were extracted from the NCBI protein database. For sequence similarity searches, PHI and PSI Blast were used (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple protein sequence alignments were performed by applying two different alignment programs, namely ClustalX, version 1.81, (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) (Jeanmougin *et al.*, 1998) with the following parameters: gap opening 10.0, gap extension 0.20, and the gonnet protein weight matrices; and Kalign, version 2 (http://msa.cgb.ki.se/cgi-bin/msa.cgi) with gap opening 11.0, gap extension 0.85 and terminal gap 0.45 were set as parameters (Lassmann *et al.*, 2005). Colour scheme: the highest similarity is presented on a blue background, high similarity on green, and low similarity on a yellow background. For abbreviations, gene assignments, and accession numbers of cyanobacterial species see Table 1. Arrows indicate essential active site residues.

hydrogen-bonded to a conserved asparagine (Asn153) and the proximal histidine is hydrogen-bonded to the carboxylate side chain of the nearby aspartate residue which, in turn, is hydrogen-bonded to the nitrogen atom of the indole group of the nearby tryptophan residue (Fig. 3A). However, the X-ray structures also revealed features unique to KatG. In the vicinity of the active site, novel covalent bonds are formed among the side chains of three distal residues including the conserved Trp122 (Fig. 3A). In particular, both X-ray crystallization data (Yamada *et al.*, 2002; Carpena *et al.*, 2003; Bertrand *et al.*, 2004) and mass spectrometric analysis (Donald *et al.*, 2003; Jakopitsch *et al.*, 2003) have confirmed the existence of a covalent adduct between Trp122, Tyr249, and Met275. Sequence alignment suggests that all these structural features are fully conserved in cyanobacterial KatGs (Fig. 2).

Other KatG-typical features also found in cyanobacterial proteins are three large loops, two of them showing highly conserved sequence patterns (Zamocky *et al.*, 2001) and constricting the access channel of H_2O_2 to the prosthetic haem *b* group at the distal side. The channel is characterized by a pronounced funnel shape and a continuum of water

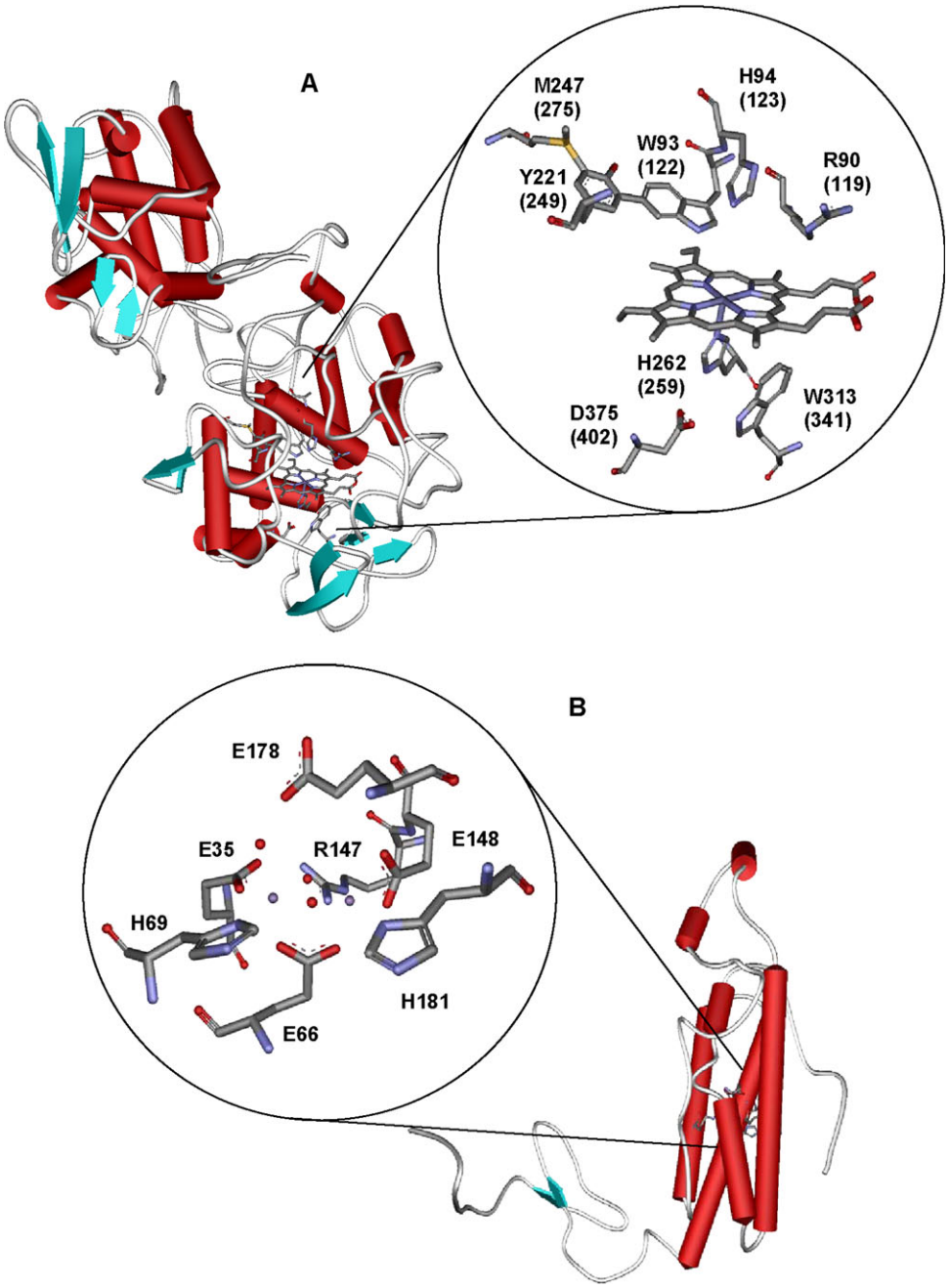

Fig. 3. (A) KatG monomer and assignment of secondary structure elements as well as the active site of dimeric catalase–peroxidase from the cyanobacterium *Synechococcus* PCC7942 (accession code 1UB2) (Wada *et al.*, 2002) (*Synechocystis* numbering in parentheses). Each subunit is composed of two distinct sequence-related N- and C-terminal domains. Only the N-terminal domain contains haem *b*. Active site residues include the proximal triad His–Trp–Asp and the distal triad His–Arg–Trp, as well as the unique covalent adduct Trp–Tyr–Met. (B) Monomeric structure and view of the dinuclear manganese complex at the active site of dimeric manganese catalase from *Lactobacillus plantarum* (accession code 1JKU) (Barynin *et al.*, 2001). In the monomeric structure, all secondary structure elements and the prosthetic group are assigned. In the active site structure, all coordinating ligands are depicted. Manganese ions are represented by violet spheres, and coordinated solvent is represented in red spheres. The figures were built using SWISS-PDB Viewer (www.expasy.ch/spdbv).

molecules. At the narrowest part of the channel, which is similar but longer and more restricted than that in typical (monofunctional) peroxidases, two highly conserved residues, namely Asp152 and Ser335, control the access to the distal haem side. Together with a conserved glutamate residue at the entrance, both acidic residues seem to be critical for stabilizing the solute matrix and orienting the water dipoles in the channel. Exchange of both residues affects the catalase but not the peroxidase activity (Jakopitsch *et al.*, 2005).

This extensive distal site hydrogen-bonding network causes KatGs to differ from typical peroxidases. Typically, the

catalase but not the peroxidatic activity is very sensitive to mutations that disrupt this network (Heering *et al.*, 2002; Jakopitsch *et al.*, 2004). Moreover, the integrity of this network is crucial for the formation of distinct protein radicals that are formed upon incubation of KatG with peroxides (Ivancich *et al.*, 2003). Mutational analysis clearly underlined the importance of the KatG-typical covalent adduct tryptophan–tyrosine–methionine for H_2O_2 dismutation. Its disruption significantly decreased the catalase but not the peroxidase activity. Similarly, all other mutations so far performed in the haem cavity or substrate channel of a KatG affected the oxidation but not the reduction reaction of H_2O_2 (Smulevich *et al.*, 2006), which is the initial reaction in all haem catalases and peroxidases.

Dismutation of H_2O_2 according to Reaction 3 follows a two-step mechanism. Heterolytic cleavage of H_2O_2 (i.e. compound I formation) follows Reaction 4. It is important to note that the exact electronic structure of formed compound I, that actually oxidizes the second H_2O_2 according to Reaction 5 (i.e. compound I reduction), is still speculative (Jakopitsch *et al.*, 2007). Conventional compound I is a oxoiron(IV) porphyrin π-cation radical species [$^{\cdot+}$Por Fe(IV)=O] that in KatG could be in equilibrium with an alternative (H_2O_2-oxidizing?) compound I [$^{+}$AA Por Fe(IV)=O], with AA being a so far not localized amino acid radical (Jakopitsch *et al.*, 2007). One role of the KatG-specific tryptophan–tyrosine–methionine adduct might be to provide the (at least transient) radical site [$^{\cdot+}$MYW Fe(IV)=O] that quenches the porphyryl radical according to Reaction 4 and participates in H_2O_2 oxidation (Reaction 5). Similar to typical peroxidases, compound I formation is clearly assisted by the conserved distal residues His123 and Arg119 (Smulevich *et al.*, 2006), but the exact role of specific distal amino acids in H_2O_2 oxidation is still speculative.

$$\mathrm{Por\,Fe(III)} + \mathrm{H_2O_2} \rightarrow \left[{}^{\cdot+}\mathrm{Por\,Fe(IV)=O} \leftrightarrow {}^{\cdot+}\mathrm{AA\,Por\,Fe(IV)=O}\right] + \mathrm{H_2O} \qquad \text{Reaction 4}$$

$$\left[{}^{\cdot+}\mathrm{Por\,Fe(IV)=O} \leftrightarrow {}^{\cdot+}\mathrm{AA\ Por\ Fe(IV)=O}\right] + \mathrm{H_2O_2} \rightarrow \mathrm{Por\ Fe(III)} + \mathrm{H_2O} + \mathrm{O_2} \qquad \text{Reaction 5}$$

Both k_{cat} and K_M values of KatGs are significantly lower compared with typical catalases. Apparent values range from 3500 s^{-1} to 7500 s^{-1} and from 3.7 mM to 8 mM (Smulevich *et al.*, 2006), respectively, and the two so far kinetically investigated cyanobacterial representatives fall within this range (Mutsuda *et al.*, 1996; Jakopitsch *et al.*, 1999). In contrast to typical catalases and MnCats, the catalase activity of KatG has a sharp maximum activity at ~pH 6.5.

KatG is a bifunctional enzyme. It oxidizes typical artificial peroxidase substrates such as *o*-dianisidine, guaiacol, or ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)]. The pH profile of the peroxidase activity of KatG has a maximum within pH 5–6 depending on the nature of the one-electron donor. Additionally, KatGs have been reported also to have halogenation (Jakopitsch *et al.*, 2001) and NADH oxidase (Singh *et al.*, 2004) activity.

The naturally occurring peroxidase substrate is unknown. In physiological conditions, it is well known that KatG from *Mycobacterium tuberculosis* can activate the anti-tuberculosis drug isoniazid (Zhao *et al.*, 2006). Due to the restricted access, only small peroxidase substrates can enter the main entrance channel. A second access route, found in monofunctional peroxidases, approximately in the plane of the haem, is blocked by the KatG-typical loops. However, another potential route that provides access to the core of the protein, between the two domains of the subunit, has been described (Carpena *et al.*, 2003). It has been speculated that this could be the binding site for substrates with extended, possibly even polymeric, character. In any case, neither the naturally occurring one-electron donor(s) nor the function of a high peroxidase activity in an enzyme with catalase activity is known either in prokaryotic or in eukaryotic KatGs. A reasonable role at low peroxide concentration could be the reduction of inactive compound II species to ferric KatG.

It has been proposed that KatG in *Synechocystis* PCC6803 has a protective role against environmental H_2O_2 generated in the ecosystem and that this protective role is most apparent at a high cell density of the cyanobacterium (Tichy and Vermaas, 1999). Deletion of the *katG* gene in *Synechocystis* PCC6803 resulted in a mutant strain with normal phenotype and resistance to H_2O_2 and methyl viologen indistinguishable from those of the wild type. Nevertheless the rate of H_2O_2 decomposition in the Δ*katG* mutant was ~30 times lower than in the wild type (Tichy and Vermaas, 1999). Apparently the residual H_2O_2-scavenging capacity was more than sufficient to deal with the rate of H_2O_2 production by the cell. In the Δ*katG* mutant, H_2O_2 degradation was light dependent and could be stimulated by addition of a thiol such as dithiothreitol. The authors concluded that this activity might derive from a thiol-specific peroxidase, for which thioredoxin is the physiological electron donor. This has been underlined by a bioinformatic analysis of the genome of *Synechocystis* PCC6803 (Stork *et al.*, 2005) that revealed the presence of five ORFs with similarity to Prxs (see below and also Table 2).

Similarly, investigation of *Synechococcus* sp. PCC7942 (Miller *et al.*, 2000; Perelman *et al.*, 2003) has also demonstrated the existence of two classes of H_2O_2-degrading enzymes, namely a (cytosolic) KatG, that is essential for survival and elimination of relatively high concentrations of externally added H_2O_2, and light-dependent peroxidase(s) that are essential during excessive radiation (see also Table 2). Despite addition of ^{18}O-labelled H_2O_2 in the light, only $^{16}O_2$ production was observed, indicative of $H_2^{16}O$ oxidation by PSII and formation of a photoreductant. However, in the dark, added $H_2^{18}O_2$ led to release of $^{18}O_2$ that could be completely blocked by NH_2OH, an inhibitor of KatG (Miller *et al.*, 2000). This also suggests a temporal difference in activity of Prxs and KatG.

Manganese catalase: MnCats (non-haem or di-manganese catalases; EC 1.11.1.6) represent only a minor gene family with catalase activity that is also found in cyanobacteria.

With the exception of *G. violaceus* PCC7421, only diazotrophic species contain at least one ORF with similarity to MnCat. Two paralogues are found in heterocyst-forming *N. punctiforme* (that also contains a typical catalase) and *Nostoc* PCC7120 (Table 2), and three paralogues exist in the genome of *Cyanothece* sp. PCC7424. Interestingly, with the exception of *G. violaceus* and *Cyanothece* sp. CCY0110, cyanobacteria contain either a *katG* gene or gene(s) encoding MnCat(s), and ~50% of the investigated species contain neither a typical catalase, nor a KatG or MnCat (e.g. all *Prochlorococcus marinus* strains, *Microcystis aeruginosa*, *Synechococcus* el. BP-1 etc., compare with Table 2). Only in all genomes of heterocyst-forming diazotrophic cyanobacteria known so far was at least one gene encoding MnCat detected (Table 2).

A recently performed phylogenetic analysis of bacterial MnCats (Zamocky *et al.*, 2008*a*) reveals the presence of five distinct and well segregated clades. The present analysis (Fig. 4), that includes all available cyanobacterial genes, demonstrates that cyanobacterial MnCats are found in clade 3 (with Firmicutes and proteobacteria) as well as in clade 4 (with the genus *Bacteroides*) that was separated rather early from the common ancestor. It is interesting to note that some authors argued for a role for di-manganese catalase in still anoxygenic (cyano?)bacteria as a forerunner of Mn-containing PSII (McKay and Hartman, 1991; Lane, 2002). It was noted that the active site structure of MnCat reveals similarity to the tetranuclear manganese complex of the oxgygen-evolving centre of PSII (Blankenship *et al.*, 1998).

So far, neither an expression study nor a functional or structural analysis of a cyanobacterial MnCat is found in literature. However, multiple sequence alignment (Fig. 5) reveals a rather high identity mainly in the active site residues with two enzymes of known crystal structure,

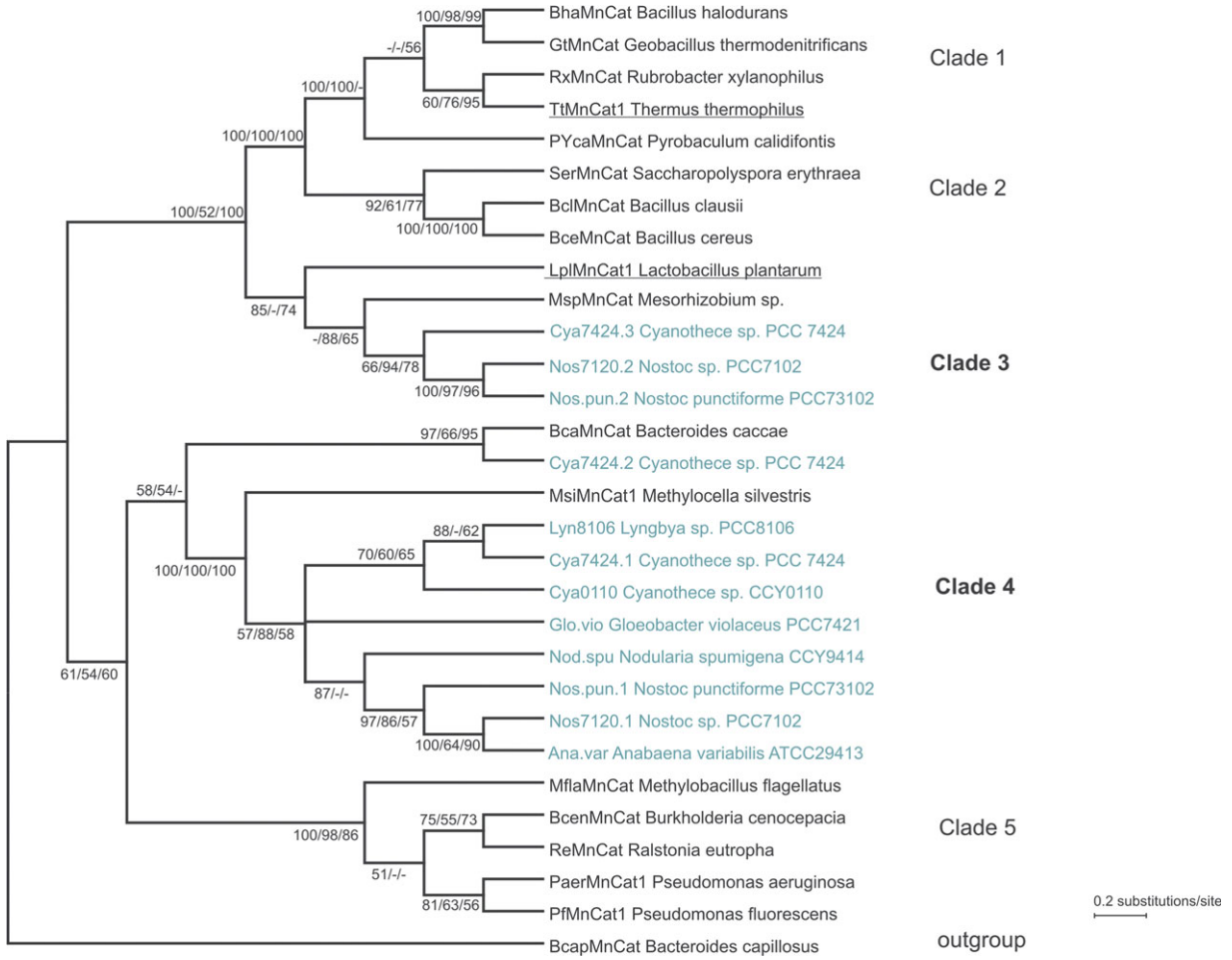

Fig. 4. Reconstructed phylogenetic tree of 30 manganese catalases rooted with an outgroup. Presented is the tree obtained with the MEGA4 package (Tamura *et al.*, 2007). The Neighbor–Joining method was applied with 1000 bootstrap replications and the Jones–Taylor–Thornton model of amino acid substitution. Very similar trees were also obtained with the maximum parsimony method (within the MEGA4 package, 1000 bootstraps) and with the maximum likelihood method (from Phylip package 3.68 http://evolution.gs.washington.edu/phylip.html using 100 bootstraps). Numbers on the branches represent bootstrap values as obtained from NJ/MP/ML, respectively (only values >50% are presented, lower values are denoted '-'). Cyanobacterial manganese catalases are highlighted in cyan. For abbreviations, gene assignments, and accession numbers of cyanobacterial sequences, see Table 1. Sequences with known 3D structures [*Thermus thermophilus* (TtMnCat1) and *Lactobacillus plantarum* (LplMnCat1)] are underlined. The scale bar indicates the frequency of substitutions per site.

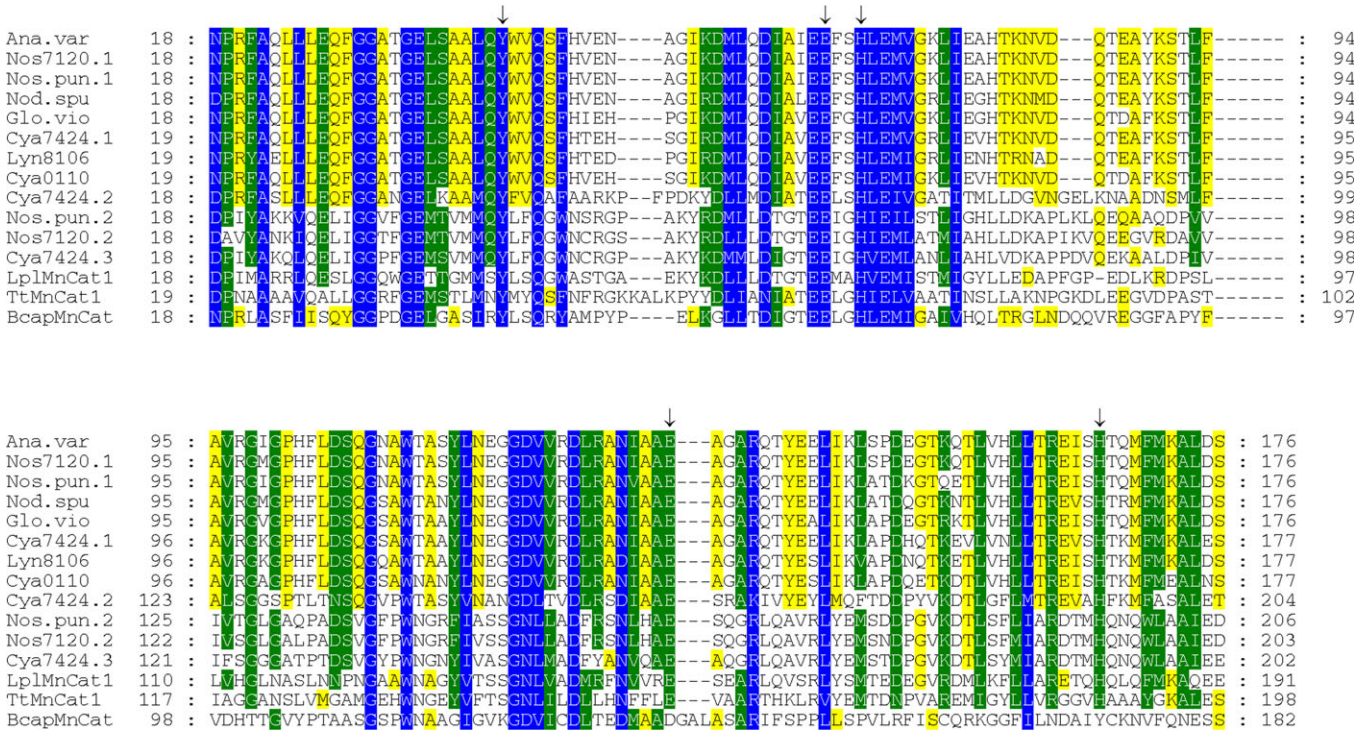

Fig. 5. Selected parts of the multiple sequence alignment of all cyanobacterial manganese catalases sequenced so far including manganese catalases of known crystal structure [*Thermus thermophilus* (TtMnCat1) and *Lactobacillus plantarum* (LplMnCat1)]. Colour scheme: the highest similarity is presented on a blue background, high similarity on green, and a low similarity on a yellow background. Arrows indicate conserved residues involved in ligation of the binuclear manganese centre as well as an essential outer sphere tyrosine that is important in maintanance of an intact hydrogen-bonding network. For abbreviations, gene assignments, and accession numbers of cyanobacterial strains, see Table 1.

namely the MnCats from *Thermus thermophilus* (Antonyuk *et al.*, 2000) and *Lactobacillus plantarum* (Barynin *et al.*, 2001). These proteins contain six identical subunits (~30 kDa) each with a bridged binuclear Mn centre located within a conserved closely packed four-helix bundle domain (Fig. 3B). The ligands for the di-manganese centre are almost invariantly conserved among all known sequences (Fig. 3B). A bridging glutamate (Glu66 in *L. plantarum* numbering) anchors the two ions in the binuclear cluster. Each Mn ion is further coordinated by one histidine (His69 to Mn1 and His181 to Mn2) and one glutamate (Glu35 to Mn1 and Glu148 to Mn2) bound to opposite faces of the cluster (Whittaker *et al.*, 2003) (Fig. 3B). These essential ligands are highly conserved, forming typical signatures of MnCat sequence (Fig. 5), whereas the environments of these ligands differ slightly. The manganese core is completed by two solvent-derived oxygen atom bridges.

Access to the binuclear centre is via a central channel that extends the full width of the hexamer, with branches into each subunit. Each di-manganese centre is embedded in a network of hydrogen bonds that radiate from the metal centre towards the outer sphere environment. The length (~15 Å) and narrowness of these channels provide a restricted access to the only substrate, H_2O_2. Mutational analysis of an essential outer sphere tyrosine (Tyr42), which is conserved in all sequences so far available, clearly demonstrated the importance of an intact hydrogen-bonding network also in MnCats (Whittaker *et al.*, 2003). In the variant Tyr42Phe, a solvent bridge was broken and an 'open' form of the di-manganese cluster was generated, thereby considerably influencing the catalytic turnover, pH optimum, and interaction with H_2O_2.

Though following the same overall reaction (Reaction 3), the mechanism of the catalase reaction in MnCat differs significantly from that of KatGs. The di-manganese cluster is equally stable in either Mn^{2+}–Mn^{2+} or Mn^{3+}–Mn^{3+} oxidation states. Upon isolation, the protein is frequently in a mixture of both oxidation states. Full reduction to Mn^{2+}–Mn^{2+} is mediated by hydroxylamine, whereas reoxidation to the homogenous Mn^{3+}–Mn^{3+} state can be achieved by molecular oxygen at pH >7.0 (Whittaker *et al.*, 2003). The catalase reaction can be written as a two-stage process. During catalytic turnover, the active site has to accommodate both the reduced and the oxidized state of the di-manganese core. The Mn^{2+}–Mn^{2+} cluster is expected to polarize the O–O bond, favouring a heterolytic cleavage of the peroxidic bond and release of water (Reaction 6) (Whittaker *et al.*, 2003). H_2O_2 oxidation and dioxygen release occur by a simple electron transfer (Reaction 7). In principal there is no temporal order to the reduction and oxidation stages.

$$H_2O_2 + Mn^{2+} - Mn^{2+}\left(2H^+\right) \rightarrow Mn^{3+} - Mn^{3+} + 2\,H_2O$$

Reaction 6

$$H_2O_2 + Mn^{3+} - Mn^{3+} \rightarrow Mn^{2+} - Mn^{2+}(2H^+) + O_2 \quad \text{Reaction 7}$$

It is assumed that the change in the oxidation status is responsible for protonation or deprotonation of both bridging oxygens. Protonation is proposed for the reduced Mn^{2+}–Mn^{2+} state (see Reaction 6). In contrast to haem-containing catalases, no reactive intermediate is formed and both product waters are formed in one reaction (Reaction 6). There is no evidence for involvement of free radicals in MnCat turnover, and nothing is known about a peroxidatic reactivity of these oxidoreductases.

The H_2O_2 dismutation rates are lower compared with typical catalases and KatGs, which could explain that MnCats may not have become as widespread in nature. The activity varies only slightly over the pH range pH 5–10, very similar to that of typical catalases. The apparent K_M values are reported to be ~220 mM (Whittaker *et al.*, 2003), suggesting a low catalytic efficiency at low H_2O_2 concentrations. Their exact physiological role(s) and expression pattern are more or less unknown.

Peroxidases

Here, it is reasonable to distinguish between haem and non-haem enzymes. From the two haem peroxidase superfamilies, i.e. the superfamily of plant, fungal, protist, and (archae)bacterial peroxidases (Welinder, 1992), and the peroxidase–cyclooxygenase superfamily (Zamocky *et al.*, 2008*b*), two types of haem peroxidases are found. Bifunctional (class 1) KatG is the only cyanobacterial member of the first superfamily and—due to its overwhelming catalase activity—has been discussed above. Most interestingly, cyanobacterial peroxidases might be at the origin of the second (i.e. peroxidase–cyclooxygenase) superfamily since ORFs with similarity to animal peroxidases were found within the subfamily of peroxidockerins (Zamocky *et al.*, 2008). Whereas genes encoding typical catalases, KatGs, and MnCat appear to have been acquired by lateral gene transfer in cyanobacteria, there is phylogenetic evidence that proteins from the peroxidase–cyclooxygenase superfamily originate from ancient cyanobacteria (see below). Furthermore two cyanobacterial genomes show the occurrence of ORFs with similarity to putative dihaem peroxidase.

The most important group of non-haem peroxide-degrading enzymes is represented by thiol-specific peroxidases including Prxs and GPxs. All four subclasses of Prxs found in plants also occur in cyanobacteria (Table 2) (Stork *et al.*, 2005). ORFs with similarity to those of GPxs are found in the majority of the cyanobacterial genomes, but so far no GPx activity could be measured in these organisms (Tichy and Vermaas, 1999). Furthermore, few cyanobacterial genomes show the occurrence of ORFs with similarity to putative vanadium peroxidase.

Peroxidockerin-related (primordial) peroxidase: It was interesting to see that some cyanobacterial genomes contain genes that encode a putative protein with similarity to mammalian peroxidases, such as lactoperoxidase (LPO) or eosinophil peroxidase (EC 1.11.1.7). So far in six cyanobacteria the corresponding ORF of this putative peroxidase is found, that—together with peroxidockerins—constitutes an old subfamily of the peroxidase–cyclooxygenase superfamily of haem peroxidases (Zamocky *et al.*, 2008*b*). Peroxidockerins are multidomain proteins of unknown physiological role composed of a transmembrane domain, dockerin type 1 repeats, and the catalytic peroxidase domain. The homologous cyanobacterial proteins lack the non-enzymatic domains and cluster separately within this subfamily (Zamocky *et al.*, 2008).

The sequence alignment shown in Fig. 6 (together with bovine LPO and peroxidockerins of mixed origin) clearly underlines that all catalytic residues that are known to be essential in the catalysis of mammalian peroxidases (Furtmüller *et al.*, 2006) are also found in these primordial haem peroxidases. (i) The essential motif -WGQXVDHD- with the distal histidine in the immediate neighbourhood of two aspartate residues that are involved in haem to protein linkage and Ca^{2+} binding. Moreover, this region also contains a highly conserved glutamine that is essential in halide binding (Furtmüller *et al.*, 2006). (ii) A second motif with two essential residues is -RXXE- that contains the catalytic distal arginine and a glutamate that is also involved in the second haem to protein ester bond in LPO. (iii) The proximal ligand histidine and its hydrogen-bonding partner asparagine (arrows in Fig. 6).

It is completely unknown whether these proteins are expressed in cyanobacteria. However, inspection of the sequence (Fig. 6) suggests a functionality very similar to that of the corresponding mammalian peroxidases that use small anionic substrates such as halides (bromide, iodide) and thiocyanate as substrates to fulfil their physiological role, i.e. to produce hypohalous acids and hypothiocyanate according to Reaction 2. These oxidizing and halogenating agents participate in unspecific immune defence of mammals against pathogens. It is tempting to speculate whether some cyanobacterial species have also acquired the ability to produce antimicrobial compounds, possibly providing selective advantage.

Dihaem peroxidase and vanadium peroxidase: Bacterial dihaem cyrochrome *c* peroxidases (DiHCCPs) catalyse the two-electron reduction of H_2O_2 to water according to Reaction 1 by soluble one-electron donors such as cytochrome *c* and cupredoxins (Echalier *et al.*, 2006). However, unlike their eukaryotic counterparts, they possess two haem *c* prosthetic groups, that are covalently attached to a single polypeptide chain via two cysteines (-CXXCH-), and allow reduction of H_2O_2 without the need to generate (semi-stable) radicals. The two haem groups are designated (low potential, N-terminal) haem P (peroxidatic), that actually reacts with peroxides and ligands, and (high potential, C-terminal) haem E (electron transferring). In the resting (oxidized) state, haem P is coordinated by two histidine ligands and the E haem is coordinated by a histidine and a methionine (Fülöp *et al.*, 1993). Activation of the peroxidase needs reduction of haem E

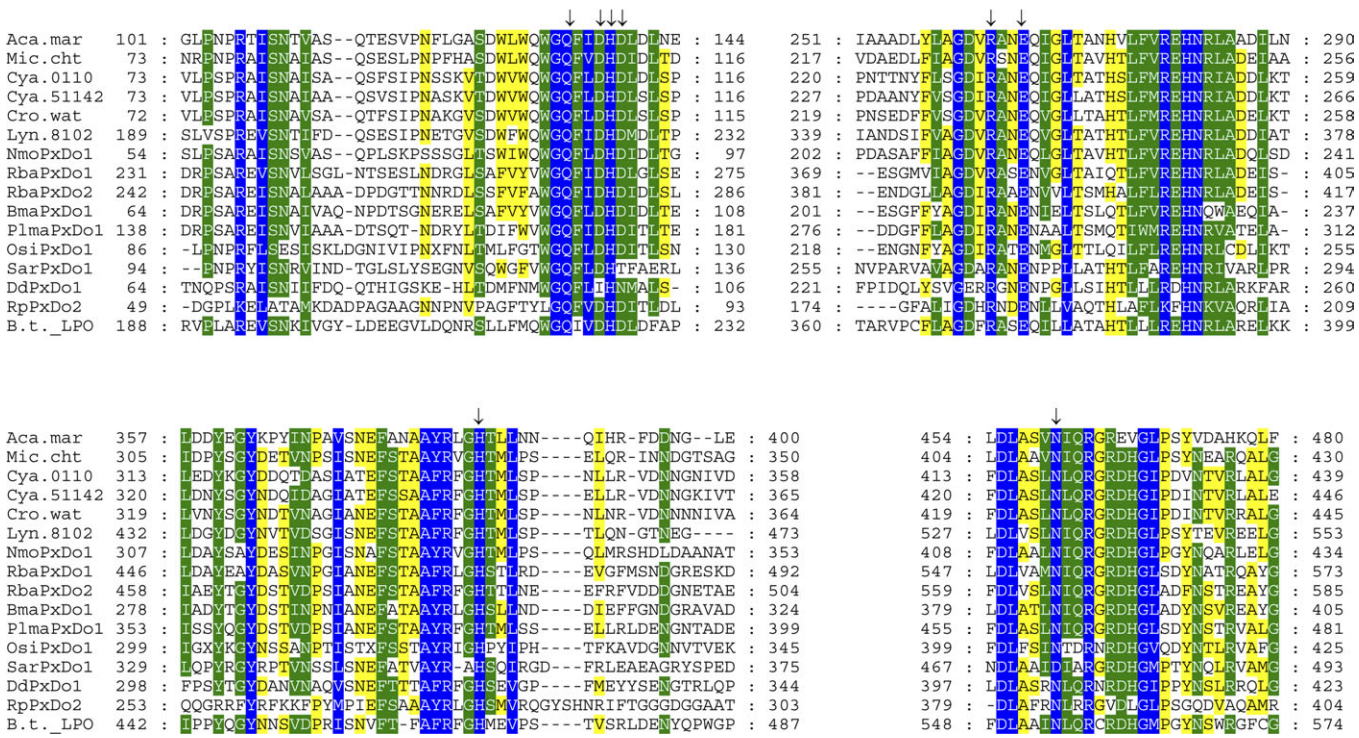

Fig. 6. Selected parts of the multiple sequence alignment of all cyanobacterial peroxidases sequenced so far from the peroxidase–cyclooxygenase superfamily of haem peroxidases including the sequence of bovine lactoperoxidase (LPO) and peroxidockerins (PxDos) of various origins (see Zamocky *et al.*, 2008*b*). Colour scheme: the highest similarity is presented on a blue background, high similarity on green, and low similarity on a yellow background. Arrows indicate conserved residues involved in haem ligation and covalent linkage as well as in catalysis as known from LPO. For abbreviations, gene assignments, and accession numbers, see Table 1.

by a small redox donor protein in order to activate haem P that includes dissociation of the coordinating distal histidine in order to allow access of the substrate(s) (Echalier *et al.*, 2006).

Three ORFs with similarity to bacterial DiHCCP were found in the genomes, one in *Anabaena variabilis* ATCC29413 and two in *A. marina* (Table 2). Analyis of the three sequences (not shown) suggests the presence of two haem *c* prosthetic groups. However, N-terminal haem P seems to lack the distal histidine, and at least in two sequences the distal coordination of haem E is unclear. Thus, the functionality of these putative proteins is not fully clear and needs further investigation. No information about (bacterial) DiHCCP-related proteins in cyanobacteria was found in the literature.

Some cyanobacterial strains in addition contain ORFs with similarity to vanadium-containing peroxidases. Vanadium-dependent enzymes are often designated as haloperoxidases (bromoperoxidase, iodoperoxidase) since they exclusively oxidize halides according to Reaction 2 and are usually found in brown and red algae, fungi, and lichen (Weyand *et al.*, 1999). They are nearly all helical homodimeric proteins that contain trigonal–bipyramidal coordinated vanadium atoms at their two active centres. The catalytically active histidine is described to be in a different environment in bromoperoxidases (-SGH-) and in iodoperoxidases (-AGH-) (Weyland *et al.*, 1999; Colin *et al.*, 2005). The present analysis demonstrates the occurrence of vanadium-dependent iodoperoxidases in at least five cyanobacterial species (Table 1). All essential structural motifs seem to be conserved (not shown). In addition, ORFs were found with the catalytic histidine located within the sequence -GGH-. Whether this has an impact on the functionality of these putative haloperoxidases is unknown (thus these proteins were set in parentheses in Table 2), as is the physiological role of vanadium peroxidases in cyanobacteria.

Peroxiredoxins: Prxs (EC 1.11.1.15) are a ubiquitous family of antioxidant enzymes which have been identified in all domains of life (Dietz, 2003; Wood *et al.*, 2003). *Synechocystis* PCC6803 was the first cyanobacterium where genes encoding Prxs as well as the expression of the corresponding proteins has been demonstrated (Yamamoto *et al.*, 1999; Kobayashi *et al.*, 2004; Dietz *et al.*, 2005; Hosoya-Matsuda *et al.*, 2005). A bioinformatic analysis of the genomes of *Synechocystis* PCC6803 and *S. elongatus* PCC7942 (Stork *et al.*, 2005) revealed the existence of five and six ORFs, respectively, with similarity to peroxide-detoxifying Prxs in plants.

All Prxs share the same basic catalytic mechanism composed of two steps. All Prxs appear to have the first step in common (Reaction 8), in which an activated cysteine (Cys-SH, the peroxidative cysteine) is oxidized to a sulphenic acid (Cys-SOH) by the peroxide substrate. The peroxide decomposition probably requires a base to deprotonate the

peroxidatic cysteine as well as an acid to protonate the poor RO^- leaving group (Wood *et al.*, 2003). All Prxs have a conserved active site arginine, which lowers the pK_a of the peroxidatic cysteine somewhat by stabilizing its thiolate form. Genenerally, Prxs have a rather low activity, but possess a broad substrate specificity. Possible substrates are H_2O_2, alkyl hydroperoxides, and peroxynitrite. Regarding detoxification of H_2O_2, it has been shown that Prxs have a K_M in the low micromolar range and thus are more efficient scavengers of trace amounts of H_2O_2 compared with enzymes with catalase activity (see above). Reductive regeneration of the oxidized catalytic thiol (Reaction 9) depends on glutathione (R′SH) or thioredoxin, glutaredoxin, cyclophilin, and tryparedoxin (Wood *et al.*, 2003; Stork *et al.*, 2005). The mechanism of Reaction 9 distinguishes the Prx subclasses.

$$\text{Cys}-\text{SH}+\text{ROOH}\rightarrow\text{Cys}-\text{SOH}+\text{ROH} \qquad \text{Reaction 8}$$

$$\text{Cys}-\text{SOH}+2\,\text{R}'\text{SH}\rightarrow\text{Cys}-\text{SH}+\text{R}'\text{SSR}' \qquad \text{Reaction 9}$$

Peroxiredoxin activity can be regulated *in vivo* by cysteine oxidation, aggregation state, phosphorylation, or limited proteolysis (Wood *et al.*, 2003; Stork *et al.*, 2005; Low *et al.*, 2008). These regulatory mechanisms have, so far, mainly been investigated in eukaryotes. It is important to mention that—besides peroxide detoxification—Prxs also have a function as regulators of redox-mediated signal transduction at least in some eukaryotes (Dietz *et al.*, 2003; Veal *et al.*, 2004). Thus Prxs are important components of both the cellular antioxidant defence system and redox homeostasis.

In plants, Prx proteins are categorized into four subclasses based on subunit composition, number, and location of the conserved cysteine residues, as well as the sequence environment of the catalytic centre. They also show differences with respect to the reductant which is predominantly used. According to the work of Stork *et al.* (2005), this categorization has been used here for the analysis of all cyanobacterial genomes so far available. Table 2 demonstrates that with a few exceptions, all cyanobacteria have at least one representative Prx for each of the four subclasses.

1-Cys peroxiredoxin. All investigated cyanobacterial genomes contain an ORF with similarity to 1-Cys Prx (Table 2). Only *A. marina* contains two paralogues. 1-Cys Prxs contain a single conserved catalytic cysteine with the N-terminal highly conserved sequence -VLFSHPXDY-TPVCTTE-. Its catalytic cycle is not fully understood. Reduction of the oxidized peroxidatic cysteine (Reaction 9) is directly by the reductant molecule. In plants it has a dual location in the cytosol and nucleus, and is preferentially expressed in the embryo and aleurone (Stacy *et al.*, 1996). Similarly to its suggested function in plants, 1-Cys Prx in cyanobacteria may be involved in protecting nucleic acids from oxidative damage, particularly under stress. With the exception of H_2O_2- and methylviologen-induced oxidative stress, in *Synechocystis* PCC6803 *1-cys prx* mRNA increased in response to all kinds of metabolic imbalances, including irradiation, salinity, and iron deficiency (Stork *et al.*, 2005). Heterologously expressed 1-Cys Prx from *Synechocystis* had only a low peroxidase activity, but disruption of the gene significantly reduced the growth rate of *Synechocystis* PCC6803 cells (Hosoya-Matsuda *et al.*, 2005).

2-Cys peroxiredoxin. All investigated species have one ORF with similarity to 2-Cys Prx (Table 2). Generally, 2-Cys Prxs, including the cyanobacterial enzymes, contain two conserved cysteines far away in the sequence within fully conserved motifs (N-terminal Cys, -FFYPLDFTFT-FVCPTE-; and C-terminal 'resolving' Cys, -VCP-). 2-Cys Prxs are homodimeric enzymes where the two subunits interact in the catalytic cycle and are linked via a disulphide bond in the oxidized form. In detail, regeneration of the oxidized peroxidatic cysteine from one subunit occurs via attack of the C-terminal 'resolving' cysteine of the second subunit, to form an intersubunit disulphide bond, which is then reduced by one of several reductant molecules. In atypical Prx (see later), the peroxidatic cysteine and its resolving cysteine are in the same polypeptide, so their reaction forms an intrachain disulphide bond.

Cyanobacterial 2-Cys Prxs are very similar to the corresponding 2-Cys Prxs in plants, that are localized exclusively in the chloroplast. The plant protein has a defined function in photosynthesis. Depending on its redox-related oligomeric state, the 2-Cys Prx cycles between a thylakoid-bound and stromal state (Konig *et al.*, 2002). In addition to ascorbate peroxidase-mediated detoxification of Mehler reaction-driven H_2O_2, a redox signalling function of 2-Cys Prx has been proposed in the context of photosynthesis (Dietz *et al.*, 2003, 2005). Sequence comparisons of 2-Cys Prx from *Synechocystis* PCC6803 and the red alga *Porphyra*, where 2-Cys Prx is still encoded in the chloroplast genome, and the higher plant 2-Cys Prx (encoded in the nucleus) has led to the hypothesis that 2-Cys Prx originated from a former cyanobacterial endosymbiont (Baier and Dietz, 1997).

Generally, in bacteria, 2-Cys Prxs are suggested to detoxify peroxides generated at low rates (Wood *et al.*, 2003). In cyanobacteria, *2-cys prx* mRNA levels were highly responsive to any type of metabolic disturbance, i.e. light, methylviologen, H_2O_2, NaCl, etc. (Stork *et al.*, 2005), which contrasts with the constitutively high level of expression in photosynthesizing plant cells. In *Synechocystis* PCC6803, the essential role of 2-Cys Prx in photosynthetic adaptation has been established in a deletion mutant that showed increased stress sensitivity and a disturbed peroxide detoxification mechanism (Klughammer *et al.*, 1998; Yamamoto *et al.*, 1999). It uses thioredoxin as reductant, and thus its activity is coupled to the photosynthetic electron transport system. Moreover, from gene disruption analysis in *S. elongatus* PCC7942, it was concluded that 2-Cys Prx is also essential for growth during excessive radiation and that under such conditions the mutant strain could not compete with the wild type (Perelman *et al.*, 2003).

Type II peroxiredoxin: About 40% of cyanobacterial genomes contain one ORF with similarity to type II Prx

(atypical 2-Cys Prx) which can use thioredoxin and glutaredoxin as the reductant and can exist in multiple isoforms localized in plants in many subcellular compartments, including one in plastids (Horling *et al.*, 2002). Cyanobacterial type II Prxs contain a fully conserved N-terminal region with one cysteine (-LPGAFTPTCSS-); however, the second (C-terminal) cysteine is not conserved in all species. It is absent in the putative type II Prxs from all *Prochlorococcus* species, in *Microcysts aeruginosa*, *Trichodesmium erythraeum*, and all *Nostocales*. However, it has been demonstrated recently (Hong *et al.*, 2008) that all the putative type II Prxs of these mentioned species (with the exception of *Prochlorococcus* species and *T. erythraeum*) are hybrids with a fused glutaredoxin domain at the C-terminus that contains the conserved -CXXC- domain. The corresponding fusion protein from *Nostoc* PCC7120 has been expressed heterologously in *Escherichia coli* and was shown to have the highest peroxidase activity toward H_2O_2 using glutathione as electron donor. The calculated k_{cat} and k_{cat}/K_M values for H_2O_2 were reported to be 48 s^{-1} and 3.3×10^6 M^{-1} s^{-1}, respectively (Hong *et al.*, 2008). Immunoblot analysis revealed its occurrence in both vegeatative cells and heterocysts. Expression of this hybrid protein is enhanced during the late phase of vegetative and heterocyst growth (Hong *et al.*, 2008).

(Non-hybrid) *Synechocystis* type II Prx has been demonstrated to be strongly up-regulated upon treatment with methylviologen, H_2O_2, and, to a lesser extent, in response to light, salt, iron, and nitrogen deprivation (Kobayashi *et al.*, 2004; Li *et al.*, 2004; Stork *et al.*, 2005). Moreover, it was shown that a Fur-type transcription factor plays a regulatory role in the induction of the gene in response to oxidative stress (Kobayashi *et al.*, 2004). Genetic disruption of the gene indicated that the gene product is essential for aerobic phototrophic growth, essential in high light (Stork *et al.*, 2005).

Peroxiredoxin Q: Peroxiredoxin Q (PrxQ, atypical 2-Cys Prx) is found in all cyanobacteria. Interestingly, in contrast to *Arabidopsis thaliana* that has only a single gene, up to four paralogues are found in cyanobacterial genomes (Table 2). Thus, this subfamily constitutes the largest group of peroxidases in cyanobacteria. PrxQs are homologues of the *E. coli* bacterioferritin co-migrating protein and function as a monomer (Kong *et al.*, 2000). In typical eukaryotic PrxQs, two cysteines are spaced apart by only a few amino acids (-CTXXXC-), and this pattern is also followed by ~48% of cyanobacterial PrxQs, whereas the other putative proteins lack the second cysteine. For example, both putative PrxQs from *Synechocystis* PCC6803 and one (out of four) protein from *S. elongatus* PCC7942 lack this second cysteine and thus represent atypical PrxQ-type enzymes. One of the *Synechocystis* proteins was hardly expressed under any conditions tested, whereas the four PrxQs from *S. elongatus* PCC7942 genes showed a time dependency and stress-specific pattern of expression (Stork *et al.*, 2005). PrxQs of higher plants function in the context of photosynthesis. They are localized to the chloroplast (Lamkemeyer *et al.*, 2003), up-regulated upon oxidative stress (Horling *et al.*, 2003), and preferentially use thioredoxins as the most efficient electron donors (Colin *et al.*, 2004). All these data support the conclusion that the plant PrxQs function in the context of antioxidant defence and in the redox homeostasis of photosynthesis. A similar role in cyanobacterial metabolism is plausible.

NADPH-dependent glutathione peroxidase-like proteins: GPx (EC 1.11.1.9 and EC 1.11.1.12) is the general name for a family of multiple isozymes that also catalyse the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols (Reaction 1) using reduced glutathione (GSH) as an electron donor ($H_2O_2 + 2\ GSH \rightarrow GSSG + 2\ H_2O$). In plants, GPxs, that are distributed in different subcellular compartments, have often been classified as the fifth group of Prxs because they can reduce peroxide with higher efficiency (sometimes exclusively) by the thioredoxin system rather than by using GSH as a reducing agent (Tanaka *et al.*, 2005). A recently performed phylogenetic analysis demonstrates the occurrence of GPxs in cyanobacteria as well as their scattered distribution inside the group of proteobacteria (Margis *et al.*, 2008). The present analysis (Table 2) demonstrates that ~70% of all cyanobacterial genomes have an ORF with homology to plant GPxs, with *Synechocystis* PCC6803 and two *Synechococcus* species having two paralogues.

At the molecular level, plant GPxs are closely related to animal phospholipid hydroperoxide glutathione peroxidase (PHGPx), and the corresponding proteins have three widely conserved cysteine residues, which are assumed to be essential for catalysis (Margis *et al.*, 2008). In cyanobacterial putative GPxs these three cysteines are found within the following conserved surroundings (from the N- to the C-terminus): -XCG-, -PCN-, and -FCX-, respectively, with the latter being absent in the *Synechocystis* PCC6803 and *Synechococcus* PCC7002 protein. It has to be mentioned that the animal enzymes may contain selenocysteine, whereas plant proteins exclusively contain cysteine.

The two GPxs from *Synechocystis* PCC6803 were overexpressed in *E. coli* and showed no peroxide-reducing activity with glutathione, but were able to use NADPH as electron donor (Tichy and Vermaas, 1999; Gaber *et al.*, 2001). This fits with the fact that arginine and lysine residues conserved in GPxs are replaced by other amino acids and that both proteins contain an NADPH-binding domain. Only unsaturated fatty acid hydroperoxides and alkyl peroxides in combination with NADPH could be used as substrates, but not H_2O_2, NADH, glutathione, ascorbate, or cytochrome *c* (Gaber *et al.*, 2001). This fits with the data reported by Tichy and Vermaas (1999) demonstrating that in a *Synechocystis* Δ*katG* mutant in the presence of 100 μM mercaptosuccinate, which is a potent and specific inhibitor of GPx, no changes in the peroxidase activity with H_2O_2 as oxidant and in the growth rate were observed.

In crude extracts of *Synechocystis*, NADPH-dependent GPx-like activity was measured using NADPH and α-linolenic acid hydroperoxide as substrates. Immunoblotting showed that both *Synechocystis* proteins are constitutively

expressed. These data suggest that GPxs in cyanobacteria seem to play a role in protection of cellular membranes from oxidative damage by reducing unsaturated fatty acids with NADPH as electron donor.

Conclusion

Prxs are the only peroxide-degrading enzymes found in all cyanobacterial genomes sequenced so far. All four classes of plant-type Prxs are found. Apparently these enzymes have acquired specific and indispensable functions in these oxygenic phototrophic organisms and are responsible for the detoxification of H_2O_2 and alkyl peroxides usually produced by the cell under normal growth conditions. Additionally, they seem to have a role in stress adaptation. It has been demonstrated that their expression is up-regulated in response to all kinds of metabolic imbalances and that their activity is coupled to photosynthetic electron transport (photosynthetic adaptation).

The majority of cyanobacterial species also contain a putative NADPH-dependent GPx-like protein that—in addition to H_2O_2 and alkyl peroxides—can also reduce fatty acid hydroperoxides produced in membranes during oxidative stress. Whether the other three (minor) groups of (putative) peroxidases (i.e. primordial haem peroxidase, dihaem cytochrome *c* peroxidase, and vanadium-dependent iodoperoxidase) found in some species are expressed and play a specific physiological role in metabolism is completely unknown at present.

It was interesting to see that genes for typical haem catalase are very unusual in cyanobacteria and that many species (~50%) even lack a gene also encoding alternative H_2O_2-dismutating enzymes. These include bifunctional KatG found primarily in non-diazotrophic species and MnCat found mainly in nitrogen-fixing organisms. As has been demonstrated at least for KatG, these H_2O_2-dismutating enzymes might have a protective role against higher concentrations of (environmental) H_2O_2 and/or when the light-dependent peroxidases have reduced activity due to limited availability of electron donor(s).

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