Suppression of pleiotropic phenotypes of a *Burkholderia multivorans fur* mutant by oxyR mutation

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INTRODUCTION

Iron is an essential element for the growth of almost all bacterial species. The solubility of iron is very low in eukaryotic hosts and aerobic natural environments, and bacteria have evolved various mechanisms to acquire iron efficiently under its limiting conditions. On the other hand, a high concentration of intracellular iron is very toxic under aerobic conditions, and intracellular ferrous iron, for example, reacts with metabolically produced hydrogen peroxide (H$_2$O$_2$), which is a representative reactive oxygen species (ROS), to generate the more toxic and reactive hydroxyl radical (OH$^-$) by the Fenton reaction (Andrews et al., 2003). The hydroxyl radical then reacts with many biomolecules, and the bacterial cell death caused by H$_2$O$_2$ is due primarily to hydroxyl-radical-mediated DNA damage (Imlay & Linn, 1986, 1988; McCormick et al., 1998). Nitric oxide (NO) is present in soil and air, and is also produced by a wide range of organisms, including bacteria (Sudhamsu & Crane, 2009; Wendehenne et al., 2001). NO is a reactive nitrogen species (RNS); it reacts with superoxide, another ROS, to generate extremely reactive peroxynitrite (Reiter et al., 2006). Thiols and iron–sulfur proteins are major targets for both ROS and RNS, and the release of iron from the latter proteins leads to not only their inactivation but also the acceleration of Fenton-reaction-mediated oxidative stress under aerobic conditions (Flint et al., 1993; Keyer & Imlay, 1996). Therefore, bacteria living in aerobic environments have sophisticated systems to enzymically remove ROS and RNS (by, for example, superoxide dismutases, catalases, peroxidases and NO and peroxynitrite reductases) as well as systems to strictly maintain intracellular iron homeostasis (Cornelis et al., 2011). The most well-studied iron homeostasis in many bacteria is mediated by the ferric uptake regulator (Fur), which functions as a global transcriptional regulator of members of the iron regulon. Under iron-replete conditions, Fur binds to ferric iron primarily to repress the

Abbreviations: qRT-PCR, quantitative RT-PCR; RNS, reactive nitrogen species; ROS, reactive oxygen species.

Four supplementary figures are available with the online version of this paper.

Fur (ferric uptake regulator) is an iron-responsive transcriptional regulator in many bacterial species, and the *fur* mutant of *Burkholderia multivorans* ATCC 17616 exhibits pleiotropic phenotypes, such as an inability to efficiently use several carbon sources, as well as high sensitivity to hydrogen peroxide (H$_2$O$_2$), paraquat (a superoxide-producing compound) and nitric oxide (NO). To gain more insight into the pleiotropic role of the Fur protein of ATCC 17616, spontaneous suppressor mutants of the ATCC 17616 *fur* mutant that restored tolerance to NO were isolated and characterized in this study. The microarray-based comparative genomic analysis and subsequent sequencing analysis indicated that such suppressor mutants had a 2 bp deletion in the *oxyR* gene, whose orthologues encode H$_2$O$_2$-responsive transcriptional regulators in other bacterial species. The suppressor mutants and the reconstructed *fur–oxyR* double-deletion mutant showed indistinguishable phenotypes in that they were all (i) more resistant than the *fur* mutant to H$_2$O$_2$, superoxide, NO and streptonigrin (an iron-activated antibiotic) and (ii) able to use carbon sources that cannot efficiently support the growth of the *fur* mutant. These results clearly indicate that the *oxyR* mutation suppressed the pleiotropic effect of the *B. multivorans* fur mutant. The *fur–oxyR* double mutants were found to overexpress the KatG (catalase/peroxidase) and AhpC1 and AhpD (alkyl hydroperoxide reductase subunits C and D) proteins, and their enzymic activities to remove reactive oxygen and nitrogen species were suggested to be responsible for the suppression of phenotypes caused by the *fur* mutation.
expression of various genes involved in iron uptake (Lee & Helmann, 2007).

The strains belonging to the genus Burkholderia (class Betaproteobacteria) are widely disseminated throughout various niches, including animals, plants, water and soils (Mahenthiralingam et al., 2008). Some strains are potentially important for the bioremediation of chemically polluted environments and the biological control of plant diseases (Coenye & Vandamme, 2003). Burkholderia cenocepa and Burkholderia multivorans are the species most often isolated from cystic fibrosis patients (Mahenthiralingam et al., 2005), and the latter species is especially prevalent in diverse natural environments, probably due to its broad metabolic capacity to use a number of carbon and nitrogen sources (Lessie et al., 1996). B. multivorans ATCC 17616 is a soil-derived strain whose extraordinary metabolic versatility has been well characterized. Our previous identification and characterization of a fur mutant of ATCC 17616 indicated that the Fur protein is pleiotropically involved in iron homeostasis, removal of ROS, catabolism of carbon sources and tolerance to NO (Yuhara et al., 2008). To gain greater insight into the pleiotropic role of the Fur protein of ATCC 17616, spontaneous suppressor mutants of the ATCC 17616 fur mutant that restored tolerance to NO were obtained in this study. Such suppression also led to the restoration of tolerance to ROS and the capacity for carbon source catabolism, and was ascribed to a mutation in the oxyR gene, a LysR-family transcriptional regulator gene that functions mainly to modulate the adaptive response to H₂O₂ stress in most proteobacteria and some Gram-positive bacteria. The suppressor mutant was found to overexpress constitutively the KatG (catalase/peroxidase) and AhpCD (alkyl hydroperoxide reductase subunits C and D) proteins. Such overproduction was suggested to account for the suppression of phenotypes exhibited by the fur mutation.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this study are listed in Table 1. Escherichia coli cells were cultivated at 37 °C and B. multivorans cells at 30 °C. The liquid media used were Luria–Bertani (LB) broth (Ausubel et al., 1991), 1/3 LB broth (3.3 g Bacto Tryptone, 1.7 g yeast extract and 5 g NaCl per litre), 1/10 LB broth (1 g Bacto Tryptone, 0.5 g yeast extract and 5 g NaCl per litre) and M9 minimal medium (Ausubel et al., 1991) supplemented with an appropriate carbon source at a concentration of 0.2%. Solid media were prepared by the addition of 1.5% agar. A concentrated (100 mM) acidified nitrite solution was prepared by dissolving equal molar amounts of anhydrous sodium nitrite and concentrated (100 mM) acidified nitrite solution was prepared by dissolving equal molar amounts of anhydrous sodium nitrite and citric acid monohydrate in deionized water, and sterilized with a 0.2 μm pore-size sterile filter. Such freshly prepared acidified nitrite was added as a NO-generator to the medium at a final concentration of 1 mM. The production of siderophores was monitored on Chrome Azurol S (CAS) agar plates (Schwyn & Neilands, 1987; Yuhara et al., 2008). When needed, IPTG and X-Gal were used at final concentrations of 0.5 mM and 40 μM, respectively. Antibiotics added to the media were as follows: ampicillin (Ap) at 100 μg ml⁻¹, tetracycline (Tc) at 10 μg ml⁻¹, kanamycin (Km) at 50 μg ml⁻¹ and gentamicin (Gm) at 10 μg ml⁻¹ for E. coli and Km at 100 μg ml⁻¹, Tc at 50 μg ml⁻¹ and Gm at 30 μg ml⁻¹ for B. multivorans.

Basic DNA and RNA manipulation. Established protocols were used for the preparation of genomic and plasmid DNA, DNA digestion with restriction endonucleases, ligation, standard agarose gel electrophoresis, DNA sequencing and transformation of E. coli and B. multivorans cells (Komatsu et al., 2003; Sambrook & Russell, 2001). PCR was performed with Ex Taq DNA polymerase (TaKaRa) or KOD plus DNA polymerase (Toyobo). The primers used in this study are listed in Table 2. To measure the amount of mRNA for each gene in the B. multivorans cells, an RNase Mini kit (Qiagen) was used to prepare total RNA from mid-exponential-phase cells in liquid culture. Reverse transcription was carried out using a ReverTra Ace (Toyobo) kit. The resulting cDNA sample was used to perform a quantitative RT-PCR (qRT-PCR) analysis using an Option 2 system (BIO-RAD) and SYBR Premix Ex Taq (TaKaRa) in a reaction volume of 10 μl. 16S rRNA was used as the internal standard.

Identification of suppressor mutation sites by DNA microarray analysis. Comparative genomic hybridization using a high-density oligonucleotide tiling array technique was carried out with a NimbleGen system (Roche) to detect the mutation sites that were introduced into the DF1 genome. The tiling array was designed to contain approximately 1950000 29-mer oligonucleotide probes with every seven-base overlap across the entire 7008810 bp sequence of ATCC 17616 (Yuhara et al., 2008) on both strands. The image extraction and subsequent processing and the detection of differences in hybridization signal intensities were carried out by the NimbleScan and NimbleGen SignalMap software.

DNA sequencing and sequence analysis. The sequencing reaction was carried out using a BigDye terminator kit, version 3 (Applied Biosystems), and the sequencing was performed with an ABI PRISM 310 sequencer (Applied Biosystems). Nucleotide sequences were analysed with the GENETYX program ver. 15 (SDC). The BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/) were used for the analysis of sequence homology.

Construction of plasmids, allelic exchange mutagenesis of ATCC 17616 and complementation analysis. Allelic exchange mutagenesis of the ATCC 17616 genome was carried out using pEX18Tc, which has a sacB gene as a counter-selective suicide marker (Hoang et al., 1998). To delete the oxyR gene from the ATCC 17616 genome, approximately 1 kb regions located up- and downstream of oxyR were amplified by PCR using ATCC 17616 genomic DNA as a template and two primer sets (OxyRuf_HindIII and OxyRur, and OxyRdf and OxyRdr _BamHI, respectively) (Table 2). The two amplified fragments were phosphorylated by T4 polynucleotide kinase (Takara) and ligated by using a DNA Ligation kit (Takara). The ligated products were then amplified by PCR using OxyRuf_HindIII and OxyRdr_BamHI, and the amplified fragment was digested with HindIII and BamHI, and cloned between the HindIII and BamHI sites of pEX18Tc. The resulting plasmid in E. coli DH5α (Table 1) was introduced into ATCC 17616 and DF1 by triparental mating using E. coli HB101(pRK2013) as a helper strain, and the transconjugants able to grow on a 1/3 LB agar plate containing Tc and Ap were selected. Subsequently, the Tc-sensitive derivatives of transconjugants able to grow on a 1/10 LB agar plate containing 5% sucrose were selected. The expected double-crossover-mediated homologous recombination in such derivatives was confirmed by PCR.

Expression of the oxyR gene in the ATCC 17616-derived strains was carried out by using pBBR1MCS5 as a vector. The oxyR gene was amplified by PCR using OxyRuf_EcoRI and OxyRdr_BamHI (Table 2), and inserted into the multi-cloning site of the vector so that the oxyR...
gene was transcribed from the vector-specific lac promoter. The resulting plasmid, pCO1, was introduced into the ATCC 171616 derivatives by electroporation.

Assay of the sensitivity of *B. multivorans* cells to acidified nitrite and oxidative stress. The sensitivity of cells to acidified nitrite was examined by measuring their growth rates as described previously (Yuhara et al., 2008). To investigate the cellular sensitivity to oxidative stress, approximately $1 \times 10^8$ cells were spread on 1/3 LB agar plates. Sterilized filter paper discs were placed on each plate, and a 10 μl aliquot of 500 mM paraquat (a superoxide producer) or 30% H$_2$O$_2$ was spotted on each disc. After incubating the plates for 36 h,

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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</thead>
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<td><strong>E. coli strains</strong></td>
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<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 Δ(lac)U169 (ø80lac ΔM15)</td>
<td>Sambrook <em>et al.</em> (1989)</td>
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<tr>
<td>HB101</td>
<td>hsdR20 recA13 ara-14 proA2 lacI1 galK2 rpsL20 xyl-5 mtl-1 supE44</td>
<td>Maniatis <em>et al.</em> (1982)</td>
</tr>
<tr>
<td><strong>B. multivorans strains</strong></td>
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<tr>
<td>ATCC 17616</td>
<td>Soil isolate; type strain</td>
<td>ATCC</td>
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<tr>
<td>DF1</td>
<td>Cm$^r$; ATCC 17616 fur deletion mutant</td>
<td>Yuhara <em>et al.</em> (2008)</td>
</tr>
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<td>SOF8</td>
<td>Spontaneous suppressor mutant of DF1 that restored tolerance to acidified nitrite</td>
<td>This study</td>
</tr>
<tr>
<td>SOF11</td>
<td>Spontaneous suppressor mutant of DF1 that restored tolerance to acidified nitrite</td>
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<td>DO1</td>
<td>ATCC 17616 oxyR deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>DFO1</td>
<td>Cm$^r$; ATCC 17616 fur and oxyR deletion mutant</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pEX18Tc</td>
<td>Tc$^r$ sacB oriT; suicide vector for gene replacement carrying the pUC18-derived multiple-cloning sites</td>
<td>Hoang <em>et al.</em> (1998)</td>
</tr>
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<td>pRK2013</td>
<td>Km$^r$ Tra$^+$; ColE1 derivative carrying all of the conjugal transfer genes from RP4</td>
<td>Figurski &amp; Helinski (1979)</td>
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<tr>
<td>pBBR1MCS5</td>
<td>Gm$^r$ derivative of pBBR1</td>
<td>Kovach <em>et al.</em> (1995)</td>
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<td>pME6041</td>
<td>Km$^r$; shuttle vector able to replicate in <em>E. coli</em> and <em>B. multivorans</em></td>
<td>Heeb <em>et al.</em> (2000)</td>
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<td>pCF1</td>
<td>Km$^r$ pME6041 derivative with the KpnI–PstI and fur-containing fragment from pFur</td>
<td>Yuhara <em>et al.</em> (2008)</td>
</tr>
<tr>
<td>pCO1</td>
<td>Gm$^r$; pBBR1MCS5 carrying oxyR</td>
<td>This study</td>
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### Table 2. Primers used in this study

Restriction sites are underlined.

<table>
<thead>
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<th>Primers</th>
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<td>OxyRf_HindIII</td>
<td>GGGAAGCTTGTACGAGAATCCACGACTGC</td>
<td>Upstream region of oxyR</td>
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<td>OxyRf</td>
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<td>Downstream region of oxyR</td>
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<tr>
<td>OxyRdr_BamHI</td>
<td>GGGGATCCGCGAGAATCCACGACTGC</td>
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<tr>
<td><strong>Complementation analysis</strong></td>
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<td>OxyRf_EcoRI</td>
<td>GGGAATCCCAGGAAACTTGGCCAAACG</td>
<td>Construction of pCO1</td>
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<tr>
<td>OxyRr_BamHI</td>
<td>GGGGATCCGCGGATCTAAGTGTTG</td>
<td></td>
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<tr>
<td><strong>qRT-PCR</strong></td>
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<td>GGCGAACGGGTGATATAC</td>
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<tr>
<td>16S rRNA-R</td>
<td>CATCCGTAGTACGTAGTACGG</td>
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<tr>
<td>katG-F</td>
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<td>katG-R</td>
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<td>ahpC1-F</td>
<td>GCCTTTCAATACATACAGAGG</td>
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<td>ahpC1-R</td>
<td>CACGAACGGATGAGCTTGCCTACC</td>
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<td>ahpD-F</td>
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<td>ahpD-R</td>
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<tr>
<td>ahpC2-F</td>
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<tr>
<td>ahpC2-R</td>
<td>TGTCGTCGTTGCCCTTGTGC</td>
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</table>
the diameters of growth inhibition zones around the discs were measured.

Carbon assimilation test. Cells grown to late-exponential phase in 1/3 LB broth were washed three times with M9 minimal solution, and resuspended in the same solution to prepare the suspension with OD_{660} 1.0, which corresponded to a c.f.u. value of approximately 10^7 ml^{-1}. This suspension was serially diluted 100-fold to obtain the suspensions with c.f.u. values of 10^2 ml^{-1}, 10^4 ml^{-1} and 10^6 ml^{-1}. A 5 μl portion of each diluted suspension was spotted onto a 1/3 LB agar plate and M9 minimal agar plate containing an appropriate carbon source. The c.f.u. value was assessed after incubation for 4 days.

Determination of cellular iron amounts. The total amount of cellular iron was determined by the colorimetric ferrozine assay (Riemer et al., 2004). The bacterial cells at mid-exponential phase in 1/3 LB were washed twice with M9 minimal media, resuspended in 100 μl of the same solution, and mixed with 100 μl 10 mM HCl and 100 μl iron-releasing reagent (a freshly mixed solution of equal volumes of 1.4 M KCl and 4.5% KMnO4). This mixture was incubated for 2 h at 60 °C and cooled to room temperature, and then its iron amount was determined by the protocol described by Lindgren et al. (2009). The total iron amount in the cells was quantitatively standardized by the amount of total cellular protein, which was determined using a Bradford colorimetric assay system (Bio-Rad).

The amount of intracellular free iron was estimated by the streptonigrin sensitivity assay described by Justino et al. (2007). Streptonigrin is an aminopurine antibiotic, and its toxicity is proportional to the intracellular free iron content. The overnight culture of cells was diluted 100-fold for transfer to fresh 1/3 LB medium supplemented with 1 mg streptonigrin ml^{-1}. The OD_{660} value of this culture was measured after incubation for 16 h, and the sensitivity to streptonigrin was expressed as a percentage of the OD_{660} value of the control culture without the supplement of streptonigrin as 100 %.

Protein analysis. Cells at mid-exponential phase were disrupted by sonication. Their lysate was recovered by centrifugation at 13 000 g for 15 min, and was separated by using 12.5% (w/v) SDS-PAGE. For determining the N-terminal amino acid sequences of proteins, the lysate was concentrated by 0–60% or 75–90% (w/v) ammonium sulfate precipitation, and dialysed in PBS buffer. The precipitated protein fraction was separated by 7.5% or 12.5% (w/v) SDS-PAGE, blotted on Immobilon-PSQ (Millipore), and stained by using Cooamassie brilliant blue. Relevant protein spots were excised, and their N-terminal amino acid sequences were determined by using the Edman degradation at Genostaff (Tokyo).

RESULTS

Identification of a spontaneous suppressor mutation that enhances resistance to acidified nitrite on DF1

To elucidate how Fur regulates cellular tolerance to acidified nitrite (a NO producer), we attempted to isolate the suppressor mutants of DF1 (=ATCC 17616 Δfur) that were more tolerant than DF1 to acidified nitrite. The suppressor mutants formed colonies 24 h after the DF1 cells were plated on 1/3 LB agar plates containing 1 mM acidified nitrite. However, a significantly high frequency [1.1 × 10^{-4} per cell (n=3)] of the formation of such colonies hampered our ability to easily clone a genomic region(s) covering the suppressor mutation site(s) by conventional genetic methods. Because of this difficulty, the tiling-array-based hybridization technique (Albert et al., 2005) using the DF1 genome as a reference was employed to identify the suppressor mutation site(s) in the two independent suppressor mutants, SOF8 and SOF11. These two derivative strains carried the common suppressor mutation within the oxyR gene (BMULJ_00577 with a size of 957 bp) on chromosome 1 (Fig. S1a, available with the online version of this paper). Subsequent nucleotide sequencing confirmed the 2 bp deletion (at nucleotide positions 300 and 301) in the oxyR gene (Fig 1a and Fig. S1b).

In the presence of H_2O_2, the E. coli OxyR protein is activated through the formation of a disulfide bond between two cysteine residues (at positions 199 and 208) (Zheng et al., 1998), and the oxidized protein activates the transcription of antioxidant genes, including katG (for catalase), ahpCF (for alkyl hydroperoxide reductase), gorA (for glutathione reductase) and grxA (for glutaredoxin I) (Zheng & Storz, 2000). The ATCC 17616 OxyR protein also had such cysteine residues (at positions 199 and 208). The 2 bp deletion in the oxyR gene in SOF8 and SOF11 was predicted to give rise to a mutated 354 aa protein lacking the two conserved cysteine residues but having the intact N-terminal 99 aa sequence of the wild-type OxyR protein.

Phenotypes of the Δfur ΔoxyR double mutants in comparison with single Δfur and ΔoxyR mutants

To clarify whether or not the loss of the regulatory function(s) of the SOF8- and SOF11-encoded OxyR derivatives was responsible for the tolerance to acidified nitrite, the oxyR genes in the DF1 and ATCC 17616 genomes were deleted to construct DFO1 and DO1, respectively. DFO1 and SOF8 were
more resistant to acidified nitrite than DF1, and the introduction of pCO1 (=pBBR1MCS5::oxyR) significantly increased the sensitivity of DFO1 (Fig. 2). These results indicated that the inactivation of OxyR in DF1 led to the resistance to acidified nitrite stress. The overexpression of oxyR from the vector-derived promoter in DFO1(pCO1) (Fig. S2) might have been responsible for the phenotype of hypersensitivity to NO stress in DFO1(pCO1) (Fig. 2). On the other hand, the sensitivity of DO1 and DO1(pCO1) to acidified nitrite was only slightly higher than that of ATCC 17616 (Fig. 2). Therefore, the involvement of OxyR function in the tolerance to NO was detectable in the fur mutation background.

The other phenotypes that DF1 exhibited (increased sensitivity to oxidative stress, reduced carbon assimilation ability and constitutive siderophore production) (Yuhara et al., 2008) were also examined by using DFO1 and DO1. As shown in Fig. 3, the sensitivity of DFO1 to paraquat (a superoxide generator) and H₂O₂ was similar to that of ATCC 17616, and the supply of pCO1 to DFO1 gave rise to the sensitive phenotypes indistinguishable from those of DF1. The ability to use four carbon and energy sources (glucose, succinate, citrate and fumarate) was measured on the basis of c.f.u. on an M9 minimal agar plate. This ability was increased in SOF8 and DFO1, and the introduction of pCO1 returned the c.f.u. of the latter strain to the level of DF1 (Fig. 4). DO1 showed phenotypes indistinguishable from ATCC 17616 with respect to the sensitivity to oxidative stress and the ability to use the four carbon sources.

Siderophore production by ATCC 17616, DF1, SOF8, DFO1 and DO1 was estimated from the sizes of the orange haloes on the CAS agar plates under iron-replete conditions. Whereas ATCC 17616 and DO1 formed small haloes (Yuhara et al., 2008), the remaining three strains formed large haloes (Fig. S3), indicating that the oxyR mutation in the fur mutant does not affect siderophore production.

**Fig. 2.** Cellular sensitivity to acidified nitrite. The sensitivity to acidified nitrite was analysed by measuring the OD₆₅₀ values of cultures in 1/3 LB supplemented with 1 mM acidified nitrite. Error bars represent the SDs from at least three independent experiments. The strains used are ATCC 17616 (WT), DF1, SOF8, DO1, DO1(pCO1), DFO1 and DFO1(pCO1).

**Fig. 3.** Cellular sensitivity to ROS. Cells grown in 1/3 LB medium were spread onto 1/3 LB agar plates, onto which were placed discs supplemented with 10 μl of 500 mM paraquat (a) or 30% H₂O₂ (b), and incubated at 30 °C for 36 h. Mean ± SD values for the diameter of the inhibition zones from three independent experiments are indicated.

**Determination of intracellular iron amounts**

The proteins containing iron–sulfur clusters are major targets of ROS and RNS that cause the clusters to release iron, leading to the inactivation of the proteins. Such released free iron further accelerates the production of ROS by the Fenton reaction under aerobic conditions (Flint et al., 1993; Keyer & Imlay, 1996). Taking into consideration that DF1 exhibited higher sensitivity to oxidative and nitrosative stress than ATCC 17616 and DFO1, the amounts of intracellular free iron in these three strains as well as in related strains were measured by a streptonigrin sensitivity assay (Justino et al., 2007). DF1 and DFO1(pCO1) were more sensitive to streptonigrin than ATCC 17616, DO1 and DFO1 (Table 3), indicating that (i) the Δfur mutation alone led to the increase in the amount
of intracellular free iron, (ii) the disruption of OxyR function in the Δfur strain reduced the amount of intracellular free iron to a level close to that in the wild-type strain and (iii) the oxyR mutation alone did not affect the amount of intracellular free iron. The increase in siderophore production in DF1 (Yuhara et al., 2008) might have resulted in the increase in the amount of total intracellular iron. However, our colorimetric ferrozine assay (Riemer et al., 2004), which allows quantification of total iron in cells, indicated that the total cellular iron concentrations in ATCC 17616 and DF1 were very similar [7.18 ± 1.5 and 7.07 ± 1.5 nmol (mg protein)⁻¹, respectively].

### Overexpression of KatG, AhpC1 and AhpD by oxyR inactivation

The ATCC 17616, DF1, SOF8, DFO1, DFO1(pCO1), DO1 and DO1(pCO1) cells were cultivated to the mid-exponential phase in 1/3 LB broth, and their crude extracts were subjected to SDS-PAGE. Comparison of the protein profiles in these strains revealed the overexpression of three protein bands in the absence of the functional oxyR gene (in SOF8, DFO1 and DO1 cells) (Fig. 5). The N-terminal amino-acid sequences of the overexpressed bands were STEXK (X: unclear), MKTVG and MEFID. All of the deduced amino acid sequences encoded by the ATCC 17616 genome were searched for these sequences. The three proteins were identified as catalase/peroxidase, alkyl hydroperoxide reductase subunit C and alkyl hydroperoxide reductase subunit D, encoded by **katG** (BMULJ_00578), **ahpC1** (BMULJ_01915) and **ahpD** (BMULJ_01916), respectively. All three genes are located on chromosome 1.

The **katG** gene in ATCC 17616 was located just downstream of oxyR, and the gene orders of the regions covering the two genes were conserved in other *Burkholderia* strains. Furthermore, the amino acid sequence of the ATCC 17616 OxyR protein showed 98 and 94 % identities with those from *B. cenocepacia* J2315 and *Burkholderia pseudomallei* P844, respectively, and the ATCC 17616 KatG protein showed 91 and 87 % identities with the experimentally analysed KatG proteins from *B. cenocepacia* C5424 [a strain presumed to be very similar to J2315 with respect to the overall genome sequence (Lefebre et al., 2005; Mahenthiralingam et al., 2000)] and *B. pseudomallei* P844 (Loprasert et al., 2003a, 2003b), respectively. The KatG proteins from C5424 (designated KatB in the original manuscript) and P844 have been indicated to play protective roles against hydrogen peroxide and various types of oxidants, respectively.

The **ahpC1** and **ahpD** genes from ATCC 17616 were suggested to form an operon because no canonical promoter sequence was found around the intergenic 68 bp region, and the AhpC1 and AhpD proteins were concomitantly overexpressed in the oxyR mutant background (Fig. 5). The ATCC 17616 chromosome 2 encodes an additional copy of the alkyl hydroperoxide reductase system consisting of AhpC2 and AhpF, but their amino
acid sequences are distinct from those of AhpC1 and AhpD. The AhpC proteins in many bacteria are known to be peroxide-scavenging enzymes that belong to the cysteine-based peroxiredoxins and have reducing activities against hydrogen peroxide, organic peroxides and peroxynitrite, whereas the AhpD and AhpF proteins are disulfide oxidoreductase partners of AhpC proteins and act as efficient electron donors for these partners (Wood et al., 2003). Various other Burkholderia strains with completely sequenced genomes also have two alkyl hydroperoxide reductase systems, and the amino acid sequences of the AhpC1 and AhpC2 proteins from ATCC 17616 showed 100 and 93 % identities with the corresponding proteins from B. pseudomallei K96243. The AhpC protein from B. pseudomallei strain P844 has been reported to be involved in the resistance to acidified nitrite (Loprasert et al., 2003a). There are no reports describing the Burkholderia AhpD proteins.

Transcriptional analysis of katG, ahpC1, ahpD and ahpC2

The RNA samples prepared from the ATCC 17616, DF1, DF1(pCF1), SOF8, DFO1, DFO1(pCO1), DO1 and DO1(pCO1) cells were subjected to qRT-PCR analysis of the katG, ahpC1 and ahpD genes as well as the ahpC2 gene, which has been indicated to be a putative OxyR-regulated gene in B. cenocepacia J2315 (Peeters et al., 2010) (Fig. 6). The transcription levels of katG, ahpC1 and ahpD were much higher in SOF8, DFO1 and DO1 than in the other five strains (Fig. 6a–c, white bars). This pattern was similar to that obtained from SDS-PAGE analysis (Fig. 5), indicating that the overexpression of KatG, AhpC1 and AhpD was defined at the transcriptional level. Furthermore, the higher mRNA levels of these three genes in the oxyR mutant cells indicated the direct or indirect transcriptional repression of the three genes by the OxyR protein. The qRT-PCR analysis further showed that the transcription levels of katG, ahpC1 and ahpD in the H2O2-exposed wild-type strain were similar to or lower than those of the H2O2-exposed oxyR mutants (Fig. 6a–c, black bars). Therefore, the H2O2-mediated role of OxyR in activating the transcriptional control of these three genes was not observed under our experimental conditions. On the other hand, the mRNA level of ahpC2 was much higher in the H2O2-treated wild-type strain than that in the H2O2-treated oxyR mutants (Fig. 6d), indicating the direct or indirect transcriptional activation of this gene by the OxyR protein.

DISCUSSION

We showed in this study that the phenotype of sensitivity to acidified nitrite of the B. multivorans ATCC 17616 fur mutant can be suppressed by an additional oxyR mutation with a null function. Furthermore, these suppressor mutants exhibited more tolerance to ROS and streptonigrin and more availability of some carbon sources than the fur mutant, indicating strong associations among iron concentration, stress responses to NO and ROS and carbon assimilation activity. This is the first report, to our knowledge, that directly showed genetically the functional relationship between fur and oxyR. There have been some reports showing that Fur and OxyR regulated common loci, such as the Salmonella enterica serovar Typhimurium mntH gene for a primary influx transporter of Mn2+ ion (Kehres et al., 2002), the E. coli suf operon for assembly of iron–sulfur cluster proteins (Lee et al., 2004, 2008; Outten et al., 2004) and the Pseudomonas aeruginosa pvdS gene for an extracytoplasmic (ECF) sigma factor for the regulation of iron homeostasis (Ochsner et al., 1995; Wei et al., 2012). The E. coli OxyR protein activates the transcription of the fur gene (Zheng et al., 1999), and the region upstream of the fur gene indeed had the conserved consensus sequence, the OxyR box, to which the OxyR protein can bind (Fig. S4) (Zheng et al., 1999). Since no canonical OxyR box was present in the region upstream of the B. multivorans ATCC 17616 fur gene, its transcriptional regulation by OxyR is unlikely. The conserved consensus sequence, the Fur box, to which the Fur protein can bind (Yuhara et al., 2008), was absent at the regions upstream of the ATCC 17616 oxyR, katG and ahpC1D genes, and direct transcriptional regulation of these genes by Fur is also unlikely.

Our observations in this study showed that the KatG, AhpC1 and AhpD proteins and their mRNAs were overexpressed in the ΔoxyR mutant of B. multivorans ATCC 17616 in the absence of H2O2 (Figs 5 and 6), suggesting that the OxyR protein under normal aerobic conditions represses the transcription of the katG gene and the ahpC1D operon. The
transcriptome analysis of *B. cenocepacia* J2315 indicated high levels of transcriptional induction of the *katG* and *ahpC2* genes by exposure to exogenously added H2O2 (Peeters et al., 2010), but did not provide direct evidence that OxyR played a role in triggering the induction. The *katG* transcription of *B. pseudomallei* P844 has been reported to be repressed and activated by OxyR in the absence and presence, respectively, of H2O2 (Loprasert et al., 2003b). Indeed, the transcription of the *katG* genes in P844 and some other bacteria strains such as *E. coli* and *Xanthomonas campestris* has been indicated to be directly regulated by the OxyR proteins (Jittawuttipoka et al., 2009; Loprasert et al., 2003b; Tao et al., 1991). Since the OxyR box-like sequences are located just upstream of the *katG* gene and the *ahpC1D* and *ahpC2F* operons in *B. multivorans* ATCC 17616 and in the corresponding regions in *B. cenocepacia* J2315 (Fig. S4), it is most likely that the transcriptional regulation of these *Burkholderia* genes and operons in response to H2O2 is mediated directly by OxyR proteins. The transcription of the *ahpC2* gene indicated activation by OxyR in the presence of H2O2 (Fig. 6d), and the transcriptional responses of the *ahpC1* and *ahpC2* genes were considerably different (Fig. 6b, c, d) in *B. multivorans* ATCC 17616. The mechanism or mechanisms leading to such differences are at present unclear. More detailed *in vitro* analysis of the *B. multivorans* ATCC 17616 OxyR protein to characterize the binding properties to each target promoter region will be needed to clarify the molecular mechanism underlying the OxyR-mediated regulation.

The Δfur mutant exhibited enhanced extracellular siderophore production. This phenotype is expected to promote the cellular uptake of iron, but the total iron amounts in ATCC 17616 and the Δfur mutant were similar. On the other hand, the amount of free iron in the cells was increased in the Δfur mutant (Table 3). Although the details of the molecular mechanism(s) that governs the concentration of free intracellular iron are at present unclear, the following are plausible scenarios. In the Δfur mutant, the overexpression of *bfld* (BMULJ_2179, encoding bacterioferritin-associated ferredoxin) has been observed (Yuhara et al., 2008), and would be expected to lead to the release of free iron from the intracellular iron storage protein, bacterioferritin. The superoxide dismutase and

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**Fig. 6.** Relative mRNA levels of *katG*, *ahpC1*, *ahpD* and *ahpC2*. The qRT-PCR analysis was performed by using RNA samples prepared from ATCC 17616, DF1, DF1(pCF1), SOF8, DFO1, DFO1(pCO1), DO1 or DO1(pCO1) cells that were treated with 500 μM H2O2 for 10 min at the mid-exponential phase (black) or were not treated (white). Relative mRNA levels of *katG* (a), *ahpC1* (b), *ahpD* (c) and *ahpC2* (d) normalized by the 16S rRNA levels are shown. The means ± SD obtained from at least three independent experiments are indicated for each strain.
catalase activities were lower in the Δfur mutant than in the wild-type (Yuhara et al., 2008), suggesting higher levels of metabolically produced superoxide and hydrogen peroxide in the Δfur mutant. The increases in both free iron and hydrogen peroxide give rise to large increases in more reactive hydrogen radicals by the Fenton reaction under aerobic conditions. The increased level of superoxide also reacts with the endogenously produced NO to generate a higher level of more-reactive peroxynitrite. These higher levels of ROS and RNS thereafter promote the release of free iron from various iron–sulfur proteins that include, for example, fumarase and aconitase of TCA cycle enzymes. The free iron thus released further stimulates the Fenton reaction and accelerates the cellular oxidative and nitrosative stress. On the other hand, the ΔoxyR mutant produced large amounts of katG-encoded catalase/peroxidase and ahpC1D-encoded alkyl hydroperoxide reductase constitutively without the addition of exogenously added hydrogen peroxide. The two enzymes convert hydrogen peroxide and various peroxidated substrates to nontoxic compounds, and the AhpC1 protein might have, as indicated by the AhpC proteins from some other bacterial species (Bryk et al., 2000), peroxynitrite reductase activity for detoxification of the substrate. These constitutively and highly expressed enzymic activities in the Δfur ΔoxyR mutant will contribute to the direct and/or indirect removal of ROS and RNS, thus preventing the release of iron from the iron–sulfur proteins. More detailed genetic and biochemical characterizations of KatG and AhpC1 will be important for future studies.

In separate experiments, we obtained spontaneous suppressor mutants of DF1 that apparently restored the normal colony-forming ability on an M9 minimal agar plate containing either succinate or citrate, and such suppressor mutants exhibited phenotypes similar to those of SOF8 with respect to the resistance to ROS and acidified nitrate (data not shown). However, some of these suppressor mutations were not mapped in the oxyR gene, and did not overproduce KatG, AhpC1 or AhpD. The identification of such suppressor mutation sites and their further analysis will provide additional clues to clarify the relational mechanisms governing the cellular iron homeostasis, carbon utilization and removal of ROS and RNS in B. multivorans.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES


Edited by: J. Cavet