Detoxification of hydrogen peroxide and expression of catalase genes in *Rhodobacter*

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The two related facultatively photosynthetic bacteria *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* show different sensitivities against peroxide stress. *R. sphaeroides* is able to tolerate higher concentrations of H_2O_2 and exhibits higher catalase activity than *R. capsulatus*. The *katE* gene of *R. sphaeroides* and the *katG* gene of *R. capsulatus* are strongly induced by H_2O_2 . This induction depends on the presence of the OxyR protein, which is able to bind to the promoter regions of these genes. In addition to *katE* *R. sphaeroides* harbours the *katC* gene, which shows no significant response to H_2O_2 but is induced in stationary phase.

INTRODUCTION

The production of reactive oxygen species (ROS) is an inherent consequence of an aerobic lifestyle for living organisms. Well-balanced defence systems are required to guarantee survival of aerobically living cells by removing ROS (Storz & Zheng, 2000), keeping the cellular environment reduced, and repairing oxidation-generated damage. Most organisms that can grow in the presence of oxygen harbour catalases and/or peroxidases which scavenge the H_2O_2 that is produced endogenously by the respiratory chain or is generated in the environment. H_2O_2 can damage enzymes by oxidizing thiol groups and iron-sulfur centres, and by conversion to hydroxyl radicals it can produce mutagenic and lethal lesions (Storz & Imlay, 1999). Two types of catalase, Mn-catalase and haem catalase, are presently known (Ueda *et al.*, 2003), which catalyse the destruction of H_2O_2 with very high K_{cat}/K_s values to ensure rapid removal of these reactive molecules. H_2O_2 detoxification in *Escherichia coli* is mainly due to two distinct types of catalase. The *katG* gene encodes a bifunctional catalase hydroperoxidase I (HPI) that harbours both catalase and peroxidase activity. HPI is transcriptionally induced by the positive activator OxyR, which directly senses oxidative stress (González-Flecha & Demple, 1997). The *katE* gene encodes the monofunctional HP II (which harbours only catalase activity) and its transcription requires a functional *rpoS* gene, which is activated in stationary-phase cells or upon various types of starvation (González-Flecha & Demple, 1997). OxyR is not involved in the regulation of *katE* in *E. coli* (Schellhorn, 1994).

Bacteria of the genus *Rhodobacter* are facultatively photosynthetic bacteria. At low oxygen tension the formation of photosynthetic complexes is induced, which allows the

bacteria to perform anoxygenic photosynthesis if no oxygen is available. As long as oxygen is present *Rhodobacter* species produce ATP by aerobic respiration. The molecular mechanisms underlying the rapid adaptation to changes in oxygen concentration have been extensively studied in the past (reviewed by Gregor & Klug, 1999, 2002; Zeilstra-Ryalls & Kaplan, 2004). In its natural environment *Rhodobacter* occasionally needs to tolerate very high oxygen concentrations which are produced by micro-organisms performing oxygenic photosynthesis. While some previous studies have addressed the role of superoxide dismutase and thioredoxins in defence against oxidative stress in *Rhodobacter* (Cortez *et al.*, 1998; Pasternak *et al.*, 1999; Li *et al.*, 2003a, b), little is known about the role of catalases and their regulation. In this study we have addressed the ability of two *Rhodobacter* species to detoxify H_2O_2 and have studied the expression of catalase genes in order to understand better how *Rhodobacter* can deal with high oxygen concentrations in its environment.

METHODS

Bacterial strains, plasmids and growth conditions. All strains and plasmids used in this study are listed in Table 1.

E. coli cultures were grown in Luria–Bertani (LB) medium at 37 °C with continuous shaking at 180 r.p.m. *Rhodobacter* cultures were cultivated at 32 °C with continuous shaking at 140 r.p.m. in a malate minimal salt medium (Drews, 1983). For aerobic growth, 20 ml of culture were incubated in 100 ml baffled Erlenmeyer flasks (about 7.7 mg l⁻¹ dissolved oxygen). For semi-aerobic incubation, 40 ml of culture were shaken in 50 ml Erlenmeyer flasks (about 0.85 mg l⁻¹ dissolved oxygen). Ampicillin (200 µg ml⁻¹), spectinomycin (10 µg ml⁻¹) and tetracycline (20 µg ml⁻¹) were added to the medium as needed.

Construction of oxyR deletion mutants of *R. capsulatus* and *R. sphaeroides*. *R. capsulatus* strain SBoxyR::ΩSp and *R. sphaeroides* strain 2.4.1oxyR::ΩSp were generated by transferring the

Abbreviation: ROS, reactive oxygen species.

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Description*	Source/reference
Strain		
<i>E. coli</i>		
M15 (pREP4)	Host strain for protein over-expression	Qiagen
SM10	<i>recA thi thr leu</i> ; chromosomal RP4-2 (Tc::Mu)	Simon <i>et al.</i> (1983)
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (lac-proAB)</i>	New England Biolabs
<i>Rhodobacter</i>		
<i>R. sphaeroides</i> 2.4.1	Wild-type	van Neil (1941)
<i>R. capsulatus</i> SB1003	Wild-type	Yen & Marrs (1976)
2.4.1oxyR	2.4.1 oxyR:: Ω Sp ^r	This study
SBoxyR	SB1003 oxyR:: Ω Sp ^r	This study
2.4.1oxyR(p2.4.1oxyR)	2.4.1oxyR harbouring p2.4.1oxyR	This study
SBoxyR(pSBoxyR)	SBoxyR harbouring pSBoxyR	This study
Plasmid		
pPHU281	Tc ^r , <i>lacZ'</i> mob(RP4)	Hübner <i>et al.</i> (1991)
pHP45 Ω	Ap ^r , Sp ^r , source of Ω Sp ^r cassette	Prentki <i>et al.</i> (1991)
pQE32	His-tag vector for cloning <i>oxyR</i> gene	Qiagen
pRK415	Tc ^r	Keen <i>et al.</i> (1988)
TkatGup	0.36 kb <i>Bam</i> HI– <i>Stu</i> I DNA fragment containing SB1003 <i>katG</i> promoter sequence cloned into T-vector (Promega), Ap ^r	This study
pQEoxyR	0.95 kb <i>Bam</i> HI– <i>Kpn</i> I DNA fragment containing the SB1003 <i>oxyR</i> gene cloned into pQE32 (Qiagen) for <i>oxyR</i> over-expression, Ap ^r	This study
pPHU Δ SBoxyR	0.75 kb fragment containing the upstream and downstream regions of the <i>R. capsulatus oxyR</i> gene cloned into pPHU281	This study
pPHU Δ SBoxyR:: Ω Sp	2.0 kb omega spectinomycin cassette cloned into pPHU Δ SBoxyR	This study
pPHU Δ 2.4.1oxyR	0.75 kb fragment containing the upstream and downstream regions of the <i>R. sphaeroides oxyR</i> gene cloned into pPHU281	This study
pPHU Δ 2.4.1oxyR:: Ω Sp	2.0 kb omega spectinomycin cassette cloned into pPHU Δ 2.4.1oxyR	This study
p2.4.1oxyR	2.1 kb fragment containing the upstream region of <i>oxyR</i> and the <i>oxyR</i> gene of 2.4.1 cloned into pRK415	This study
pSBoxyR	1.4 kb fragment containing the upstream region of <i>oxyR</i> and the <i>oxyR</i> gene of SB1003 cloned into pRK415	This study

*Sp^r, Spectinomycin-resistant; Tc^r, tetracycline-resistant; Ap^r, ampicillin-resistant.

suicide plasmids pPHUS Δ oxyR:: Ω Sp into *R. capsulatus* SB1003 and pPHU2.4.1 Δ oxyR:: Ω Sp in *R. sphaeroides* 2.4.1, respectively, and screening for insertion of the omega (Ω)-spectinomycin cassette into the chromosome by homologous recombination. Briefly, parts of the *oxyR* genes of *R. capsulatus* SB1003 or *R. sphaeroides* 2.4.1, together with upstream and downstream sequences were amplified by PCR using oligonucleotides SBoxyupEco (5'-GTGTTC-GAATTCCCCGCC-3'), SBoxy315Bam (5'-GAAGATAGGATCCAA-ATCGTCG-3') and SBoxy500Bam (5'-GAAGATAGGATCCAA-TCGTCG-3'), SBoxydownPst (5'-CAGGCGCTGCAGAGGGCG-3') for SB1003 and 2.4.1oxyupEco (5'-CGAATTCTGGTTGTCGGCG-ATC-3'), 2.4.1oxy290Bam (5'-GGGATCCCGCCAGATTGAC-3'), 2.4.1oxy600Bam (5'-CGGATCCGCTGCGGCGGTGGCGC-3') and 2.4.1oxydownPst (5'-CCTGCAGGACGGCCGCGTGA-3') for 2.4.1.

The amplified PCR fragments were cloned into the *Eco*RI–*Bam*HI and *Bam*HI–*Pst*I sites of the suicide plasmid pPHU281, respectively, generating plasmids pPHUS Δ oxy and pPHU2.4.1 Δ oxyR. A 2.0 kb *Bam*HI fragment containing the Ω -spectinomycin cassette from pHP45 Ω was inserted into the *Bam*HI site of pPHUS Δ oxy and pPHU2.4.1 Δ oxyR to generate pPHUS Δ oxyR:: Ω Sp and

pPHU2.4.1 Δ oxyR:: Ω Sp, respectively. Both pPHUS Δ oxyR:: Ω Sp and pPHU2.4.1 Δ oxyR:: Ω Sp were transferred into *E. coli* strain SM10, and mobilized from SM10(pPHUS Δ oxyR:: Ω Sp) or SM10(pPHU2.4.1 Δ oxyR:: Ω Sp), into either *R. capsulatus* SB1003 or *R. sphaeroides* 2.4.1 wild-type strain by diparental conjugation. Conjugants were selected on malate minimal salt agar plates containing 10 μ g spectinomycin ml⁻¹. Southern blot analysis of chromosomal DNA was carried out to confirm the double crossover event of the Ω -spectinomycin cassette into the *Rhodobacter* chromosome. By insertion of the Ω -spectinomycin cassette, 181 bp of the 915 bp *R. capsulatus oxyR* gene and 247 bp of the 926 bp *R. sphaeroides oxyR* gene, respectively, were deleted.

Complementation of the *oxyR* deletion mutants of *R. capsulatus* and *R. sphaeroides*. For complementation of the *oxyR* deletion mutant of *R. capsulatus*, a 1.4 kb PCR fragment containing the entire *oxyR* gene along with approximately 450 bp of the upstream sequence was amplified using the oligonucleotides SBoxyRup450Eco (5'-GACGTTGTCTGGGCCAGGAATTCAGC-3') and SBoxydownKpn (5'-GGGGTACCTGGCCTCGGTCAGATTG-3'). Following digestion with *Eco*RI and *Kpn*I, the PCR fragment was cloned into the corresponding sites of pRK415, resulting in

plasmid pSBoxyR. For complementation of the *oxyR* deletion mutant of *R. sphaeroides*, a 1.2 kb PCR fragment containing the entire *oxyR* gene along with 100 bp of the upstream sequence was amplified using the oligonucleotides 2.4.1oxyupEco (5'-CGAATTCTGGTTGTCGGCGATC-3') and 2.4.1oxydownPst (5'-CCTGCGGACGGCCGCGTGA-3'). After digestion with *EcoRI* and *PstI*, the PCR fragment was cloned into the corresponding sites of pRK415 resulting in plasmid p2.4.1oxyR. To complement the *oxyR* deletion in SB1003 and 2.4.1, respectively, plasmids pSBoxyR and p2.4.1oxyR were transferred into *E. coli* strain SM10 and conjugated into the SBoxyR or 2.4.1oxyR strains by diparental conjugation.

Enzyme assays. *Rhodobacter* cells were disrupted by brief sonication, and crude extract was used for enzyme assays. Protein concentration was determined according to Bradford (1976). For total catalase measurement, 30–300 µg of total protein were diluted to 1 ml with water. H₂O₂ (0.5 ml of 59 mM H₂O₂ freshly diluted in 50 mM potassium phosphate buffer, pH 7.0) was added, and the absorbance of the samples at 240 nm was measured every 10 s for 1 min. The initial linear rates were used to calculate the activities. Specific activity of catalase (µM H₂O₂ decomposed per minute per milligram of total protein) was calculated using an extinction coefficient of 43.6 M⁻¹ cm⁻¹ (Hochman & Shemesh, 1987).

For analysis of H₂O₂ detoxification, cells were grown exponentially to an OD₆₆₀ of 0.5 and H₂O₂ was added to the desired final concentration. Aliquots were drawn at various time points, and the amount of H₂O₂ was determined by oxidation of Fe²⁺ in the presence of xylene orange and measuring the absorbance at 560 nm. The absorbance of standard concentrations of H₂O₂ served to quantify the amount of H₂O₂ for each experiment. As previously described by Perelman *et al.* (2003), the ability to decompose H₂O₂ is strongly dependent on cell density. Therefore, all of the analyses presented in this study were performed at a fixed cell density.

Measurement of resistance to H₂O₂. Exponentially grown cultures of *Rhodobacter* strains (0.2 ml) were diluted into 2 ml of pre-warmed top agar (0.7% agar) and layered onto minimal malate agar plates. Filter paper disks were placed on the surface of the agar, and 5 µl of different concentrations (0.001–0.2 M) H₂O₂ were applied on the filter disk. Zones of inhibition were measured after overnight incubation at 32 °C.

RNA extraction and quantitative real-time RT-PCR analysis. *Rhodobacter* strains in exponential phase (OD₆₆₀ = 0.5), grown under semi-aerobic conditions, were treated with H₂O₂ (1 mM final concentration) and at certain time-points cells were collected into centrifugation bottles and pelleted by centrifugation. Total RNA was isolated by the hot phenol method, quantified by spectrophotometric analysis (absorbance at 260 nm), and 60 ng of total RNA were used for quantitative real-time PCR.

The following primers were designed for gene amplification, *katE*: katE-A (5'-CTATCCGCTGATCGAGGT-3') and katE-B (5'-GTGCGCATAGGAGAAGAC-3'); *katC*: katC-A (5'-GGATGCGGCGATGCTAGCCGCAAC-3') and katC-B (5'-GAGGGTTCGCCGCCAGC-GTCGCC-3'); *katG*: katG-A (5'-GCCTCGGTGCGCGATGTGAT-3') and katG-B (5'-CACC GGCTCCAGCAGTCAA-3'); *rpoZ* genes: 2.4.1rpoZ-A (5'-TTTCGAGCTGGTGATGCT-3') and 2.4.1rpoZ-B (5'-ACTCGATCTGGGTCTGG-3'); SBropZ-A (5'-GATGATCTGCGGAGCGTCT-3') and SBropZ-B (5'-CCTTGCGCGTCCATCAA-TGC-3').

The *rpoZ* gene (encoding the ω -subunit of RNA-polymerase) of *R. capsulatus* and *R. sphaeroides*, respectively, was used to normalize expression values for all other genes. The One-Step RT-PCR kit (Qiagen) was used for reverse-transcription-PCR following the manufacturer's instruction, except that a total volume of 15 µl was

used. SYBR Green was used to monitor amplification and to quantify the amount of PCR products using the Rotor-Gene 3000 real-time PCR cyclor (LTF). Relative expression of *kat* and *rpoZ* mRNA were calculated after the method of Pfaffl (2001).

Expression and isolation of the *R. capsulatus* OxyR protein.

Oligonucleotides SBoxystartBam (5'-CCCCGGATCCTCTCGATGA-AACAGC-3') and SBoxydownKpn (5'-GGGGTACCTGGCCTCG-GTCAGATTG-3'), which hybridize to the 5' and 3' regions of the *oxyR* gene, respectively, were used to amplify the *oxyR* coding region. The 950 bp PCR product was digested with *Bam*HI and *Kpn*I and ligated into pQE32 to generate pQEoxyR, which was transformed into *E. coli* JM109. The correct construct as confirmed by sequencing (using a Genetic Analyser 310 sequencer; ABI) was transformed into *E. coli* M15(pREP4) for overexpression of His-tagged OxyR. For this purpose M15(pREP4 pQEoxyR) was grown in 500 ml of LB medium to an OD₆₀₀ of 0.7–0.8 and induced with 1 mM IPTG for 4–5 h at 32 °C. Following harvest, cells were resuspended in lysis buffer (50 mM Tris, pH 7.5; 250 mM NaCl; 3 mM imidazole; 1 µg lysozyme µl⁻¹ and 0.1 mM PMSF) and disrupted by a brief sonication. The lysate was centrifuged at 12 000 r.p.m. for 20 min at 4 °C. The supernatant was loaded onto Ni-NTA agarose and incubated at 4 °C for 4–5 h. Proteins were washed with washing buffer (0.05 M Tris, pH 7.5 and 0.25 M NaCl) containing 20–50 mM imidazole and eluted with imidazole at a concentration between 80 and 100 mM. Aliquots of these fractions were analysed on SDS 15% polyacrylamide gels, using the buffer system of Laemmli (1970). Fractions containing purified OxyR protein were pooled by using Centricon-10 columns (Amicon) and washed in Z buffer (50 mM HEPES, pH 8.0; 0.5 mM EDTA, pH 8.0; 10 mM MgCl₂ and 300 mM KCl).

Gel chromatography (FPLC). The purified OxyR protein in a 2 ml volume was applied to a Superdex-200 HR 16/60 gel filtration column (Pharmacia Biotech) equilibrated with Z buffer. For reducing conditions 200 mM DTT was added to the Z buffer. The protein was eluted with Z buffer and aliquots of the column fractions were analysed by SDS 10% PAGE using the buffer system of Laemmli (1970).

Determination of OxyR redox status by using AMS. For *in vitro* modification of free thiol groups, 4-acetamido-4'-maleimidyldistibene-2',2'-disulfonic acid (AMS; Molecular Probes) was used. The addition of AMS leads to the alkylation of free thiol groups, present in the reduced but not in the oxidized OxyR. The addition of the high-molecular-mass AMS moiety to the reduced but not to the oxidized protein allows separation of the two forms by gel electrophoresis. The purified OxyR protein was first treated with freshly prepared H₂O₂ (1 mM) or DTT (200 mM) at room temperature for 30 min. The protein was then mixed with 10% trichloroacetic acid (TCA). Precipitated protein was collected by centrifugation (10 000 r.p.m., 10 min). After complete removal of the supernatant, the pellet was dissolved in a buffer containing 0.1% SDS; 50 mM Tris/HCl, pH 8.0 and 15 mM AMS (apart from the non-AMS-modified sample which was dissolved in the same buffer without AMS), and incubated for 2 h at 37 °C. The samples were loaded on SDS 10% PAGE and visualized by silver staining.

Gel mobility-shift assay. Binding of the OxyR protein to the *katG* and *katE* upstream region, respectively, was determined by gel mobility-shift assay. For this, a DNA fragment containing the *katG* or the *katE* promoter region was generated by PCR. The following oligonucleotides were used to generate the PCR fragments. *katG*: katGupBspEI (5'-CGTCCGGACCCGCGGCACCATC-3') and katGStuI (5'-AGGCCTTCATCGCGCCATGCAT-3'); *katE*: katEupEcoRI (5'-GTAGAATTCTCTGCCGAG-3') and katEPstI (5'-GTCTGCACTGCGCGCGCCGCGCGT-3'). The PCR fragments with a length of 350 bp were cloned into T-vector (Promega), and

isolated from the vector by using enzymes *BspEI/StuI* and *EcoRI/PstI*, respectively. The restricted DNA fragments were then radioactively labelled in a fill-in reaction with [α - 32 P]dCTP using the Klenow fragment.

Binding reactions were carried out in a final volume of 20 μ l and contained an appropriate amount of protein, [α - 32 P]CTP-labelled DNA probe, and binding buffer (10 mM Tris/HCl, pH 8.0; 50 mM NaCl; 1 mM DTT; 1 mM EDTA and 5% glycerol). Since purified OxyR protein is predominantly in the oxidized form, we added 200 mM DTT to the binding reaction to reduce OxyR. Binding incubations were carried out for 30 min at room temperature (25 °C) before the samples were loaded onto a 4% polyacrylamide gel in 0.5 $\times$ Tris Borate EDTA (TBE) buffer and run at 130 V for 2 h.

DNaseI footprinting assay. A *Bam*HI–*Stu*I DNA fragment containing the *katG* promoter region was 5'-end labelled at the *Bam*HI site as follows. Plasmid *TkatGup* (Table 1) was linearized with *Bam*HI, dephosphorylated using alkaline phosphatase (NEB) and labelled with polynucleotide kinase (NEB) and [γ - 32 P]ATP. The DNA was then digested with *Stu*I and the 361 bp *Bam*HI–*Stu*I fragment was purified from a 6% non-denaturing polyacrylamide gel. The end-labelled DNA fragment (approx. 10 000 c.p.m.) was incubated with different amounts of purified OxyR protein, using the same reaction conditions as described for the gel mobility-shift assay. After 30 min of incubation at room temperature, 5 μ l of a buffer containing 0.01 M CaCl₂ and 0.01 M MgCl₂ were added. DNaseI (1 μ l, 0.1 U μ l⁻¹; Promega) was then added for 2 min at room temperature. The reaction was stopped by adding 250 mM EDTA. After phenol/chloroform extraction, the samples were dissolved in formamide dye and loaded onto a 6% sequencing gel. The DNA fragment containing the *katG* promoter region was sequenced with the primer SB*katG*300seq (5'-GCACCATCGCCGCGCCAG-3') by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the T7 sequencing kit from USB.

RESULTS

R. sphaeroides 2.4.1 is more resistant to H₂O₂ and shows faster H₂O₂ detoxification than *R. capsulatus* SB1003

In order to compare the sensitivity of the two related *Rhodobacter* strains to H₂O₂ we performed inhibition zone assays. Fig. 1(a) shows the mean sizes of inhibition zones at the different concentrations of H₂O₂ which had been applied to the filters. *R. sphaeroides* 2.4.1 is clearly more resistant to H₂O₂ at lower concentrations and can tolerate higher concentrations of the agent. While *R. capsulatus* SB1003 showed significant growth inhibition when 12.5 mM H₂O₂ was spotted on the filter, *R. sphaeroides* 2.4.1 only showed inhibition when 25 mM H₂O₂ was applied. We also monitored the detoxification of H₂O₂ added to exponentially growing cultures of *R. sphaeroides* or *R. capsulatus* (Fig. 1b). Both strains showed faster removal of H₂O₂ when grown aerobically compared to semi-aerobic growth. While the two *Rhodobacter* strains showed identical rates of H₂O₂ detoxification under aerobic conditions, semi-aerobically grown *R. sphaeroides* cultures were clearly more efficient than semi-aerobic *R. capsulatus* cultures. Similar results were obtained in the measurement of the basal catalase activity (Table 2). While an activity of about 200 μ M decomposed H₂O₂ min⁻¹ (mg total protein)⁻¹ was

Fig. 1. Effect of H₂O₂ on wild-type strains of *Rhodobacter*. (a) Inhibition of growth of SB1003 (open bars) and 2.4.1 (filled bars) by H₂O₂. The values give the mean of five experiments with maximal deviation. (b) Time-course of H₂O₂ decomposition by aerobic (solid lines) or semi-aerobic (dashed lines) cultures of wild-type SB1003 (□) and 2.4.1 (■). H₂O₂ (3.5 mM) was added to the cultures at time-point 0. Each point represents the mean of four independent experiments. Error bars indicate maximal deviation.

determined for aerobically grown *R. sphaeroides* cells in exponential phase, an activity of only 40 μ M decomposed H₂O₂ min⁻¹ (mg total protein)⁻¹ was determined for *R. capsulatus* cells grown under identical conditions. In contrast to that, semi-aerobically grown cells of *R. sphaeroides* showed a reduced catalase activity of about 130 μ M decomposed H₂O₂ min⁻¹ (mg total protein)⁻¹ and cells of *R. capsulatus* an activity of 10 μ M decomposed H₂O₂ min⁻¹ (mg total protein)⁻¹. These results indicate that H₂O₂-scavenging enzymes are present in higher amounts or show higher activity under aerobic growth conditions.

The *oxyR* gene product is required for the H₂O₂-dependent response

To study the involvement of the global stress regulator OxyR in the H₂O₂-dependent response, we constructed *oxyR* deletion mutants of both *Rhodobacter* strains (see Methods). The deletion of the *oxyR* gene resulted in higher sensitivities of the mutants after exposure to H₂O₂ in

Table 2. Catalase activities of wild-type, *oxyR* mutant strains and *oxyR*-complemented strains of *R. capsulatus* and *R. sphaeroides* grown under aerobic or semi-aerobic conditions

Strain	Catalase activity* exponential phase		
	Aerobic	Semi-aerobic	Semi-aerobic, H ₂ O ₂ -induced†
2.4.1	209.9 ± 29.8	129.9 ± 33.9	251.9 ± 19.4
2.4.1 <i>oxyR</i>	43.3 ± 9.6	28.0 ± 10.8	38.0 ± 9.6
2.4.1 <i>oxyR</i> (p2.4.1 <i>oxyR</i>)	119.4 ± 7.8	76 ± 2.5	132.3 ± 30.5
SB1003	40 ± 11.1	10 ± 4.3	42.6 ± 4
SBoxyR	2.2 ± 1.4	0.9 ± 1.3	0.7 ± 0.3
SBoxyR(pSBoxyR)	27 ± 2.5	8.9 ± 2.2	28 ± 2

*Catalase activities are given in $\mu\text{M H}_2\text{O}_2$ decomposed min^{-1} ($\text{mg total protein}^{-1}$) and are means of at least seven independent experiments, the error range represents standard deviation.

†Exponentially growing cells were treated with 250 $\mu\text{M H}_2\text{O}_2$ for 30 min prior to harvest.

comparison to the wild-types (Fig. 2a, b). The zones of growth inhibition were significantly larger for the *oxyR* mutants. The intact *oxyR* gene of 2.4.1 and SB1003, respectively, located on plasmids p2.4.1*oxyR* and pSBoxyR could restore the *oxyR* deletion phenotype (Fig. 2a, b). Moreover, the basal level of total catalase activity was reduced in both *oxyR* mutant strains (fivefold decrease in 2.4.1*oxyR* under aerobic and semi-aerobic conditions compared to the wild-type 2.4.1; 18- and 11-fold decrease, respectively, for SBoxyR under aerobic and semi-aerobic conditions compared to the wild-type SB1003) (Table 2). The increase in catalase activity after H₂O₂ treatment in the *oxyR* mutants was also reduced (1.3-fold increase in 2.4.1*oxyR* compared to 1.9-fold increase in 2.4.1 wild-type and 1.3-fold decrease in SBoxyR compared to 4.3-fold increase in SB1003 wild-type) (Table 2). The complemented *oxyR* mutant strains showed catalase activities that were clearly higher than that of both *oxyR* mutants, but the activities did not reach wild-type levels. In the complemented strains, the *oxyR* gene is present in 1–2 copies *in trans*, which may lead to altered expression compared to the chromosomally located wild-type gene. Both *oxyR* mutants exhibit kinetics of H₂O₂ removal which were clearly retarded when compared to the kinetics observed for the isogenic wild-type strains (Figs 2c and 1b). These data reveal an involvement of the *oxyR* gene product in the response to H₂O₂. Similar observations regarding the essential role of OxyR in the peroxide response have been made in other bacteria, such as *E. coli*, *Haemophilus influenzae* and *Agrobacterium* (González-Flecha & Demple, 1997; Maciver & Hansen, 1996; Nakjarung *et al.*, 2003). The *R. capsulatus* mutant SBoxyR was more severely affected in its ability to detoxify H₂O₂ than the *R. sphaeroides oxyR* mutant. While 2.4.1*oxyR* was able to remove 3.5 mM H₂O₂ from the medium within 5 min, SBoxyR was unable to remove this amount of H₂O₂ even after 5 min. After addition of 3.5 mM H₂O₂ the growth of the *R. capsulatus oxyR* mutant was completely inhibited, while both wild-type strains, as well as the 2.4.1*oxyR* mutant, showed growth inhibition for a period of 60 min after the addition

of H₂O₂, but were able to grow after that time of adaptation (data not shown).

H₂O₂-dependent *katG* expression in *R. capsulatus* and *katE* gene expression in *R. sphaeroides* are mediated by the OxyR protein

Analysis of the completed genome of *R. sphaeroides* 2.4.1. (<http://genome.ornl.gov/microbial/rsph>) revealed the presence of two genes encoding catalases, RSP2779 and RSP2380. The RSP2779 gene product shows 45 % identity to the *E. coli* hydroxyperoxidase (HP) II, which is encoded by the *E. coli katE* gene. The RSP2380 gene product shows 51 % identity to the *E. coli* HPII protein but even higher identity to the *katC* gene products of *Agrobacterium* or *Sinorhizobium*. Like other KatC proteins it harbours a C-terminal protease domain. The almost completed genome of *R. capsulatus* SB1003 (data taken from: www.ergo-light.com/ERGO) contains only one gene with significant similarity to a catalase/peroxidase, *katG*. The *katG* gene product shows 53 % identity to the *E. coli* HPI protein, which is encoded by the *E. coli katG* gene. We investigated the expression of these genes after addition of H₂O₂ by real-time RT-PCR. The expression of the *katE* gene of *R. sphaeroides* as well as expression of the *katG* gene of *R. capsulatus* were strongly induced after addition of H₂O₂ to exponentially growing cultures (Fig. 3a, b). High expression levels were already observed 1 min after the addition of H₂O₂. These data fit well with the fast kinetics of H₂O₂ detoxification observed for *Rhodobacter* cultures. Oxygen itself also exerted an effect on *katE* and *katG* gene expression in *R. sphaeroides* and *R. capsulatus*, respectively (Fig. 4). The basal expression of both *katE* and *katG* were reduced about fivefold under semi-aerobic conditions. No change could be observed for the basal expression of *katC* when the cells were grown under aerobic or semi-aerobic conditions (Fig. 4).

The known ability of OxyR to positively regulate the expression of catalases in *E. coli* (Storz & Zheng, 2000) prompted us to determine whether the OxyR protein of

Fig. 2. Sensitivity of *Rhodobacter oxyR* mutants to H_2O_2 . Growth inhibition by H_2O_2 of (a) *R. capsulatus* [open bars, wild-type SB1003; filled bars, *SBoxyR*; hatched bars, complemented mutant *SBoxyR*(p*SBoxyR*)] and (b) *R. sphaeroides* [open bars, wild-type 2.4.1; filled bars, *2.4.1oxyR*; hatched bars, complemented mutant *2.4.1oxyR*(p*2.4.1oxyR*)]. The values give the mean of five experiments with maximal deviation. (c) Time-courses of H_2O_2 decomposition by aerobic (solid lines) or semi-aerobic (dashed lines) *oxyR* mutant strains *SBoxyR* (●) and *2.4.1oxyR* (▲). At time-point 0 3.5 mM H_2O_2 was added to the cultures. Each point represents the mean of four independent experiments. Error bars indicate maximal deviation.

Rhodobacter is also involved in the regulation of the H_2O_2 -dependent expression of catalases. The expression of *katE* in *R. sphaeroides* and *katG* in *R. capsulatus* were both dependent on OxyR (Fig. 3a, b). Without the addition of H_2O_2 the expression levels in the *oxyR* mutant strains were similar to that of the parental wild-type strains. The

Fig. 3. Expression of *kat* genes in wild-type strains (open bars), *oxyR* mutant strains (filled bars) and complemented mutant strains (hatched bars). H_2O_2 was added to exponentially grown cultures to a final concentration of 1 mM, samples were taken at the indicated times. Total RNA was isolated and the level of *kat* expression was monitored by real-time RT-PCR analysis. (a) Expression of *katG* in *R. capsulatus* strains. (b) Expression of *katE* in *R. sphaeroides* strains. (c) Expression of *katC* in *R. sphaeroides* strains. Values represent the fold change of the *kat* gene expression compared to that of the control at time-point 0. The data represent the mean of three independent experiments. Error bars indicate maximal deviation.

H_2O_2 -inducibility of *kat* expression was regained by provision of a functional *oxyR* gene *in trans* (Fig. 3a, b). These data imply that the *oxyR* gene product is important for regulation of the genes encoding *katE* and *katG*, respectively, in *Rhodobacter*.

Regulation of *katC* gene expression

In contrast to the gene encoding *katE* of *R. sphaeroides*, the *katC* gene showed only a weak response when H_2O_2 was

Fig. 4. Effect of oxygen tension on the expression of *katC*, *katE* and *katG*. Cultures of strains SB1003 (wild-type *R. capsulatus*) and 2.4.1 (wild-type *R. sphaeroides*) were grown under aerobic or semi-aerobic conditions to exponential phase. Total RNA was isolated and expression of *kat* genes (*katG* in *R. capsulatus* and *katE* and *katC* in *R. sphaeroides*) was analysed by real-time RT-PCR. Filled bars represent the fold change of the *kat* expression under semi-aerobic growth compared to the expression under aerobic conditions (=1; open bars). Values are means and maximal deviation of three independent experiments.

added to exponentially growing cultures of *R. sphaeroides* (Fig. 3c). The low H_2O_2 -dependent induction of *katC* expression in *R. sphaeroides* was independent of OxyR (Fig. 3c). In contrast to the *katE* and *katG* basal expression levels, respectively, oxygen tension had no effect on the basal *katC* expression level (Fig. 4). To determine whether expression of the *katC* gene is induced in the stationary phase, as it was reported for *Sinorhizobium meliloti* (Sigaud *et al.*, 1999), expression of the gene was measured by real-time RT-PCR during growth (Fig. 5a, b). An 11- to 38-fold increase in expression of *katC* was observed when cells reached stationary phase, suggesting a function of the *katC* gene product in this growth stage. This finding is consistent with the results of Terzenbach & Blaut (1998), who observed a twofold higher catalase activity in *R. sphaeroides* cells grown in stationary phase. Expression of *katE* did not increase after cells entered stationary phase (data not shown).

Reduced and oxidized OxyR bind to the *kat* promoter region

In *E. coli*, OxyR exerts its effect on gene expression by binding to promoter regions of target genes (Kullik *et al.*, 1995). Some promoter regions bind only oxidized or reduced OxyR, other promoter regions can bind both forms, but only the oxidized form activates transcription (Kullik *et al.*, 1995). To understand the regulation of *kat* genes by various forms of OxyR better, the DNA-binding ability of the *Rhodobacter* OxyR protein was tested. To do this, the *oxyR* gene of SB1003 was cloned and overexpressed

Fig. 5. Real-time RT-PCR analysis of *katC* expression during stationary phase. (a) Bacterial growth (expressed as log OD₆₆₀) was monitored, samples were taken after 0, 8, 12, 16, 24, 29 and 32 h of growth. (b) Total RNA was isolated and the expression of *katC* was measured by real-time RT-PCR. Values were calculated as fold change of the expression of *katC* compared to that of the control at time-point 0. The data represent the mean of three independent experiments. Error bars indicate maximal deviation.

with an N-terminal His₆-tag in *E. coli*. The isolated protein showed specific binding to the *katG* promoter region, no matter whether it was in its reduced or oxidized form. However, different types of DNA–protein complexes were formed by reduced and oxidized OxyR (Fig. 6a). At high concentrations, OxyR may bind to the *katG* promoter as an oligomer leading to the C2 band in the gel-shift experiment.

To test whether these different types of DNA–protein complexes are due to different sized DNA–protein complexes or rather to different conformation of the complexes, we performed gel filtration assays with purified protein. The purified protein was loaded on a Superdex-200 gel filtration column, and the fractions were analysed by SDS-PAGE. The oxidized protein eluted predominantly in fractions that most likely correspond to a tetramer of the 32.2 kDa OxyR protein (128.8 kDa) (Fig. 7a). We also examined the size of the protein when the column was loaded and eluted in the presence of 200 mM DTT. As shown by AMS modification, 200 mM DTT reduced the

Fig. 6. Binding of reduced and air-oxidized OxyR to the *kat* promoter region. To generate reduced conditions, 200 mM DTT was added to the binding reactions. Ox and red indicate oxidized and reduced OxyR, respectively. (a) Binding of OxyR to the *katG* promoter region. C1_{ox}, complex 1 of DNA and oxidized OxyR; C1_{red}, complex 1 of DNA and reduced OxyR; C2, complex 2. The following amounts of OxyR were added to the reaction: 10 ng (lanes 1 and 5), 20 ng (lanes 2 and 6), 50 ng (lanes 3 and 7) and 100 ng (lanes 4 and 8). (b) Binding of OxyR to the *katE* promoter region. C1, complex of DNA and oxidized or reduced OxyR; C2, complex 2. The following amounts of OxyR were added to the reaction: 10 ng (lanes 1 and 5), 20 ng (lanes 2 and 6), 50 ng (lanes 3 and 7), 100 ng (lanes 4 and 8). P represents unbound probe, c represents the control without addition of OxyR. (c) DNaseI footprinting assay with oxidized and reduced (in the presence of 200 mM DTT) OxyR binding to the *katG* promoter region. The regions protected by OxyR are indicated by the brackets. The heavy brackets indicate the reduced extended footprint of OxyR, the light brackets indicate the shorter footprint of oxidized and reduced OxyR. The positions of the footprints are labelled with respect to the *katG* translational start site. (d) Sequence of the *katG* and *oxyR* promoters. The starts of the *katG* and the *oxyR* coding sequences are indicated by arrows. Binding sites of oxidized and reduced OxyR are denoted by black bars.

Fig. 7. (a) Analytical gel filtration profile of oxidized (thin line) and reduced (heavy line) OxyR protein. The purified OxyR protein was passed through a Superdex-200 HR 16/60 column equilibrated with Z buffer. To generate reducing conditions, 200 mM DTT was added to the Z buffer. The flow rate was 1 ml min⁻¹, and the absorbance of the eluted protein was monitored at 280 nm. (b) The elution fractions were analysed by SDS-PAGE. The elution position of the peak fraction for the standard protein immunoglobulin G (150 kDa) under oxidizing and reducing conditions is indicated. (c) Redox status of OxyR. OxyR protein (125 ng) was reduced or oxidized with 200 mM DTT or 1 mM H₂O₂. Samples were mixed with 10% TCA and then treated with AMS. Separation and visualization of OxyR was achieved by SDS-PAGE and silver staining. Control: OxyR without DTT, H₂O₂ and AMS treatment. The addition of AMS leads to the alkylation of free thiol groups, present in the reduced OxyR. The addition of the high molecular mass AMS moiety to the reduced but not to the oxidized protein allows separation of the two forms by gel electrophoresis.

OxyR protein completely (Fig. 7c). The elution profile observed under reducing conditions was identical to that in the absence of DTT (Fig. 7a, b). Protein eluted from the

column in presence of DTT still resulted in band C1red in the gel shift (not shown), confirming that OxyR was still reduced. These data indicate that oxidized as well as reduced OxyR binds as a tetramer, but that the conformation of the DNA-protein complexes differ.

Since the OxyR proteins of both *R. capsulatus* and *R. sphaeroides* show a high homology to each other (43 % identity, 56 % similarity), we also tested the binding capacity of the *R. capsulatus* OxyR protein to the *katE* promoter region. We observed a binding of both the reduced and oxidized protein to the *katE* promoter region, but no different DNA-protein complexes were observed (Fig. 6b).

In order to define the binding sites of reduced and oxidized OxyR to the *katG* promoter region better, we performed footprint analysis. Oxidized OxyR protein bound to AT-rich sequences close to the translational start of *oxyR* (Fig. 6c, d). When reduced OxyR was applied, the footprint was significantly extended. A long DNA stretch comprising sequences well within the *oxyR* gene was protected from DNaseI digestion.

DISCUSSION

R. sphaeroides and *R. capsulatus* are related bacteria living in the same habitats. In their natural environment, mostly lakes and ponds, they are exposed to changing light and oxygen conditions. Although the responses of the two species to changes in the environment are similar, some differences can be observed. *R. sphaeroides* harbours almost no bacteriochlorophyll under high oxygen tension, while *R. capsulatus* assembles low amounts of photosynthetic complexes. The latter strategy allows a faster adaptation to decreasing oxygen concentrations and a faster switch to anoxygenic photosynthesis when light is present. In this study, we observed a different performance of the two *Rhodobacter* species under peroxide stress conditions and investigated its molecular basis. Moreover, we could show that environmental stimuli influence the ability of the two *Rhodobacter* strains to cope with this peroxide stress.

Our results reveal that the two *Rhodobacter* strains show significant differences in their ability to cope with peroxide stress, which is correlated to strong differences in catalase activities. Both strains showed much faster detoxification of H₂O₂ under aerobic growth conditions than during growth under low oxygen tension, again correlating to higher catalase activities in both strains when grown under high oxygen tension. The basal expression of genes encoding catalases as determined by real-time RT-PCR was indeed higher under aerobic conditions, in both wild-type strains. Growing at high oxygen tension, bacteria will produce more ROS than during growth at low oxygen tension. Therefore, bacteria adapt to the presence of high oxygen levels by increasing the expression of genes involved in the detoxification of ROS, such as catalases. These higher expression levels result in higher resistance to peroxide stress at high oxygen levels. The assumption that the higher resistance of

R. sphaeroides to H_2O_2 is caused by the presence of two catalases, KatE [encoded by RSP2779 (*katE*)] and KatC [encoded by RSP2380 (*katC*)] was not supported by our findings. While the expression of *katE* was strongly induced by oxygen or peroxide stress, expression of *katC* remained unaffected (Fig. 3b, c and Fig. 4). The possibility that peroxidases could be responsible for the higher resistance of 2.4.1 can be excluded, since *R. sphaeroides* does not harbour peroxidase activity (Terzenbach & Blaut, 1998). Hochman & Shemesh (1987), however, observed two different peroxidase activities in *R. capsulatus*. Thus, the higher resistance of *R. sphaeroides* to peroxide stress in comparison to *R. capsulatus* is probably not due to the presence of additional enzymes, but rather to the high activity of KatE. Nevertheless, even the low expression levels of *katC* might contribute to increase the resistance of *R. sphaeroides* to H_2O_2 .

A key regulator of the response to H_2O_2 is the OxyR transcriptional regulator, which induces the expression of antioxidant activities in response to H_2O_2 stress (Storz & Imlay, 1999). *oxyR* mutants of both *Rhodobacter* species were more sensitive to the exposure to H_2O_2 and showed slower kinetics in the detoxification of this agent compared to the parental strains, confirming a role of OxyR in the oxidative stress response of *Rhodobacter*. While very little detoxification of H_2O_2 was observed in strain SBoxyR, the *R. sphaeroides* mutant 2.4.1oxyR was able to detoxify 3–5 mM H_2O_2 within 300 s, but with slower kinetics than the isogenic wild-type (Figs 1b and 2c). This indicates that OxyR is more important for defence against H_2O_2 in *R. capsulatus* than in *R. sphaeroides*. Unexpectedly, we found that the *oxyR* mutation resulted in a decrease of total catalase activity in both *Rhodobacter* species. This finding is in contrast to observations in *E. coli*, where the deletion of *oxyR* did not reduce the basal activity of catalases during exponential growth, but only in cells induced with H_2O_2 (Visick & Clarke, 1997). The lower levels of catalase activity in strain SBoxyR compared to strain 2.4.1oxyR are in agreement with the very poor detoxification of H_2O_2 by the *R. capsulatus* mutant. The addition of H_2O_2 to semi-aerobically grown wild-type cultures resulted in an induction of catalase activity, whereas no induction was observed in both *oxyR* mutants. This again is in agreement with findings in other bacterial systems, where OxyR acts as an activator of H_2O_2 -inducible genes. Our observations suggest an effect of OxyR on the catalase activity in unstressed cells as well as an important role of OxyR in the adaptation of *Rhodobacter* to H_2O_2 .

In many bacteria, genes encoding hydroperoxidases (HP) are members of the OxyR regulon. Gene expression analysis in *oxyR* mutant strains revealed that both *katG* of *R. capsulatus* and *katE* of *R. sphaeroides* are regulated by the OxyR protein. In *E. coli* the *katG* gene is strongly induced by H_2O_2 , while the *katE* gene is not (Schellhorn, 1994). A regulatory effect of OxyR on *katG* expression has been shown for many bacteria (Loprasert *et al.*, 2003; Ochsner *et al.*, 2000). Our observation of fast kinetics in the

induction of *kat* gene expression by H_2O_2 as well as the reduction 30 min after addition of H_2O_2 can be explained by the very fast kinetics of activation of OxyR by H_2O_2 . Aslund *et al.* (1999) were able to examine the kinetics of OxyR oxidation and reduction *in vivo* and *in vitro*. OxyR oxidation by H_2O_2 was completed within 30 s, and the half-time of deactivation was 10–30 min. Compared to *katE* and *katG* expression, respectively, expression of *katC* was not affected by OxyR in *R. sphaeroides*. This is in agreement with results for the *E. coli katE* gene (Schellhorn, 1994). Both the *E. coli katE* gene (Schellhorn, 1994) and the *R. sphaeroides katC* gene are induced during stationary phase. The expression of the *E. coli katE* gene is known to be regulated by the stationary phase sigma factor RpoS, while the regulator of the *R. sphaeroides katC* gene is presently unknown. So far, no homologue of *rpoS* has been reported for the α -subclass of proteobacteria, which includes the *Rhodobacter* species (Rava *et al.*, 1999; Roop *et al.*, 2003).

The *oxyR* family is widespread among prokaryotes and nearly all known *oxyR* genes share overlapping promoters with other genes (Kim & Mayfield, 2000). The majority of genes located adjacent to *oxyR* are involved in oxidative stress protection, such as *aphC*, *dps* and *oxyS*, and are regulated by OxyR (Nakjarung *et al.*, 2003). In both *Rhodobacter* species the *katE* and *katG* genes, respectively, are located adjacent to the *oxyR* gene on the chromosome. Both genes are separated by approximately 100 nt (*katE/oxyR* 101 nt; *katG/oxyR* 98 nt) of untranslated region and are transcribed divergently. Thus, the *oxyR* and *katE* or *katG* genes, respectively, share a common upstream DNA sequence and may also share *cis* regulatory elements. This *kat/oxyR* gene organization is found in many α -proteobacteria [*Rhizobium etli* (del Carmen Vargas *et al.*, 2003); *Brucella abortus* (Kim & Mayfield, 2000); *Agrobacterium tumefaciens* (Nakjarung *et al.*, 2003)], suggesting a general mechanism of regulation of these genes in α -proteobacteria.

Since it is reported that the OxyR protein regulates expression of genes by direct binding to the promoter region (reviewed by Schell, 1993), we tested the ability to bind DNA of the *R. capsulatus* OxyR protein. The OxyR protein showed strong binding to the *katG* promoter region and our data indicate that different conformations of DNA–protein complexes are formed with oxidized or reduced OxyR. Footprint analysis of the *E. coli* OxyR protein showed that OxyR binding is different under oxidizing and reducing conditions (Kullik *et al.*, 1995). As described for *E. coli* (Kullik *et al.*, 1995), the oxidized and reduced forms of *R. capsulatus* OxyR were predominantly tetrameric as revealed by size exclusion chromatography. As a member of the LysR family of bacterial regulators, OxyR acts as an activator of a regulon of genes and a repressor of its own expression (Storz & Altuvia, 1994). Toledano *et al.* (1994), looking in *E. coli*, found that only oxidized OxyR binds *katG*, *ahpC*, *dps* and *gorA* promoters, whereas both the oxidized and the reduced protein bind the *oxyRS* promoter. They proposed that, by remodelling its DNA contacts, OxyR

can impose opposite regulatory effects on the divergent *oxyR* and *oxyS* promoters.

Our results show that oxidized as well as reduced OxyR binds to the *katG/oxyR* promoter region, but the reduced protein makes extended contacts to the DNA compared to the oxidized protein. Our data also show that *katG* expression is strongly increased by H₂O₂, and that H₂O₂ leads to oxidation of the OxyR protein (Fig. 7c). Without the addition of H₂O₂, the level of *katG* expression is similar in the wild-type and the *oxyR* mutant. Thus OxyR acts as an activator of *katG* expression in the presence of H₂O₂. We propose that this activation includes the release of OxyR from binding sites within the *oxyR* gene. Our findings also suggest that reduced OxyR has little repressing effect on *katG* expression, which is low in the absence of oxidized OxyR.

Future work will be aimed at the identification of additional OxyR-regulated genes and additional OxyR-binding sites to understand better the OxyR signalling mechanism in *Rhodobacter*.

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