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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Molecular mechanism involved in the response to hydrogen peroxide stress in *Acinetobacter oleivorans* DR1

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Abstract Two-dimensional gel electrophoresis was conducted to investigate the effect of H_2O_2 on whole protein expression in *Acinetobacter oleivorans* DR1. Functional classification of 13 upregulated proteins using MALDI-TOF mass spectrometry showed relationships with oxidative stress, energy production and conversion, nucleotide and amino acid metabolism, membrane-related, ion transport, and chaperone-related functions. Alignment of OxyR-binding regions from *Pseudomonas aeruginosa* and *Escherichia coli* with promoters of identified proteins revealed that only *ahpC*, *ahpF*, and *trxB* (thioredoxin-disulfide reductase) genes, along with a newly found *oprC* (putative outer membrane receptor protein) gene, have OxyR-binding sites. The *oxyR* and *ahpC* mutants were more sensitive to H_2O_2 and showed growth defects in both nutritional and *n*-hexadecane-amended media. Four catalases present in the genome of *A. oleivorans* DR1 were not detected, which led us to confirm the expression and activity of those catalases in the presence of H_2O_2. The expression patterns of the four catalase genes differed at different concentrations of H_2O_2. Interestingly, the promoters of both known OxyR-controlled *katG* gene (AOLE_17390) and putative small catalase gene (AOLE_09800) have OxyR-binding sites. Gel-shift assay confirmed OxyR binding to the promoter regions of newly identified OxyR-controlled genes encoding OprC and a putative catalase. Hierarchical expression and OxyR-binding of several OxyR-controlled genes suggested that concentration is an important factor in inducing the set of genes under H_2O_2 stress.

Keywords H_2O_2 · OxyR · Oxidative stress · Proteomics · *Acinetobacter*

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Introduction

Bacteria have systems for sensing and removing the endogenous or exogenous oxidants they are exposed to (Wei et al. 2012). Oxygen can accept electrons from cellular reductants or reactive oxygen species (ROS) (Cabiscol et al. 2000; Imlay 2013). Moreover, ROS are produced by general aerobic metabolism, or exposure to redox-cycling drugs or phagocytosis during infection (Wei et al. 2012), and are injurious species that react with and damage intracellular components (Imlay 2003). Superoxide ($O_2^{\bullet-}$) is generated when an oxygen molecule accepts an electron from a donor such as a flavoprotein. Similarly, hydrogen peroxide (H_2O_2) is produced when two electrons are received by an oxygen molecule. Superoxide and H_2O_2 can inactivate enzymes that disrupt iron-sulfur clusters (Kuo et al. 1987; Dubbs and Mongkolsuk 2012). H_2O_2 reacts with ferric iron, forming the highly toxic hydroxyl radical ($HO^{\bullet}$) through the Fenton reaction. This hydroxyl radical is a strong oxidant that can react with almost every molecule within a cell including DNA, RNA, proteins, and lipids. The oxidation of DNA by hydroxyl radicals induces DNA lesions or mutations that have deleterious effects on the cell (Imlay 2003, 2013).

Bacteria have oxidative stress defense systems that protect against ROS-mediated damage (Zheng et al. 1998). Several in-depth studies have attempted to explain the mechanisms underlying the antioxidant defense system by using *Escherichia coli* as a model organism (Farr and Kogoma 1991). In *E. coli*, two major redox-sensing proteins, SoxR and OxyR, are activated upon oxidation (Imlay 2013). These transcriptional regulators modulate gene expression involved in the oxidative stress response and defense (Pomposiello and Demple 2001; Imlay 2013). Activated SoxR regulates the expression of SoxS, which then controls the expression of genes including *sodA* (manganese superoxide dismutase), *fpr* (ferredoxin-NADP⁺ reductase), *zwf* (glucose-6-phosphate dehydrogenase), *nfo* (DNA repair endonuclease IV), and *fumC* (fumarase C) (Pomposiello and Demple 2001; Martin and Rosner 2003; Mukhopadhyay et al. 2004). H_2O_2 activates OxyR, which is a LysR-type transcriptional regulator, through the oxidation of two conserved cysteine residues (Cys 199 and Cys 208). Oxidized OxyR then regulates the expression of defensive genes, such as *katG* (hydrogen peroxidase I), *ahpCF* (alkyl hydroperoxidase), *gorA* (glutathione reductase), *grxA* (glutaredoxin I), *oxyS* (a small regulatory RNA), and *dps* (a nonspecific DNA-binding protein) (Zheng et al. 1999, 2001). OxyR homologues have been found in various bacteria; in particular, OxyR regulates the expression of two major catalases (KatA and KatB), AhpC (peroxiredoxin), and TrxB (thioredoxin reductase) in *Pseudomonas putida* (Fukumori and Kishii 2001; Hishinuma et al. 2006, 2008; Kim and Park 2014). Recently, the presence of the *oxyR-recG* operon and its function in the antioxidant system have been reported in *P. putida* and *Pseudomonas aeruginosa* (Ochsner et al. 2000; Yeom et al. 2012).

Studies of the mechanisms of oxidative stress defense in *Acinetobacter* species have mainly focused on pathogenic *Acinetobacter baumannii*, but nonpathogenic *Acinetobacter* species can easily encounter ROS generated both endogenously and in their natural habitat (e.g., soil). The bacterial oxidative stress response in soil-borne *Acinetobacter* species has only rarely been explored and is therefore a valuable line of study. Thus far, there are a few published reports on transcriptional regulation of the oxidative stress defense system in *Acinetobacter* species (Longkumer et al. 2014). In *A. baumannii*, the expression of a novel glutathione *S*-transferase gene was found to be OxyR-dependent (Longkumer et al. 2014).

Acinetobacter oleivorans strain DR1 has been isolated from a rice paddy (Jung et al. 2010). We previously found that *A. oleivorans* DR1 survival upon exposure to antibiotics was related to upregulation of oxidative stress response genes, as demonstrated by transcriptome analyses (Kim et al. 2013; Hong et al. 2014). Although *Acinetobacter* species have been widely studied recently, the regulation of genes controlled by OxyR in *Acinetobacter* species has received little attention.

Therefore, the goals of this study were to understand the molecular mechanism involved in the response to H_2O_2 stress and to identify the target genes regulated by OxyR. We focused on protein profiling using two-dimensional gel electrophoresis (2-DE) and MALDI-TOF MS analyses in the presence of H_2O_2, and direct binding of OxyR to the target promoters was examined using a DNA-protein binding affinity assay. Target genes regulated by OxyR were newly identified in *A. oleivorans* DR1, and the physiological functions of OxyR were demonstrated.

Materials and methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are shown in Table 1. A diesel-degrading *A. oleivorans* DR1 was isolated from the soil of a Korea University paddy field (Deokso, Gyeonggi-Do, Korea); its genome has been completely sequenced in a previous study (Jung et al. 2010). *A. oleivorans* DR1 was grown at 30 °C in nutrient broth with aeration by shaking. *E. coli* was grown at 37 °C in Luria-Bertani (LB) medium. When required, kanamycin (50 μg/ml) was added to the media. Growth was monitored by measuring the optical density of the cultures at 600 nm (OD_{600}) using a BioPhotometer (Eppendorf, Hamburg, Germany). The complete genome sequence of strain DR1 can be found in GenBank (accession no. CP002080). Strain DR1 was deposited in the Korea Collection for Type Cultures (KCTC 23045) and the Japan Collection of Microorganisms (JCM 16667).

Construction of the *oxyR* and *ahpC* mutants in *A. oleivorans* DR1

The primers used in this study are listed in Table 1. Disruption of the *oxyR* and *ahpC* genes was achieved using a single-crossover recombination method with the suicide vector pVIK112 (Kalogeraki and Winans 1997). The fragment encompassing the partial *oxyR* gene of *A. oleivorans* DR1 was amplified using *oxyR*-F and *oxyR*-R primers. A 485-bp PCR product for *oxyR* was digested using *Eco*RI and *Kpn*I restriction enzymes. A 384-bp PCR product for *ahpC* (amplified using *ahpC*-F and *ahpC*-R primers) was digested using *Sma*I and *Kpn*I restriction enzymes. Fragments were subsequently inserted into the pVIK112 vector via ligation and then pVIK112-*oxyR* and pVIK112-*ahpC* were transformed into *E. coli* S17-1λpir. Constructed plasmids were then introduced into *A. oleivorans* DR1 by electroporation. To ensure that homologous recombination occurred in *A. oleivorans* DR1, PCR verification was conducted using the *oxyR* OE-F/MCS-R and *ahpC* OE-F/MCS-R primer pairs. The MCS-R primer was designed based on the sequence of the pVIK112 plasmid

Table 1 Bacterial strains, plasmids, and primers used in this study

Strain/plasmid/primer	Description/sequence (5′-3′)	Source/reference
Strains		
Acinetobacter oleivorans DR1	Wild type, diesel oil degrader	Jung et al. (2010)
ΔoxyR	*oxyR* (encoded by AOLE_14380) mutant; insertion of pVIK112-*oxyR* into DR1	This study
ΔahpC	*ahpC* (encoded by AOLE_13380) mutant; insertion of pVIK112-*ahpC* into DR1	This study
DR1 (pRK415)	Insertion of pRK415 into DR1	Park and Park (2011)
DR1 (pRK415-AOLE_09800)	Insertion of pRK415-AOLE_09800 into DR1	This study
Escherichia coli BL21(DE3)	F- ompT hsdSB (rB-mB-) gal dcm (DE3)	Invitrogen (Carlsbad, CA, USA)
Escherichia coli BL21(DE3) (OxyR)	pET-28a(+)-*oxyR* into *E. coli* BL21(DE3)	This study
Escherichia coli BL21(DE3) (AOLE_09800)	pET-28a(+)-AOLE_09800 into *E. coli* BL21(DE3)	This study
E. coli Top10	F⁻ ara D 139 Δ(ara, leu) 7697 ΔlacX74 galU galK rpsL (Str^R) deoR ø80dlacZΔM15 endA1 nupG recA1 mcrA Δ(mrr–hsdRMS mcrBC)	Invitrogen (Carlsbad, CA, USA)
Plasmids		
pVIK112	R6K, *oriV*, suicide vector, *lacZ* fusion	Kalogeraki and Winans (1997)
pVIK112-*oxyR*	Km^r, internal *oxyR* fragment in pVIK112	This study
pVIK112-*ahpC*	Km^r, internal *ahpC* fragment in pVIK112	This study
pRK415	Broad-host-range vector, Tet^R, Mob$^+$	Yin et al. (2003)
pRK415-AOLE_09800	Insertion of the full-length AOLE_09800 region in pRK415	This study
pET-28a(+)	Protein overexpression with His taq	Novagen (Madison, WI, USA)
pET-28a(+)-*oxyR*	*oxyR* fragment region in pET-28a(+)	This study
pET-28a(+)-AOLE_09800	AOLE_09800 gene in pET-28a(+)	This study
Primers		
oxyR and *ahpC* mutants, OxyR overexpression		
oxyR OE-F	CGCCATATGATGGCTGCATTACCCTCA	
oxyR OE-R	CGCGAGCTCGGCAAATATGGTTCAGTCGCAGTT	
oxyR-F	CGCGAATTCTAGGCGAACAAGTCGTTGCT	
oxyR-R	CGCGGTACCACTTGCTTTTAAACGGTTGTCGT	
ahpC OE-F	CGCCCCGGGTCGAATACAGTGTTTGGGGACT	
ahpC-F	CGCCCCGGGTGCTTACCACAACGGCCAAT	
ahpC-R	CGCGGTACCCCGATACCACCAGCGTTGAG	
AOLE_09800 pRK-F	CGCCTGCAGAATATTTTTGCACTGAGGAGAT	
AOLE_09800 pRK-R	CGCTCTAGATTATGGTTAGCAGATTTTATTGAT	
AOLE_09800 OE-F	CGCGGATCCATGTCAAATTTTATGCAG	
AOLE_09800 OE-R	CGCCTCGAGAATTTCGAGGGTGCAGTTGTTAG	
MCS-R	ACCATGGTCATAGCTGTTTCCTG	
EMSA		
AOLE_09800 proF	CATTTTGTGAGTTAGTTATC	
AOLE_09800 proR	ATTTGACATTTATTATCTCC	
AOLE_11385 proF	ACTTGTCATTATGGTAGTCATTGC	
AOLE_11385 proF(2)	CTTAATTGCTGGAGCGGTATT	
AOLE_11385 proR	ACTCCAATGCATCCAAATTCTTTCA	
AOLE_13380 proF	CGCTCTAATGCCCTTCAGGT	
AOLE_13380 proF(2)	TACTATAACTGTCATTTGAT	
AOLE_13380 proR	TCAAGCTCATCATTGTCTCCA	
AOLE_15340 proF	CACTCGACACCGCAGTCAT	
AOLE_15340 proF(2)	GTTTAGAGATGTGAAGAACT	
AOLE_15340 proR	TAACCGAGAATGACGAGCACT	
AOLE_15340 proR(2)	TGAAAATTATACGTGAATTT	

Table 1 (continued)

Strain/plasmid/primer	Description/sequence (5′-3′)	Source/reference
AOLE_18500 proF	TACGAAGACGGGGATCAGGA	
AOLE_18500 proR	GCATGAAATTCAACCATGCCG	
qRT-PCR		
AOLE_09800-F	TAAAGCGCATGGTTGTGTTCG	
AOLE_09800-R	ATTCCGCGTGCATCTTTGTC	
AOLE_11385-F	TAATTGGTGGTGGACCTGCG	
AOLE_11385-R	TCGGCAGCAAACTTAGGACC	
AOLE_13380-F	TCCAGCTGACTTCACTTTCGT	
AOLE_13380-R	GAGTCCAAGTTGGGTCACCA	
AOLE_15340-F	GCATGTGCAACATGTGATGGT	
AOLE_15340-R	TTGCCTTCTTTTTCTTTGGCA	
AOLE_18500-F	CGCGATGGCAATGGAGGTAA	
AOLE_18500-R	AGCACCGTATGATTGAAAACGC	

Underlined sections indicate restriction sites for cloning

(Yeom et al. 2010b). When the *oxyR* OE-F and MCS-R primer pairs were used, a 712-bp PCR product was observed in the *oxyR* mutant cells, indicating that the vector sequence was inserted in the *oxyR* region. When the *ahpC* OE-F and MCS-R primer pairs were used, a 502-bp PCR product was observed in the *ahpC* mutant cells, indicating that the vector sequence had been inserted in the *ahpC* region.

Construction of putative catalase-overproducing *A. oleivorans* DR1 strain

Overproduction of AOLE_09800-encoded protein in *A. oleivorans* DR1 was carried out using a broad-host-range vector pRK415 (Yin et al. 2003). The region of AOLE_09800 was amplified using the AOLE_09800 pRK-F and AOLE_09800 pRK-R primers. The amplified fragments were cloned into the *Pst*I and *Xba*I cloning site of pRK415. The constructed vector was transformed to *A. oleivorans* DR1, yielding DR1 (pRK415-AOLE_09800).

Protein sample preparation and two-dimensional gel electrophoresis for proteomics

Two independent *A. oleivorans* DR1 cultures were grown overnight in nutrient broth and diluted 100-fold. When the diluted cells reached the exponential phase ($OD_{600} \sim 0.4$), they were exposed to H_2O_2 (1 mM) for 60 min. Then, bacterial cell pellets were collected and washed twice with ice-cold phosphate-buffered saline. Protein profiling of each sample (control and H_2O_2 treatment) was performed in duplicate. 2-DE was performed as previously described (Kang and Park 2010). Cell pellets were sonicated for 10 s using a Sonoplus ultrasonic homogenizer (Bandelin electronic, Berlin, Germany) in the sample lysis solution comprising: 7 M urea; 2 M thiourea containing 4 % (w/v) 3-[(3-cholamidopropy) dimethyammonio]-1-propanesulfonate (CHAPS), 1 % (w/v) dithiothreitol (DTT), and 2 % (v/v) Pharmalyte; and 1 mM benzamidine. All chemicals in the sample lysis solution were purchased from Sigma-Aldrich (St Louis, MO, USA). Proteins were extracted for 1 h at room temperature with vortexing. After centrifugation at $15,000g$ for 1 h at 15 °C, insoluble material was discarded and the soluble fraction was used for 2-DE. Protein loading was normalized by the Bradford assay.

Immobilized pH gradient dry strips were equilibrated for 12–16 h with the reswelling solution containing 7 M urea, 2 M thiourea containing 2 % CHAPS, 1 % DTT, and 1 % Pharmalyte, and then loaded with 800 μg of the sample. All chemicals in the reswelling solution were purchased from Sigma-Aldrich. Isoelectric focusing was performed at 20 °C using a Multiphor II electrophoresis unit and an EPS 3500 XL power supply (Amersham Biosciences, Piscataway, NJ, USA) following the manufacturer's instructions. For isoelectric focusing, the voltage was linearly increased from 150 to 3,500 V over 3 h for sample entry, followed by maintenance at 3,500 V, with focusing complete after 96 kVh. Prior to the two-dimensional separation, strips were incubated for 10 min in equilibration buffer (50 mM Tris-Cl, pH 6.8, containing 6 M urea, 2 % sodium dodecyl sulfate (SDS), and 30 % glycerol), first with 1 % DTT and then with 2.5 % iodoacetamide. The equilibrated strips were placed onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (20× 24 cm, 10–16 %). SDS-PAGE was performed using a Hoefer DALT 2D system (Amersham Biosciences) following the manufacturer's instructions. Two-dimensional gels were run at 20 °C for 1,700 Vh and then stained with Coomassie brilliant blue G250 (Bio-Rad, Hercules, CA, USA) and SYPRO® Ruby (Invitrogen, Carlsbad, CA, USA). After

electrophoresis, the gel was placed into a clean container and agitated on an orbital shaker with 400 ml of fixative solution (40 % ethanol, 10 % acetic acid) for 600 min. After fixing, the gel was hydrated with 400 ml of rehydration solution (5 % ethanol, 5 % acetic acid) for 30 min. Rehydration was performed twice more with fresh rehydration solution. SYPRO® Ruby gel stain (400 ml) was then added, followed by agitation on an orbital shaker for 2 h. The gel was then transferred to a clean container and washed with 400 ml of wash solution for 30 min. Fluorescence image acquisition was performed with a CCD camera (DIVERSITY, Syngene, Frederick, MD, USA) with a Cy3 emission filter for 20 s of exposure time. Quantitative analysis of digitized images was performed using PDQuest (version 7.0, Bio-Rad) software according to the protocols provided by the manufacturer. The quantity for each spot was normalized against the total valid spot intensity. Protein spots were selected for significant expression when the variation in intensity deviated over 1.5-fold with respect to the control or normal sample.

Protein identification by mass spectrometry

For protein identification by peptide mass fingerprinting (PMF), protein spots were excised, digested with trypsin (Promega, Madison, WI, USA), mixed with α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile/0.1 % TFA, and subjected to MALDI-TOF analysis (microflex LRF 20, Bruker Daltonics, Billerica, MA, USA). Spectra were collected from 300 shots per spectrum over m/z range 600–3,000 and calibrated by two-point internal calibration using trypsin auto-digestion peaks (m/z 842.5099, 2211.1046). The peak list was generated using Flex Analysis 3.0 (Bruker Daltonics). The threshold used for peak-picking was as follows: 500 for minimum resolution of monoisotopic mass, and 5 for S/N. The search program MASCOT, developed by Matrixscience (http://www.matrixscience.com/), was used for protein identification by peptide mass fingerprinting. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic masses, and a mass tolerance of ±0.1 Da. The PMF acceptance criteria were based on probability scoring. The differentially expressed protein spots were identified by MALDI-TOF mass spectrometry and their detailed information is shown in Table S1.

OxyR and catalase protein purification

To demonstrate the direct regulation of candidate genes by OxyR, we expressed and purified OxyR. The *oxyR* region was amplified by PCR using the *oxyR* OE-F and *oxyR* OE-R primers. The fragment was cloned into the *Nde*I and *Sac*I cloning sites of the pET-28a(+) vector. The

constructed vector was conjugated to *E. coli* Top10 and introduced via transformation into *E. coli* BL21 for over-expression. For OxyR purification, *E. coli* BL21 (DE3) cells harboring pET-28a(+)-*oxyR* were grown to the exponential phase ($OD_{600} \sim 0.4$) at 37 °C with aeration. The cell cultures were grown at 30 °C for 4 h after induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and then harvested. To purify the putative catalase (AOLE_09800), PCR amplification using AOLE_09800 OE-F/R primers was performed. The fragment was cloned into the *Bam*HI and *Xho*I restriction enzyme sites of the pET-28a(+) vector. The constructed vector was transformed into *E. coli* BL21 (DE3). For catalase purification, *E. coli* BL21 (DE3) cells harboring pET-28a(+)-AOLE_09800 were grown to the exponential phase at 37 °C with aeration in LB medium. Then, the cell cultures were grown with 0.5 mM $FeCl_2$ at 30 °C for 3 h after induction with 0.5 mM IPTG. All purification steps were performed at 4 °C using a fast protein liquid chromatography (FPLC) system (ÄKTAFPLC, Unicorn 4.0, Amersham Biosciences). *E. coli* cell pellets were resuspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 40 mM imidazole, pH 7.4) and disrupted by sonication. After removal of cell debris by centrifugation at $10,000 g$ for 30 min, the soluble fraction was loaded onto a nickel-nitriloacetic acid (Ni-NTA) column (1 ml, His-trap, Amersham Biosciences) equilibrated with binding buffer and the proteins were eluted with 20 ml of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, and 250 mM imidazole, pH 7.4). The eluted fractions were dialyzed by ultrafiltration with Centricon tubes (Amicon, Beverly, MA, USA) and stored at −80 °C in 10 % glycerol. SDS-PAGE was performed using 10 % polyacrylamide gels to check the level of expression and purification.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was conducted as described previously (Kim and Park 2013). The DNA probe was generated using the primer pairs listed in Table 1. The PCR product was dephosphorylated and labeled with $[\gamma\text{-}^{32}P]ATP$ and T4 polynucleotide kinase. The reaction mixture (20 μl final volume) containing the probe, protein, and loading buffer in 5× binding buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 50 %v/v glycerol, 10 mM DTT, and 375 mM KCl) was incubated for 30 min at 4 °C. The resulting complexes were analyzed by electrophoresis on 5 % poly-acrylamide gels in 0.5×Tris-borate/EDTA buffer (1.1 M Tris, 900 mM borate, 25 mM EDTA, pH 8.3) for 2 h at 120 V. Autoradiography was conducted using an IP plate (Fujifilm, Tokyo, Japan) and the Multiplex Bio-Imaging System (Fujifilm).

Quantitative reverse transcriptase-PCR

Total RNA was isolated from 5 ml of cells in the exponential growth phase ($OD_{600}\sim0.4$) using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of RNA with primers (Table 1) and used as the template for quantitative reverse transcriptase (qRT)-PCR. The PCR mixture contained 12.5 μl of iQ SYBR Green Supermix (Bio-Rad), 1 μl of each primer (0.5 μM), and 2 μl of 100-fold diluted cDNA in a total volume of 25 μl. The PCR conditions were 95 °C for 3 min, followed by 40 cycles of 45 s at 95 °C, 45 s at 61 °C, and 45 s at 72 °C. To normalize the expression of each gene, the expression level of 16S rDNA was quantified using primers that have been described previously (Watanabe et al. 2001). The quantification results were calculated from three independent cultures.

Assay of catalase activity and activity gel staining

The catalase activity was measured as described by Wayne and Diaz (1986). Whole-cell lysates were prepared from 5 ml of cells in the exponential growth phase with or without H_2O_2 treatment. The cells were lysed by sonication and centrifuged at 4 °C for 30 min (12,000g). Supernatants were loaded onto a 7.5 % native acrylamide gel and separated at 120 V for 40 min. The gel was washed extensively in distilled water and immersed in a substrate solution containing 0.01 M H_2O_2 for 10 min in darkness. Subsequently, the gel was stained with a solution containing 2 % (w/v) ferric chloride and 2 % (w/v) potassium ferric cyanide for the visualization of catalase activity.

Measurement of H_2O_2 degradation

The amount of H_2O_2 was measured as described by Seaver and Imlay (2001). A horseradish peroxidase (HRP)-based assay using Amplex Red (AR) was employed to detect H_2O_2. HRP was purchased from Sigma-Aldrich and AR was obtained from Invitrogen. In the presence of H_2O_2, horseradish peroxidase oxidizes AR to the fluorescent product, resorufin (Zhou et al. 1997). To measure H_2O_2, 100 μl of sample was mixed with 50 μl of 0.05 mg/ml AR and 50 μl of 0.05 mg/ml HRP. Fluorescence was then measured using a fluorometer (Multi-Detection Microplate Reader, HIDEX, Turku, Finland) and converted to H_2O_2 concentration using our standard curve. To detect H_2O_2 scavenging by whole cells, exponentially growing cells ($OD_{600}\sim0.4$) were collected and washed twice, and resuspended in fresh media (minimal salts basal media supplemented with 10 mM succinate) to an OD_{600} of 0.1. Then, H_2O_2 (250 μM) was added. At various time intervals, 1-ml aliquots were removed and assayed immediately for H_2O_2 measurement. To detect H_2O_2 scavenging

activity of purified catalase, 1.2 or 12 μM of purified catalases reacts with 5 μM H_2O_2 at 30 °C. At various time intervals, aliquots were assayed immediately for H_2O_2 content by the AR/HRP method.

Results

Proteomic analysis of the oxidative stress response induced by H_2O_2

To elucidate the alteration of protein profiles in the presence of H_2O_2, we conducted 2-DE PAGE and MALDI-TOF MS analyses. To determine the appropriate concentration of H_2O_2 for differential protein expression, we measured growth upon H_2O_2 treatment. A concentration of 1 mM was selected for H_2O_2 because this concentration caused slight inhibition of cell growth but did not kill the cells during 1 h of treatment at the exponential growth phase (data not shown). Protein profiling of each sample (control and H_2O_2 treatment) was performed in duplicate by 2-DE of the whole protein extract. Differentially expressed proteins were analyzed and classified. Of the more than 550 individually detectable protein spots, 54 spots were differentially expressed in response to H_2O_2 treatment (18 spots upregulated and 36 spots downregulated). Proteins upregulated in the presence of H_2O_2 were identified by peptide mass fingerprinting using MALDI-TOF MS. Several upregulated spots were derived from the same proteins (AhpC, AhpF, and PurL) (Table 2). The highest protein expression level among all the upregulated proteins was found for AhpC (peroxiredoxin; encoded by AOLE_13380; upregulated 1120.2-fold), which is involved in the detoxifying process for hydroperoxides. AhpF (alkyl hydroperoxide reductase; encoded by AOLE_11385), which can reduce oxidized AhpC for reactivation, was also highly expressed upon H_2O_2 treatment (upregulated 401.5-fold) (Table 2) (Hishinuma et al. 2006). Peroxiredoxin AhpC and thioredoxin reductase TrxB are known to be regulated by OxyR in *P. putida* (Fukumori and Kishii 2001; Hishinuma et al. 2006, 2008). According to our protein profiling analysis, TrxB (encoded by AOLE_15340) was induced by H_2O_2 along with AhpC and AhpF (Table 2). We predicted that AhpCF and TrxB may be regulated by OxyR in *A. oleivorans* DR1, which was similar to the well-characterized OxyR regulon in *E. coli* and *P. putida*.

According to the proteomic analysis using MALDI-TOF mass spectrometry, well-known oxidative stress response proteins (including AhpCF and TrxB) were highly activated to act as antioxidant enzymes in the presence of H_2O_2 in *A. oleivorans* DR1. Furthermore, proteins involved in energy production and conversion (Etfα and PntA), nucleotide metabolism (PurD and PurL),

Table 2 Proteins upregulated under 1 mM H_2O_2 treatment for 1 h

Spot no.	MW	pI	Gene	Protein	Locus taq	Fold
102	27.82	4.38	*ahpC*	Peroxiredoxin	AOLE_13380	1120.2(H)
204	37.89	4.27	*etfA*	Electron transfer flavoprotein subunit alpha	AOLE_04180	918.2(H)
712	67.85	4.55	*ahpF*	Alkyl hydroperoxide reductase subunit F	AOLE_11385	401.5(H)
713	67.7	4.58	*ahpF*	Alkyl hydroperoxide reductase subunit F	AOLE_11385	261.9(H)
508	54.9	4.38	*purD*	Phosphoribosylamine-glycine ligase	AOLE_06215	190.9(H)
8	27.48	4.57	*ahpC*	Peroxiredoxin	AOLE_13380	15.7
7	27.41	4.54	*ahpC*	Peroxiredoxin	AOLE_13380	10.7
1704	67.72	4.66	*ahpF*	Alkyl hydroperoxide reductase subunit F	AOLE_11385	7.5
5403	49.72	5.42	*pntA*	NAD(P) transhydrogenase subunit alpha	AOLE_16625	2.5
2915	157.36	4.84	*purL*	Phosphoribosylformylglycinamide synthase	AOLE_04400	2.4
2003	14.53	4.83	*lon*	ATP-dependent protease La	AOLE_14185	2
2914	154.34	4.87	*purL*	Phosphoribosylformylglycinamide synthase	AOLE_04400	2
1003	25.45	4.73	*entF*	Putative enterobactin synthetase component F	AOLE_09920	1.9
4405	51.58	5.33	*metK*	S-adenosyl methionine synthetase	AOLE_11050	1.7
6803	93.77	5.86	*oprC*	Outer membrane receptor protein	AOLE_18500	1.7
1303	43.33	4.74	*trxB*	Thioredoxin-disulfide reductase	AOLE_15340	1.6
6102	33.73	5.56	*metF*	5,10-Methylenetetrahydrofolate reductase	AOLE_05340	1.5
4809	94.97	5.32	*metG*	Methionyl-tRNA synthetase	AOLE_15780	1.5

'H' indicates proteins exclusively detected in H_2O_2-treated cells

amino acid metabolism (MetF, MetG, and MetK), membrane-related and ion transport (EntF and OprC), and chaperone-related functions (Lon) were upregulated by more than 1.5-fold under oxidative stress (Table 2). We reasoned that the proteins that were highly induced might play a role in H_2O_2-mediated oxidative stress defense. The hydroxyl radical generated from H_2O_2 is a strong oxidant that can react with cellular molecules, resulting in damage to proteins (Imlay 2003). The removal of damaged proteins is required to prevent their accumulation, which could inhibit the correct cellular metabolism upon exposure to oxidative stress (Tsilibaris et al. 2006). ATP-dependent protease (Lon) may act as protein quality controller under oxidative stress in *A. oleivorans* DR1, as identified in our proteomic analyses.

Three proteins involved in the methionine biosynthetic pathway (MetF, MetG, and MetK) were upregulated by more than 1.5-fold in the presence of H_2O_2 (Table 2). Methionine is an important amino acid because it is an initiator of protein synthesis and a substrate for elongation (Old et al. 1993). In addition, it was previously demonstrated that methionine residues in proteins may act as catalytic antioxidants (Luo and Levine 2009). Therefore, the methionine synthesis pathway plays an important role in protecting *A. oleivorans* DR1 cells under oxidative stress. Proteomic analyses might thus explain the oxidative stress response induced by $H_2O_2$ in *A. oleivorans* DR1; a detailed description of the H_2O_2-induced effect on proteins is presented in the following discussion section.

Expression of catalases in response to H_2O_2 in *A. oleivorans* DR1

Catalases are important for the degradation of H_2O_2. However, catalases were not detected in our proteomic analyses. This result may reflect the time-dependent expression of each protein under different H_2O_2 concentrations in *A. oleivorans* DR1. To confirm the induction of total catalases by H_2O_2, we conducted catalase activity-staining gel analysis. H_2O_2 (1 mM) was added to exponentially growing cells for 0, 15, 30, 45, and 60 min. The activity of the catalases was enhanced as the exposure time increased (Fig. 1a). Four putative catalase genes (AOLE_09800, AOLE_11770, AOLE_12755, and AOLE_17390) are present in the genome of *A. oleivorans* DR1. To investigate which genes are responsible for defense against H_2O_2 stress, we checked the expression of four catalase genes. Their expression patterns were different at each concentration of H_2O_2 (Fig. 1b). H_2O_2-sensing transcriptional regulator OxyR activates the expression of various genes for the defense against H_2O_2 stress. OxyR regulates the expression of catalases in *E. coli* and *P. putida* (Schellhorn 1995; Hishinuma et al. 2006). We speculated that the activation of OxyR by H_2O_2 may induce catalase activity in *A. oleivorans* DR1. To identify the OxyR-regulated catalase gene in

Fig. 1 Catalase activity and expression of catalases in the presence of H_2O_2 in *A. oleivorans* DR1. **a** Visualization of catalase activity by gel staining. Exponentially grown cells were treated with H_2O_2 (1 mM) for 0, 15, 30, 45, and 60 min and then cells were harvested. Cell extracts that contained 25 μg of protein were loaded on a 7.5 % native gel. Catalase activities were enhanced as the exposure time increased. Quantification of band intensity is indicated in the graph. **b** Expression of catalase genes subjected to various concentrations of H_2O_2. Exponentially grown cells were treated with each concentration of H_2O_2 for 15 min. **c** Measurement of H_2O_2 scavenging activity of whole cells and purified catalase. Comparison of H_2O_2 degradation ability with the plasmid harboring the AOLE_09800 gene (*left panel*). Empty vector (pRK415)-containing wild type strain was used as a control. Exponentially growing cells were treated with 250 μM of H_2O_2. At various time intervals, aliquots were assayed immediately for H_2O_2 content by the AR/HRP method. H_2O_2 scavenging activity of purified catalase (*right panel*). 1.2 or 12 μM of purified catalases reacted with 5 μM H_2O_2 at 30 °C. At various time intervals, reaction mixtures were assayed immediately via the AR/HRP method
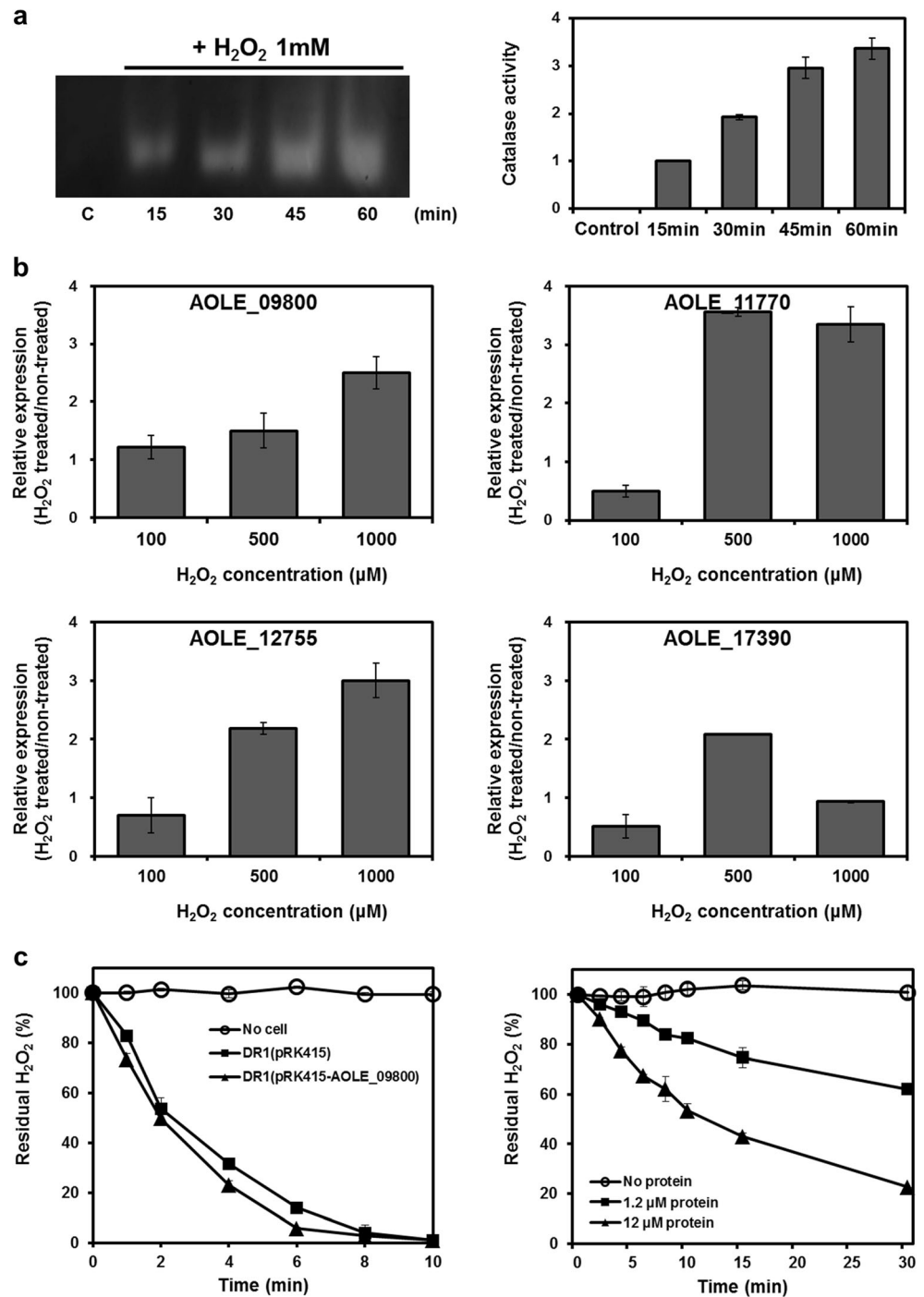
A. oleivorans DR1, we constructed the consensus OxyR-binding sequence using the OxyR box of *P. aeruginosa* and *E. coli* (AGnnnnnnnWTYnWWKnnnnnKnnnRWnA, where W indicates A or T; Y indicates C or T; K indicates G or T; R indicates G or A; and n indicates any nucleotide). Of the four putative catalase genes, AOLE_09800, annotated as a putative catalase, has a putative OxyR-binding sequence in its promoter region. AOLE_09800 genes with consensus sequences in their promoter regions were selected and subjected

to EMSA and qRT-PCR, regardless of whether OxyR functions as a transcriptional regulator. Function of AOLE_09800-encoded protein was initially monitored by its H_2O_2 scavenging activity in DR1 cells by overproduction using a plasmid pRK415. The DR1 (pRK415-AOLE_09800) strain could degrade H_2O_2 more quickly than cells containing only the empty vector (Fig. 1c). To characterize the function of AOLE_09800-encoded protein, we purified the product of AOLE_09800. Purified putative catalase could degrade

H_2O_2 (Fig. 1c). Our data suggested that the purified protein might function as a catalase in *A. oleivorans* DR1.

Construction and characterization of the *oxyR* and *ahpC* mutants

The genome of *A. oleivorans* DR1 encodes an OxyR homologue (AOLE_14380, 302 amino acids) with 33.0, 32.6, and 99.0 % amino acid identity with OxyR of *E. coli*, *P. aeruginosa*, and *A. baumannii*, respectively. The *A. oleivorans* DR1 *oxyR* gene is located next to the *rubA* and *rubB* genes for alkane degradation (Fig. 2a). OxyR can be activated by formation of an intramolecular disulfide bond between two cysteine residues (Aslund et al. 1999). These two cysteine residues (C202 and C211) are highly conserved in the OxyR protein in many bacteria including *A. oleivorans* DR1 (Fig. 2b). The sequence analysis suggested that the OxyR of *A. oleivorans* DR1 may function similarly to *E. coli* and *Pseudomonas* OxyR protein in H_2O_2 sensing, DNA binding, and transcriptional regulation.

To understand the role of OxyR under H_2O_2 stress, we constructed an *oxyR* mutant. The gene encoding AhpC, which showed highest protein expression level in our proteomic analyses, was also disrupted. The two mutants had growth defects in nutrient media (Fig. 3a). The *oxyR* mutant showed more delayed growth than the wild type and the *ahpC* mutant in the exponential phase. The reason could be that peroxides were generated by autoxidation of the medium (Seaver and Imlay 2001; Korshunov and Imlay 2010). We compared the H_2O_2-sensitivity of those strains. The *oxyR* mutant showed greater sensitivity to H_2O_2 than the *ahpC* mutant (Fig. 3a). *A. oleivorans* DR1 can degrade *n*-hexadecane (Jung et al. 2010; Kang and Park 2010), and ROS can be generated as a result of the degradation of hydrocarbon compounds (Denef et al. 2005, 2006). Thus, we tested *oxyR* and *ahpC* mutants for
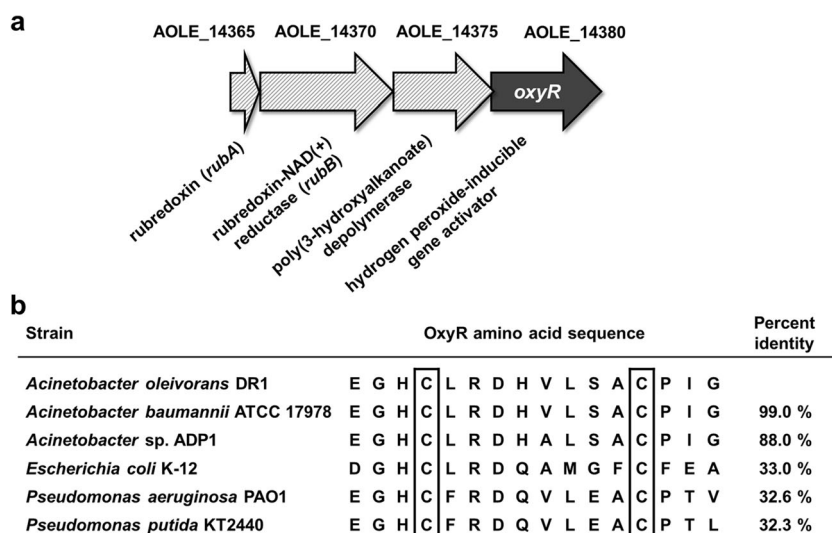
their growth on *n*-hexadecane. Cells were incubated with MSB media containing 2 % *n*-hexadecane. The *ahpC* mutant had slightly reduced growth in the presence of *n*-hexadecane, but the *oxyR* mutant exhibited severely reduced growth on *n*-hexadecane (Fig. 3b). These results demonstrated that OxyR, as a master regulator under H_2O_2 stress, is important for the elimination of ROS.

Binding of OxyR to the promoter region of target genes and gene expression analysis

To identify OxyR target genes in *A. oleivorans* DR1, we searched for the putative consensus sequence of OxyR-binding sites in the promoter region of the upregulated proteins through proteomic analyses. Four genes with consensus sequences in their promoter regions were selected and subjected to EMSA and qRT-PCR: *ahpF* (encoded by AOLE_11385), *ahpC* (encoded by AOLE_11380), *trxB* (encoded by AOLE_15340), and *oprC* (encoded by AOLE_18500). Along with these candidates, a putative catalase (encoded by AOLE_09800), which has an OxyR-binding sequence in its promoter region, was also chosen. To determine whether OxyR binds to the promoter regions of the target genes, we conducted EMSA using DNA probes (P_{AOLE_09800}, P_{ahpF}, P_{ahpC}, P_{trxB}, and P_{oprC}) with purified OxyR. The EMSA results demonstrated that OxyR retarded the migration of the probes (Fig. 4a) and indicated that the OxyR-binding site may lie in five promoter-specific regions of the target genes. To ensure that the binding of OxyR to the DNA probe was specific, the nonspecific competitor poly(dI-dC) was included in all tested binding reactions; furthermore, the addition of an excess of an unlabeled DNA probe abolished OxyR binding to the labeled fragments.

To investigate the role of OxyR in the expression of these five target genes, mRNA expression levels were compared

Fig. 2 Sequence analysis of an OxyR homologue in *A. oleivorans* DR1. **a** The *A. oleivorans* DR1 *oxyR* gene (AOLE_14380) is located next to the *rubA* and *rubB* genes. **b** Two cysteine residues (C202 and C211) are conserved in OxyR of *A. oleivorans* DR1. Amino acid sequence identities between OxyR of *A. oleivorans* DR1 and those of other bacteria are listed in the right column
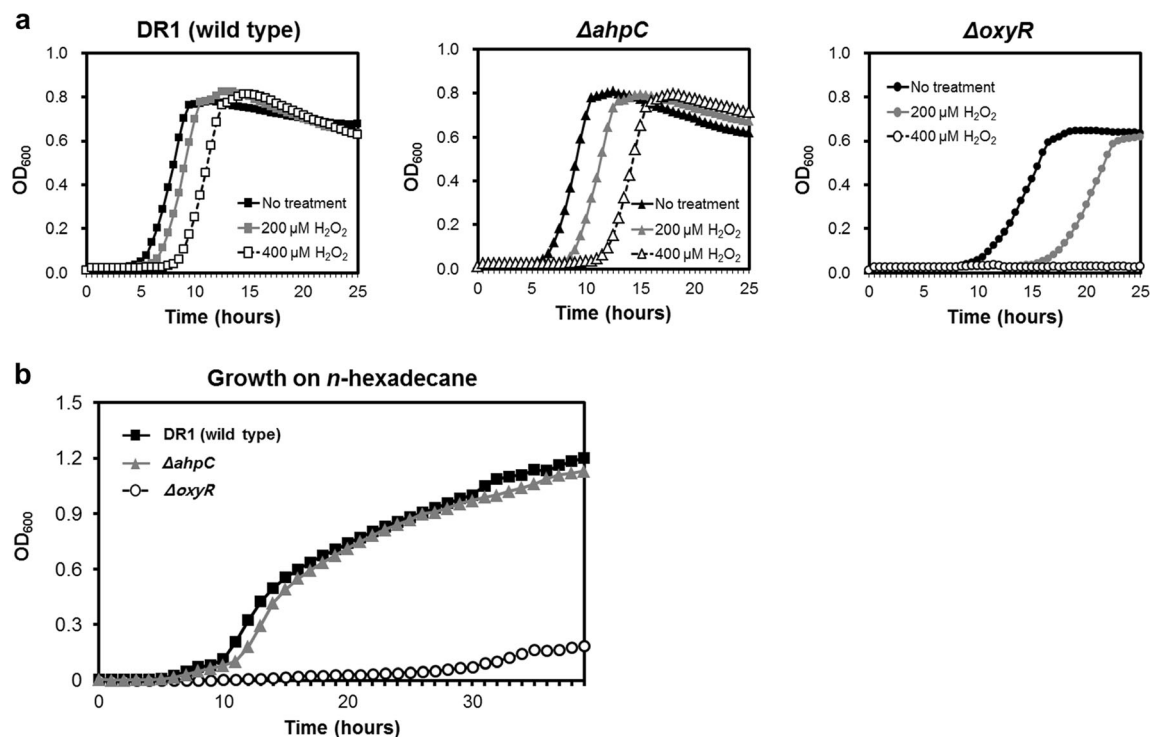
Strain	OxyR amino acid sequence																	Percent identity
Acinetobacter oleivorans DR1	E	G	H	C	L	R	D	H	V	L	S	A	C	P	I	G		
Acinetobacter baumannii ATCC 17978	E	G	H	C	L	R	D	H	V	L	S	A	C	P	I	G		99.0 %
Acinetobacter sp. ADP1	E	G	H	C	L	R	D	H	A	L	S	A	C	P	I	G		88.0 %
Escherichia coli K-12	D	G	H	C	L	R	D	Q	A	M	G	F	C	F	E	A		33.0 %
Pseudomonas aeruginosa PAO1	E	G	H	C	F	R	D	Q	V	L	E	A	C	P	T	V		32.6 %
Pseudomonas putida KT2440	E	G	H	C	F	R	D	Q	V	L	E	A	C	P	T	L		32.3 %

Fig. 3 Different growth defects of the *oxyR* and *ahpC* mutants compared with those of the wild type strain. **a** Growth curve of each strain in the nutrient broth containing H_2O_2 (200 and 400 μM). Cells were grown at 30 °C. **b** Growth on *n*-hexadecane in the wild type, the *oxyR* mutant, and the *ahpC* mutant. Cells were grown in minimal salts basal medium supplemented with 2 % *n*-hexadecane. The growth of each strain was monitored by measuring the optical density (OD) of the cultures at 600 nm

between the wild type and *oxyR* mutant by qRT-PCR. The expression of four genes increased in the presence of H_2O_2 and decreased in the absence of the *oxyR* gene (Fig. 4b). However, gene expression of *oprC* showed an opposite trend, indicating that OxyR could control the *oprC* gene as a repressor. The *oprC* expression was not increased by H_2O_2, it might be suggested that *oprC* is regulated by an additional unknown repressor. However, OprC protein was expressed in response to H_2O_2 treatment for 1 h, as observed in the proteomics analyses. We speculated that this result may reflect the time-dependent expression of mRNAs and proteins involved in the oxidative stress response of *A. oleivorans* DR1. Although the absence of OxyR decreased the expression of the putative catalase gene under H_2O_2 treatment compared with the wild type, this gene still showed increased expression in response to H_2O_2 treatment of the *oxyR* mutant. We speculate that the putative catalase might be controlled by another transcriptional regulator, as with KatG, which is regulated by RpoS in *E. coli* (Ivanova et al. 1994; Zheng and Storz 2000). Gel shift analysis and gene expression profiling demonstrated that purified *A. oleivorans* DR1 OxyR bound to the promoter regions of five target genes and may thus regulate their expression.

Among these target genes, *ahpC*, *ahpF*, and *trxB* showed hierarchical expression at different concentrations of H_2O_2. The gene expression of *ahpC* was enhanced as the

concentration of H_2O_2 increased, but high concentration of H_2O_2 slightly reduced *ahpC* expression (Fig. 5a). Unlike the expression pattern of *ahpC*, *trxB* expression remained higher at concentrations greater than 500 μM H_2O_2 (Fig. 5a). The *ahpF* gene was induced by H_2O_2, but it had a lower level of expression compared with the expression of *ahpC* and *trxB* (Fig. 5a). OxyR regulates various antioxidant defense genes and it is able to recognize and alternate target genes dependent on the cellular redox state (Toledano et al. 1994). The promoter regions of *ahpC* (K_d=5.40±0.87 μM) and *trxB* (K_d=6.49± 0.32 μM) had tighter binding affinity with OxyR than that of the *ahpF* gene (K_d=13.02±2.35 μM) (Fig. 5b). A low level of oxidized OxyR was sufficient to bind the OxyR-binding sites of *ahpC* and *trxB*, although more OxyR protein might be required to activate the promoter region of *ahpF* in *A. oleivorans* DR1. This result revealed that genes regulated by redox-sensitive global regulatory protein OxyR in *A. oleivorans* DR1 were controlled in a hierarchical manner, which indicated the extent of activation of OxyR by the concentration of H_2O_2.

Discussion

In this study, we characterized the OxyR homologue and OxyR regulon in *A. oleivorans* DR1. An *oxyR* mutant was
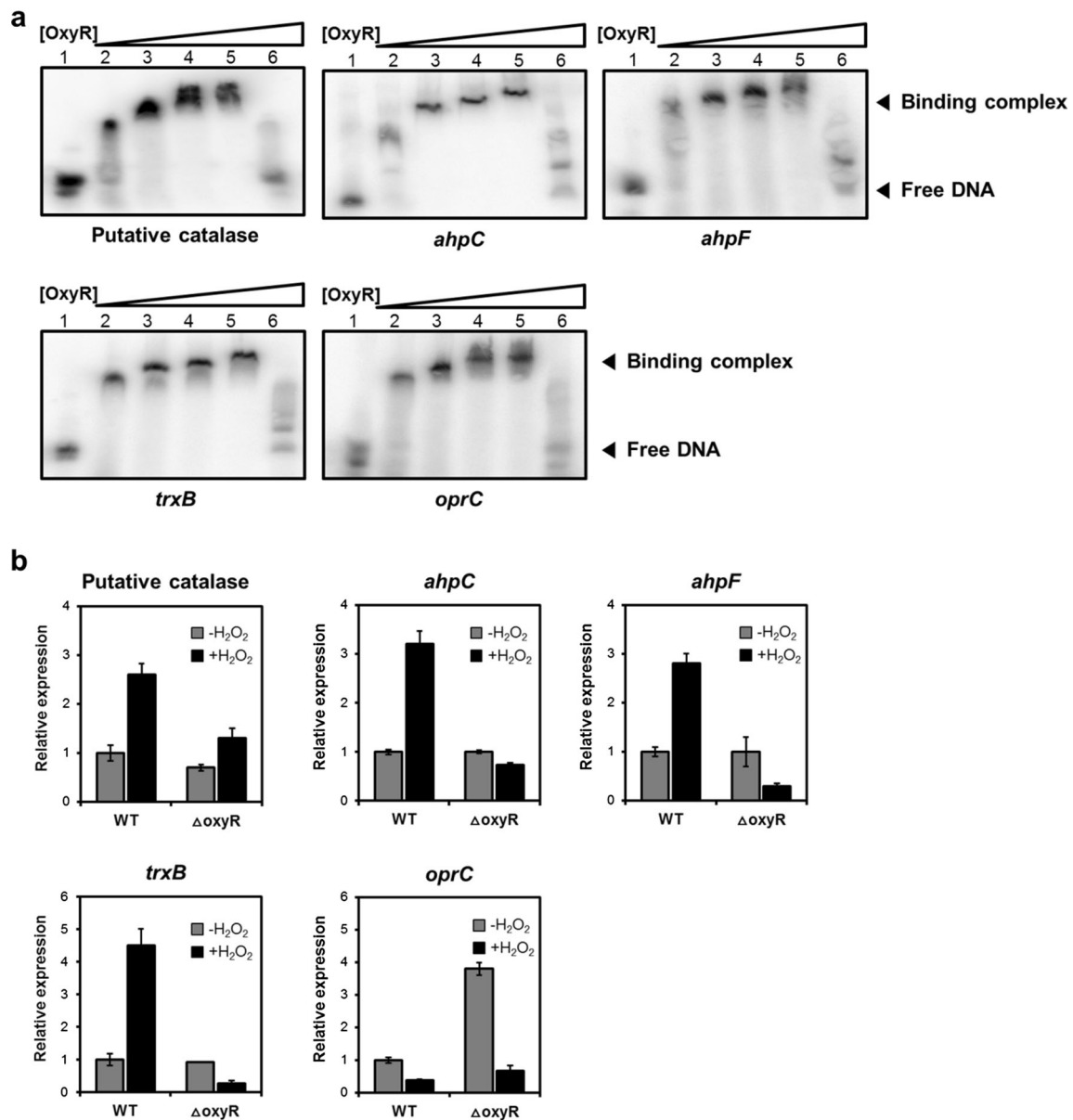
Fig. 4 Binding of OxyR to the promoter region of target genes and gene expression analysis. **a** Binding of OxyR to the promoter region of the target genes. The putative catalase, *ahpC*, *ahpF*, *trxB*, and *oprC* were controlled by OxyR via direct binding. Protein-DNA complexes and free DNA probes are indicated. *Lane 1*, free DNA (no protein); *Lane 2*, purified OxyR 4 μM; *Lane 3*, purified OxyR 8 μM; *Lane 4*, purified OxyR 16 μM; *Lane 5*, purified OxyR 32 μM; *Lane 6*, purified OxyR 16 μM with nonprobing DNA. The nonspecific competitor poly(dI-dC) was added to all binding reactions. **b** Gene expression of the putative catalase, *ahpC*, *ahpF*, *trxB*, and *oprC* in the wild type and the *oxyR* mutant determined by quantitative reverse transcriptase PCR. Comparison of gene expression between exponentially growing cells and H_2O_2 (1 mM)-treated cells. All data show the average of three replicate cultures, and standard deviations are shown

constructed to determine the physiological role of OxyR in *A. oleivorans* DR1. The *oxyR* mutant showed reduced growth and increased H_2O_2 sensitivity. To evaluate the oxidative stress response, protein profiling using 2-DE and MALDI-TOF MS analyses was conducted in the presence of H_2O_2. Functional classification of H_2O_2-induced proteins revealed that 15 proteins that are involved in the oxidative stress response, energy production and conversion, nucleotide and amino acid metabolism, membrane-related, and ion transport and chaperone-related functions may play important roles in

the oxidative stress response mechanism of *A. oleivorans* DR1. Five OxyR-controlled genes (putative catalase gene, *ahpC*, *ahpF*, *trxB*, and *oprC*) were identified through a DNA-protein binding assay and quantification of the mRNA levels.

Catalases catalyze the degradation of H_2O_2 to water and oxygen. The expression of catalase genes in *E. coli* is known to be controlled by OxyR, and *E. coli* has two types of catalases, HPI and HPII (Schellhorn 1995; Ochsner et al. 2000; Zheng and Storz 2000; Chauvatcharin et al. 2005; Jamet et al.
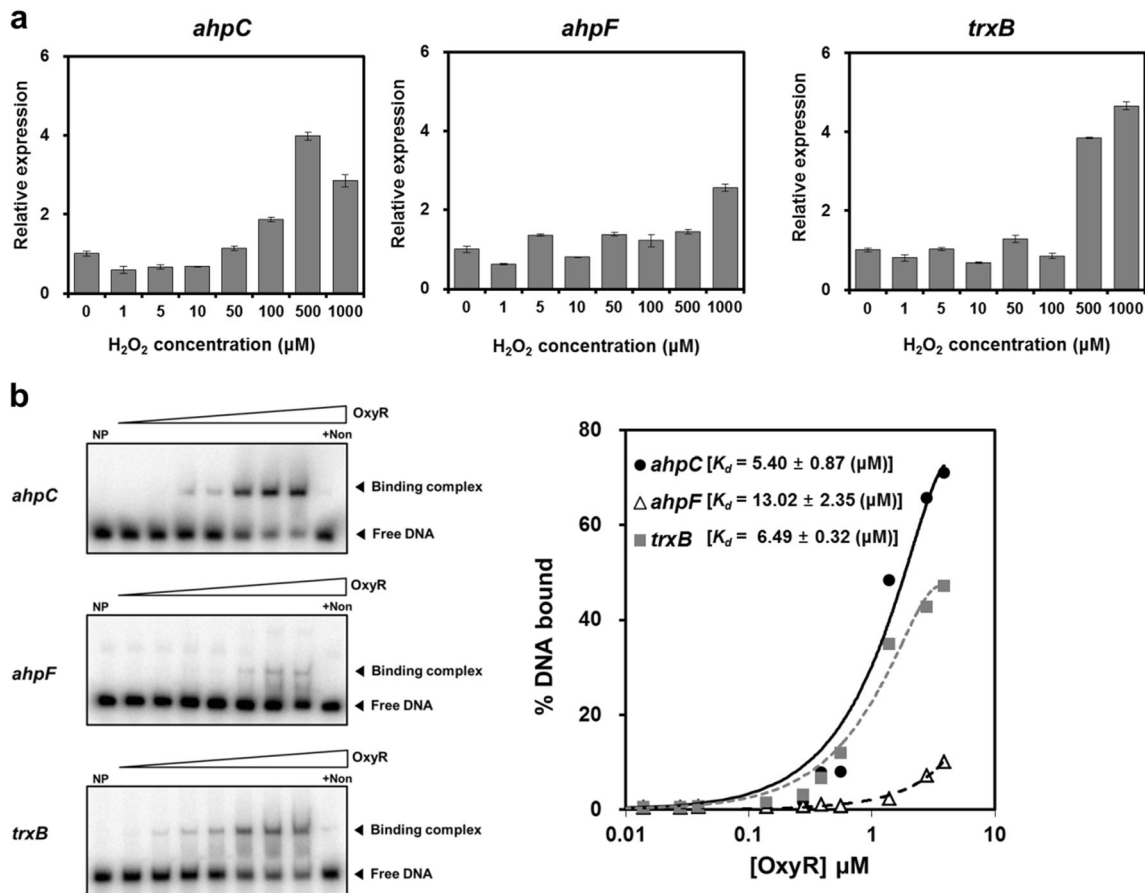
Fig. 5 Hierarchical expression of *ahpC*, *ahpF*, and *trxB* controlled by OxyR in *A. oleivorans* DR1. **a** Gene expression of *ahpC*, *ahpF*, and *trxB* in the presence of various concentrations of H_2O_2 determined by qRT-PCR. Exponentially grown cells were treated with H_2O_2 for 15 min. **b** Electrophoretic mobility shift assay (EMSA) showing OxyR concentration dependence on binding to three promoter regions. OxyR was used in the following concentrations (μM): 0, 0.14, 0.28, 0.39, 0.55, 1.38, 2.77, and 3.87. The first lane (NP) contained only probing DNA

without OxyR and the last lane (+non) contained 3.87 μM OxyR with nonprobing DNA. The DNA probe was generated using primer pairs (P_{ahpC}, AOLE_13380 proF(2)/proR; P_{ahpF}, AOLE_11385 proF(2)/proR; P_{trxB}, AOLE_15340 proF(2)/proR(2)). The nonspecific competitor poly(dI-dC) was added to all binding reactions. Plotted data were used to calculate the K_d for the OxyR binding with each promoter. All data show the average of three replicates, and one representative image is shown

2005). The H_2O_2-inducible bifunctional catalase HPI (KatG) is involved in the oxidative stress response through regulation by OxyR and RpoS (Ivanova et al. 1994; Zheng and Storz 2000). However, monofunctional HPII (KatE) is independent of OxyR. Four putative catalase genes (AOLE_09800, AOLE_11770, AOLE_12755, and AOLE_17390) are present in the genome of *A. oleivorans* DR1. Among the four catalases, the AOLE_11770-encoded catalase (712 amino acids) has 59 % amino acid identity with *E. coli* KatE and 64 % amino acid identity with *P. putida* KatE. The AOLE_17390-encoded catalase (718 amino acids) is similar to KatG of *E. coli* because it has 75 % identity with KatG in *E. coli*. The AOLE_12755-encoded catalase (354 amino acids) has 43 % amino acid identity with the PP_2887-encoded catalase of *P. putida* KT2440, but there is no similar catalase in *E. coli*. Little is known about the PP_2887-encoded catalase (Kim and Park 2014). In *Acinetobacter* species, most strains have two or more genes annotated as catalases like as *Pseudomonas* (Kim

and Park 2014). *Acinetobacter* species can live in various environments, such as soils, sediments, contaminated sites, fresh water, oceans, and human intestinal tract, because of its metabolic versatility (Jung and Park 2015). These habitat and metabolic diversities might give rise to various environmental stresses including ROS stress in *Acinetobacter* species, which leads to evolve complex regulatory systems for alleviation of ROS stress. The amino acid sequence of AOLE_09800 (385 amino acids), which was designated as a putative catalase gene in this study, showed lower than 30 % identity with the catalases in *E. coli* and *P. putida*. In addition, the amino acid sequence of this catalase (encoded by AOLE_09800) is shorter than that of well-known catalases in *E. coli* and *P. putida* (more than 700 amino acids). In a recent study, a 57.256-kDa catalase from the marine bacterium *Acinetobacter* sp. YS0810 (YS0810CAT) was identified and characterized (Fu et al. 2014). However, there is no YS0810CAT homologue in *A. oleivorans* DR1. Here, we

demonstrated direct binding of OxyR to the promoter regions of a putative catalase gene (AOLE_09800) and OxyR-dependent mRNA expression. In addition, the purified protein encoded by AOLE_09800 can degrade H_2O_2 in vitro. Therefore, the putative catalase (encoded by AOLE_09800) of *A. oleivorans* DR1 is a newly identified OxyR-regulated novel catalase.

To investigate the effect of H_2O_2 on protein expression in *A. oleivorans* DR1, protein profiling using 2-DE and MALDI-TOF MS analyses was conducted. AhpC (peroxiredoxin) was highly induced by H_2O_2, and this protein is involved in the detoxifying process for hydroperoxides. AhpF (alkyl hydroperoxide reductase) was also highly expressed under H_2O_2 treatment to reduce oxidized AhpC for reactivation. It is well known that *ahpCF* genes are co-transcribed under the control of the OxyR regulator in *E. coli* and *P. aeruginosa* (Zheng et al. 1999, 2001; Wei et al. 2012). Only one OxyR-binding site is located in the promoter region of *ahpC* (Zheng et al. 1999, 2001; Wei et al. 2012). Interestingly, *ahpC* (AOLE_13380; bp 2852427 to 2852990) and *ahpF* (AOLE_11385; bp 2445233 to 2446798) are located far from each other in the *A. oleivorans* DR1 genome. We demonstrated that the promoters of both the *ahpC* and *ahpF* gene have OxyR-binding sites and they were controlled by OxyR in a hierarchical manner in *A. oleivorans* DR1. Further individual studies on regulation of *ahpC* and *ahpF* and the difference in the OxyR-binding affinity of each target gene in *A. oleivorans* DR1 are required to extend understanding of the defense system of oxidative stress in *Acinetobacter* species.

The second highly expressed protein in our proteomic analyses was electron transfer flavoprotein subunit alpha (Etfα; encoded by AOLE_04180). The electron transfer flavoprotein (ETF) is generally known to be an electron carrier that transfers electrons from dehydrogenases to the main respiratory chain via ETF-ubiquinone oxidoreductase (Watmough and Frerman 2010). In the benzoate utilization process, ETF is upregulated in *Desulfotignum balticum* (Habe et al. 2009). Several studies have reported that oxidative stress occurs as a result of the metabolism of aromatic compounds (Denef et al. 2005, 2006). Thus, ETF could contribute to oxidative stress defense by its ability to reduce H_2O_2 and detoxify many aromatic compounds through hydroxylation, similar to other flavoproteins (Massey 2000).

The proton-translocating transhydrogenase PntAB acts as a major NADPH producer (Sauer et al. 2004). NADPH is an essential molecule in most organisms and it is required in a multitude of biochemical reactions. NADPH plays important roles in antioxidative defense mechanisms, as it is the general reducer of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, which are involved in the alleviation of oxidative stress (Minard and McAlister-Henn 2005; Ying 2008). It has also been demonstrated that conversion of NADH into NADPH via a metabolic network may be valuable as a novel antioxidative defense mechanism

(Singh et al. 2008). Proteomics analyses showed that PntA was upregulated by 2.5-fold in the presence of H_2O_2 in *A. oleivorans* DR1 (Table 2). In the facultative phototroph *Rhodobacter sphaeroides*, a transhydrogenase-deficient strain showed increased sensitivity to the oxidative stress-inducing agent diamide, which is known to change the redox state of bacterial cells (Hickman et al. 2002). Thus, transhydrogenase may be responsible for combating oxidative stress.

Two proteins identified as being induced in the presence of H_2O_2 participate in the purine biosynthesis pathway (phosphoribosylamine-glycine ligase (PurD) and phosphoribosylformylglycinamidine synthase (PurL)) (Table 2), which plays a critical role in the synthesis of RNA and DNA. The importance of purine biosynthesis for virulence in *Brucella abortus* and for colony spreading in *Bacillus subtilis* has also been confirmed (Alcantara et al. 2004; Kinsinger et al. 2005). Under toluene-induced oxidative stress, increased biosynthesis of purine was detected in *P. putida* (Segura et al. 2005). In concordance with this result, the genes involved in purine metabolism showed a response to both H_2O_2 treatment and thioredoxin reductase overexpression, which appears to comprise a mechanism for countering oxidative stress in *Lactobacillus plantarum* (Serrano et al. 2007). In addition to our proteomics data, the set of proteins involved in purine metabolism induced by oxidative stress suggests that the high energy demand needed for defenses against oxidative stress is acquired through enhancement of basic cell metabolism.

Metal ions are essential micronutrients in bacterial cellular metabolism, but excess ions that cause ROS formation are detrimental to cells (Yeom et al. 2010a). Bacteria have an adequate mechanism to maintain proper concentrations of intracellular metal ions, a process known as metal ion homeostasis. To date, little is known about the regulation system of metal ion homeostasis in *Acinetobacter* species. Nevertheless, our proteomic analyses suggest that the management of ion levels may be closely related to the oxidative stress response in *Acinetobacter*. Under H_2O_2 treatment, the putative enterobactin synthetase component F (encoded by AOLE_09920) was upregulated by 1.9-fold in *A. oleivorans* DR1 (Table 2). Enterobactin is a type of siderophore that acquires ferric ion for microbial systems primarily found in gram-negative bacteria, such as *E. coli* and *Salmonella typhimurium* (Neilands 1982). Recently, it has been reported that enterobactin can scavenge hydroxyl radicals and thereby reduce oxidative stress in *E. coli* (Adler et al. 2014). Accordingly, we speculated that siderophores produced by *Acinetobacter* could also act to alleviate oxidative stress.

An outer membrane receptor protein (encoded by AOLE_18500) in *A. oleivorans* DR1 with an OxyR-binding site in its promoter region was upregulated 1.7-fold by H_2O_2 treatment for 1 h, but its mRNA level was upregulated by more than 3.8-fold in the absence of OxyR (Table 2 and Fig. 4b). The outer membrane receptor protein (encoded by AOLE_18500)

contains a TonB-dependent copper receptor domain (TIGRFAM TIGR01778) and has 45.1 % amino acid identity with OprC in *P. aeruginosa*. OprC has shown high copper-binding activity, and the expression of OprC appears to be repressed by excess Cu^{2+} in *P. aeruginosa* (Yoneyama and Nakae 1996). These results indicate that OprC may play an important role in copper utilization. However, the molecular mechanism of OprC remains unknown and there are no published reports on OxyR regulation of OprC or the copper receptor protein. In this study, we suggest for the first time that OprC is involved in copper homeostasis and the oxidative stress response through direct regulation by OxyR in *A. oleivorans* DR1.

A. oleivorans DR1 can degrade *n*-hexadecane (Jung et al. 2010; Kang and Park 2010). The *oxyR* mutant showed growth defect in the presence of hexadecane (Fig. 3b). Degradation of hydrocarbon compounds can affect bacterial cell physiology by ROS generated during their metabolism (Denef et al. 2005, 2006). Our recent study also demonstrated that induction of genes involved in oxidative stress defense was contributable for enhanced growth on hexadecane in *A. oleivorans* DR1 (Jung et al. 2015). OxyR, a master regulator, is essential for the defense of H_2O_2 stress. The *oxyR* mutant could not control H_2O_2 defense genes and a severe growth defect was observed under hexadecane-amended condition. The only slight growth defect of the *ahpC* mutant on hexadecane suggested that there might be a compensatory mechanism for detoxifying H_2O_2 during its growth on hexadecane (Fig. 3b).

Acinetobacter species are ubiquitous in various environments (Bergogne-Bérézin and Towner, 1996). Although *Acinetobacter* species have been widely studied recently because of their clinical importance, the regulation of their genes and the molecular mechanisms of their oxidative stress response have received little attention. Here, we determined that OxyR is indeed involved in the regulation of antioxidant enzyme-encoding genes (*ahpC*, *ahpF*, and *trxB*) and two novel OxyR-controlled genes (*oprC* and the putative catalase gene) in *A. oleivorans* DR1. These findings show that OxyR functions as an important regulator in the defense against oxidative stress. *A. oleivorans* DR1 is genetically related to *A. baumannii* (Jung et al. 2011). Thus, this work provides an insight into the mechanisms underlying resistance to oxidative stress in *Acinetobacter* species including *A. baumannii*. To extend the understanding of the oxidative stress response system in *Acinetobacter* species, functional characterization of the newly identified OxyR-controlled catalase and the molecular mechanisms of other OxyR regulons in *Acinetobacter* require further investigation.

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