Superoxide dismutase-encoding gene of the obligate anaerobe Porphyromonas gingivalis is regulated by the redox-sensing transcription activator OxyR

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Inspection of the genomic DNA sequence of the oral anaerobe Porphyromonas gingivalis reveals that the micro-organism possesses the peroxide-sensing transcription activator OxyR, but not the superoxide-sensing transcription factor SoxR. Investigation of oxidative-stress-responsive proteins in P. gingivalis by two-dimensional gel electrophoresis showed that two proteins were predominantly upregulated in oxidative conditions. In a P. gingivalis oxyR mutant these two proteins were not induced by treatment with hydrogen peroxide under aerobic conditions. By N-terminal amino acid sequencing, the two proteins were found to be superoxide dismutase and alkyl hydroperoxide reductase, encoded by sod and ahpC, respectively. Northern blot and lacZ fusion analyses revealed that P. gingivalis sod and ahpC were positively regulated by OxyR. Primer extension analysis located the promoter regions of sod and ahpC, and putative −35 boxes of these promoters were found immediately adjacent to their putative OxyR-binding sequences. Moreover, the promoter regions of sod and ahpC had the ability to bind P. gingivalis OxyR protein. These results demonstrate that P. gingivalis sod is one of the OxyR regulons, suggesting that OxyR functions as an intracellular redox sensor rather than a peroxide sensor in this organism. A sod gene of Bacteroides fragilis, which is taxonomically related to P. gingivalis, is inducible by redox stresses but not controlled by its OxyR. A DNA fragment including the B. fragilis sod promoter region could bind the P. gingivalis OxyR protein; however, a putative OxyR binding sequence within the DNA fragment was 14 bases distant from a putative −35 box of its promoter.

INTRODUCTION

Reactive oxygen species such as superoxide anion radical, hydrogen peroxide and hydroxyl radical lead to oxidative damage to lipids, proteins and nucleic acids, resulting in mutagenesis and cell death (see Halliwell & Gutteridge, 1999 for a review). It seems that each organism subject to oxidative stresses has developed defence mechanisms to adapt to its environment. Several oxidative-stress defence mechanisms have been characterized in prokaryotes and eukaryotes (Halliwell & Gutteridge, 1999). Among them, Escherichia coli has been well characterized for these mechanisms. With respect to reducing reactive oxygen species, E. coli cells produce two types of superoxide dismutase (SOD), Mn-containing SOD (sodA gene product) and Fe-containing SOD (sodB gene product), which dismutate superoxide to hydrogen peroxide (Touati, 2000). E. coli also possesses two types of catalase, HPI catalase (katG gene product) and HPII catalase/peroxidase (katE gene product), which disproportionate hydrogen peroxide into water and oxygen (Iuchi & Weiner, 1996; Loewen, 1996). In addition, peroxiredoxins such as alkyl hydroperoxide reductase (AhpC, ahpC gene product), thiol-dependent peroxidase (tpx gene product) and bacterioferritin-comigratory protein (bcp gene product) are thought to confer additional defences against peroxides upon E. coli cells by reducing various organic hydroperoxides (Jeong et al., 2000; Kong et al., 2000; Schroder & Ponting, 1998).

When exposed to oxidative stresses, E. coli induces one or both of two regulatory systems, the OxyR and SoxRS regulons (Storz & Imlay, 1999; Storz & Zheng, 2000). Expression of OxyR-regulated genes (OxyR regulons) is induced by the transcription factor OxyR, activated by peroxides such as hydrogen peroxide through formation of a sulfenic acid at the cysteine residue C-199 of OxyR or an intramolecular disulfide bond between two cysteine residues (C-199 and
C-208) of OxyR, katG, ahpCF, dps (a non-specific DNA-binding protein), gorA (glutathione reductase), grxA (glutaredoxin I) and fur (ferric uptake regulator) are included in the OxyR regulon. When superoxide is generated in the cells, SoxRS-regulated genes (SoxRS regulons) are induced. The SoxRS transcription factor activates the expression of soxS in response to exposure to superoxide-generating agents and to nitric oxide. SoxS proteins positively control several genes, including sodA, zwf (glucose-6-phosphate dehydrogenase), fpr (NADPH: flavodoxin oxidoreductase), fldA (flavodoxin I), fumC (fumarase C), acnA (aconitase), nfo (endonuclease IV) and micR (a regulatory RNA).

The anaerobic Gram-negative bacterium Porphyromonas gingivalis is considered as one of the aetiologically important agents for periodontal disease (Holt & Ebersole, 2005). The primary ecological niche of this organism is the gingival crevice and its presence is associated with the development of periodontal pockets. In order to colonize and survive at these sites, the organism must possess the ability to tolerate oxygen in an air and reactive oxygen species generated by leukocytes and macrophages. In attempting to understand how P. gingivalis survives and causes lesions in the oral cavity, it is important to investigate the presence and role of oxidative stress defence mechanisms in this organism. P. gingivalis possesses the sod gene encoding Fe/Mn-containing SOD, which contributes to the relatively high aerotolerance of this organism (Amano et al., 1990; Nakayama, 1990, 1994). Dps and AhpC were found in P. gingivalis, and genetic analysis revealed that these proteins are responsible for its peroxide resistance (Ueshima et al., 2003; Diaz et al., 2004; Johnson et al., 2004). However, other genes for oxidative stress defence and regulation of their expression in this organism have been poorly characterized. Inspection of the P. gingivalis genome yielded several genes putatively involved in oxidative stress defence, including genes homologous to bcp, tpx and oxyR. It also revealed that there were no genes homologous to soxR or soxS in P. gingivalis genomic DNA (Nelson et al., 2003).

In this study, we investigated oxidative stress responses of P. gingivalis regulated by the OxyR transcriptional regulator and found that sod gene expression in this bacterium is under OxyR control. This is in contrast to the observation that SoxRS, not OxyR, regulates sodA in E. coli. This is believed to be the first report to demonstrate the presence of an OxyR-controlled sod gene.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids in this study are listed in Table 1. Cells of Porphyromonas gingivalis and Bacteroides fragilis were grown anaerobically (10% CO₂, 10% H₂ and 80% N₂) in enriched brain heart infusion (BHI) broth [containing, per litre, 37 g brain heart infusion (Difco), 5 g yeast extract (Difco), 1 g cysteine, 5 mg haemin and 1 mg vitamin K₁] (Nakayama et al., 1995) and on enriched tryptic soy (TS) agar [containing, per litre, 40 g Trypto-Soya agar (Nissui), 5 g brain-heart infusion, 1 g cysteine, 5 mg haemin and 1 mg vitamin K₁] (Ueshima et al., 2003).

Plasmid pET-22b (+) (Novagen) was used as a vector for expression of oxyR. E. coli strain BL21(DE3) was used as host for derivatives of this plasmid.

Oxidative stress conditions. Cells of P. gingivalis and B. fragilis were grown anaerobically at 37°C in enriched BHI broth. Exponential phase cultures were divided into two portions; one portion was left untreated, and the other portion was incubated aerobically for 1–2 h with or without 100 μM hydrogen peroxide or 10 μM paraquat.

Materials. The proteinase inhibitor N’-p-tosyl-L-lysine chloromethyl ketone (TLCK) was purchased from Wako Pure Chemical Co. Oligonucleotides were purchased from Invitrogen Japan.

General genetic procedures. Unless otherwise stated, standard procedures were used for the preparation and handling of DNA and RNA (Sambrook et al., 1989).

Two-dimensional gel electrophoresis (2DE). Samples were prepared using a modification of the method of Pridmore et al. (1999). Bacterial cells (1·5 x 10¹⁰ cells) were suspended in 1 ml lysis buffer (0·25%, w/v, SDS; 5%, v/v, 2-mercaptoethanol; 50 mM TLCK), vortexed, and heated at 100°C for 5 min. The following components were then added directly to the mixture to achieve the concentrations indicated: solid urea (9·5 M), Nonidet P-40 (2%, w/v), 2-mercaptoethanol (5%, v/v), Biolyte 3/10 (0·4%, v/v) (Bio-Rad) and Biolyte 4/6 (1·6%, v/v). This mixture was incubated at 37°C for 60 min with vortexing. The insoluble materials were then removed by centrifugation.

Proteins in the lysates were separated by isoelectric focusing (IEF) in an Immobiline DryStrip (Amersham Pharmacia Biotech) in the pH range from 4·0 to 7·0 and then by SDS-PAGE in a 12–14% ExcelGel SDS XL Gradient gel (Amersham Pharmacia Biotech) by using the Multiphor II System (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Gels were stained with Coomassie brilliant blue or electrophoretically transferred onto a PVDF membrane (Millipore) by the method of Matsudaira (1987).

Protein sequencing. After transfer of proteins onto the PVDF membrane, protein spots were cut out of the membrane and subjected to sequencing using a model 470A gas-phase protein sequencer (Applied Biosystems).

Primer extension assay. RNA samples were subjected to primer extension assays using 5'-labelled oligonucleotides. The oligonucleotides used were PEA1 for P. gingivalis ahpC, PEA2 for P. gingivalis sod and PEA3 for B. fragilis sod (Table 2). To label the 5' ends of the oligonucleotides, T4 DNA polynucleotide kinase and γ[32P]ATP were used. RNA samples (50 μg) were incubated with 0·5 pmol radiolabelled primer for 60 min at 60°C and then for 90 min at 37°C. After addition of dNTPs (0·5 mM each) and SuperscriptII RNase H⁻ reverse transcriptase (200 U, Gibco-BRL) the mixtures were incubated for 1 h at 42°C. The resulting products were loaded on a 5% Long Ranger (FMC BioProducts) polyacrylamide-urea gel and electrophoresed together with the DNA samples that were obtained from the DNA sequencing reaction of the corresponding double-stranded DNA with the same oligonucleotide primers.

Construction of P. gingivalis strains containing sod− lacZ and ahpC− lacZ protein fusion genes. Plasmid pKD398, which contains the sod− lacZ protein fusion gene, and KDP151 (sod− lacZ sod− oxyR) were used as the circular plasmid DNA of pKD398 into KDP143 (oxyR::Tc) following electroporation (Kikuchi et al., 2005). Proper DNA integration in KDP152 was confirmed by Southern hybridization. To construct an ahpC− lacZ protein fusion gene, the DNA region covering from the site 21 kb upstream of the start codon of the ahpC gene to a site within...
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
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<td><strong>Strains</strong></td>
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<td><em>P. gingivalis</em></td>
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<td>ATCC 32377</td>
<td>Wild-type</td>
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<td>KDP143</td>
<td>oxyR::Te'C</td>
<td>Ueshima et al. (2003)</td>
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<td>KDP147</td>
<td>ahpC+ ahpC'--lacZ Em'</td>
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<td>KDP149</td>
<td>ahpC+ ahpC'--lacZ oxyR::Te'C Em'</td>
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<td>KDP151</td>
<td>sod+ sod'--lacZ Em'</td>
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<td>KDP152</td>
<td>sod+ sod'--lacZ oxyR::Te'C Em'</td>
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<td><strong>B. fragilis</strong></td>
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<td>Stratagene</td>
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<td>BL21(DE3)</td>
<td>Host strain for expression vector pET22b(+)</td>
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<td><strong>Plasmids</strong></td>
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<td>Invitrogen</td>
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<td>pET22b(+)</td>
<td>Ap', expression vector</td>
<td>Novagen</td>
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<td>Ap', pET22b(+) containing <em>P. gingivalis</em> oxyR</td>
<td>This study</td>
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<td>pKD393</td>
<td>Ap' Em', lacZ reporter suicide/integration plasmid, containing unique EcoRI and BamHI sites at lacZ fusion sites</td>
<td>Ueshima et al. (2003)</td>
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<td>pKD395</td>
<td>Ap' Em'</td>
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<td>pKD397</td>
<td>Ap' oxyR::Te'C, containing the tetQ in pUC19</td>
<td>Ueshima et al. (2003)</td>
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<td><strong>Gene fusion</strong></td>
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<td><strong>Northern analysis.</strong> Total RNA (30 μg per lane) was separated by electrophoresis on an agarose gel containing 2-2 M formaldehyde, then transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech) in 10 × SSC (1 × SSC is 0-15 M NaCl plus 0-015 M sodium citrate). The probe DNAs used were PCR-amplified from <em>P. gingivalis</em> chromosomal DNA with oligonucleotides NH1 and NH2 for ahpC, and NH3 and NH4 for ahpF as upper and lower primers, respectively (Table 2). Random hexamer primers (Promega), dNTPs (except for dATP), BcaBEST DNA polymerase and [α-32P]dATP were used for labelling the probe DNAs. Hybridization was performed at 58 °C, and the final wash was at 58 °C in 2 × SSC/0-1% SDS.</td>
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<td><strong>Sensitivity of the <em>P. gingivalis</em> oxyR mutant to atmospheric oxygen.</strong> <em>P. gingivalis</em> cells grown in enriched BHI medium overnight were diluted twofold with fresh enriched BHI medium and then incubated aerobically at 37 °C with vigorous shaking (150 r.p.m.). To determine the number of surviving cells, cultures were withdrawn at intervals and plated on enriched TS plates after adequate dilution. The plates were incubated anaerobically for 7 days at 37 °C.</td>
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<td><strong>Construction of expression plasmids.</strong> The oxyR gene DNA was PCR-amplified using oligonucleotides EX1 and EX2, producing a 937 bp DNA with unique sites for NcoI and XhoI (Table 2). This fragment was digested with NcoI and XhoI and cloned into the same site of pET22b(+) allowing expression of the OxyR protein fused to a polyhistidine-containing tag at the C terminus (r-OxyR). The resulting plasmid was designated pETPGoxyR.</td>
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<td><strong>Expression and purification r-OxyR.</strong> <em>E. coli</em> BL21(DE3) harbouring pETPGoxyR was cultured in LB medium containing 50 μg ampicillin ml⁻¹ at 37 °C to OD₆₀₀ 0-6 and then IPTG was added to the culture at 0-4 mM, followed by an additional 2 h incubation. The cells were harvested, suspended in phosphate buffer and sonicated; cell extracts were obtained by centrifugation at 10000 g for 5 min at 4 °C. r-OxyR was purified from the cell extracts by affinity chromatography and ion-exchange chromatography. Since r-OxyR formed insoluble inclusion bodies, it was solubilized in 20 mM sodium phosphate buffer supplemented with 0-5 M NaCl and 6 M guanidine hydrochloride. It was then purified by Ni²⁺-chelate affinity chromatography using ProBond resin column (Invitrogen) in denaturing conditions according to the manufacturer’s instructions. The peak fractions were then dialysed against 10 mM Tris/HCl (pH 8.0) containing 0-1% Triton X-100 to remove urea. r-OxyR was further purified by cation-exchange chromatography using Mono-S resin column (Amersham Pharmacia Biotech).</td>
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<td><strong>Gel retardation.</strong> The DNA fragments used for gel retardation were generated by PCR amplification of bacterial chromosomal DNA with oligonucleotides GSA1 and GSA2 for <em>P. gingivalis</em> ahpC, GSA3 and GSA4 for <em>P. gingivalis</em> sod, GSA5 and GSA6 for <em>P. gingivalis</em> sod.</td>
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oxyR, and GSA7 and GSA8 for B. fragilis sod as upper and lower primers, respectively (Table 2). The DNA fragments were then digested with EcoRI and filled in with [\(\alpha\)-\(^32\)P]dATP (Amersham Pharmacia Biotech) and dTTP + dCTP using BcaBEST DNA polymerase (Takara). Gel retardation was performed essentially according to the method described by Ochsner et al. (2000). Briefly, various amounts (up to 1 \(\mu\)g) of r-OxyR were mixed with radiolabelled DNA fragments (2 ng) in the presence or absence of various amounts of the corresponding unlabelled DNA fragments and incubated in 20 \(\mu\)l reaction buffer [20 mM Bistris (pH 7.5), 20 mM boric acid, 40 mM KCl, 5 mM MgCl\(_2\), 1 mM EDTA, 1 mM dithiothreitol, 100 mg bovine serum albumin ml\(^{-1}\), 50 mg poly(dI-dC) ml\(^{-1}\), 10% (v/v) glycerol] for 15 min at 4°C. The mixture was loaded on a 6% polyacrylamide gel in running buffer [20 mM Bistris (pH 7.5), 20 mM boric acid] at 250 V for 3–4 h and subjected to autoradiography.

**RESULTS**

**P. gingivalis proteins with OxyR-dependent induction**

To investigate the effect of OxyR on oxidative stress responses of P. gingivalis, proteins in oxidatively-stressed
cells were analysed by 2DE. When cultures of the wild-type strain (ATCC 33277) were exposed to hydrogen peroxide, two proteins (P1 and P2) with the same molecular mass of 21 kDa and isoelectric points of 6.0 and 5.3 were prominently produced (Fig. 1). Similar results were obtained when cells were treated with paraquat (methyl viologen) (data not shown). Amounts of these two proteins were also increased by treatment with hydrogen peroxide.

![Image](http://mic.sgmjournals.org)

**Fig. 1.** 2DE analysis of total proteins of *P. gingivalis* cells in the oxidative condition. (a) Crude protein extracts of *P. gingivalis* ATCC 33277 (wild-type) and its oxyR mutant (KDP143) cultured in anaerobic or oxidative conditions. (b) Individual panels correspond to a magnified view of the area surrounded by the dotted line of panels in (a). Directions of IEF and SDS-PAGE are shown in the top right corner. MM, molecular mass. The arrow and arrowhead indicate protein spots P1 and P2, respectively. Three independent experiments were carried out and a typical set of results is shown.
or paraquat in another P. gingivalis strain, 381 (data not shown). On the other hand, oxidative stresses did not induce these two proteins in the OxyR-deficient mutant KDP143. The wild-type strain produced more P1 protein than the oxyR mutant even under anaerobic conditions. The oxyR mutant did not produce P2 protein in either oxidative or reduced conditions. These two proteins were further analysed.

Identification of P1 and P2 proteins
To identify P1 and P2 proteins, their N-terminal amino acid sequences were determined, resulting in the sequences MTGELISLPYAVNKL and MTPILNTVFP, respectively. Homology searches in the DDBJ/EMBL/GenBank and the Institute for Genome Research databases revealed that the N-terminal 15 amino acids of protein P1 were identical to the first 15 amino acid residues of P. gingivalis SOD (Nakayama, 1990) and the N-terminal 10 amino acids of protein P2 were identical to the first 10 amino acid residues of P. gingivalis AhpC (Nelson et al., 2003). The position of each spot in 2DE was in good agreement with the hypothetical molecular mass (MM) and the hypothetical isoelectric point (pI) of each protein (SOD: MM 21 500 ± 05 Da; pI 5 9; AhpC: MM 21 076 ± 06, pI 5 27). We therefore identified spots 1 and 2 as SOD and AhpC, respectively.

OxyR-dependent response
To find the promoter loci of the ahpC and sod genes, primer extension analysis was performed (Fig. 2). The 5’ terminus of the ahpC transcript of the wild-type strain was found in a position corresponding to the G residue 38 bases upstream of the initiation codon of ahpC. When the oxyR mutant was employed, however, no extension products from the ahpC transcript were obtained. Primer extension analysis with the sod gene revealed two transcripts (T1 and T2). The 5’ termini of the T1 and T2 transcripts were located at positions corresponding to the G residues 82 and 45 bases upstream of the initiation codon of sod, respectively, in both the wild-type and the oxyR mutant strains. The analysis also suggested that amounts of the T2 transcript of the oxyR mutant were not different from those of the wild-type strain and that amounts of the T1 transcript of the oxyR mutant were clearly less than those of the wild-type strain. Primer extension analysis with the sod gene of B. fragilis, which is taxonomically related to P. gingivalis, located the 5’ terminus of the sod transcript in a position corresponding to the G residue 63 bases upstream of the initiation codon. We compared the DNA sequences upstream of P. gingivalis ahpC, P. gingivalis sod and B. fragilis sod to the consensus DNA sequence of the P. gingivalis promoter (Jackson et al.,

![Fig. 2. Determination of the transcription initiation sites of P. gingivalis ahpC and sod (P. g. ahpC and P. g. sod, respectively) and that of B. fragilis sod (B. f. sod). Total RNA isolated from P. gingivalis ATCC 33277 (wild-type), its oxyR mutant (KDP143) and B. fragilis ATCC 25285 under aerobic conditions with hydrogen peroxide was used as the template for reverse transcription. The corresponding DNA sequences are shown on the left. The transcriptional start sites are indicated by arrowheads.](image-url)
and determined putative -10 and -35 boxes of those promoters (Fig. 3a). Putative OxyR-binding sequences were also identified upstream of the ahpC and sod promoters of P. gingivalis and of the sod promoter of the B. fragilis. Four ATAG elements spaced at 10 bp intervals comprise the binding sites for oxidized E. coli OxyR (Toledano et al., 1994; Zheng et al., 2001), and such elements were found within the ahpC and sod promoters of P. gingivalis and the sod promoter of B. fragilis. The number of bases matching with the consensus OxyR-binding sequence (16 bp) was 13, 10 and 10 in P. gingivalis ahpC, P. gingivalis sod and B. fragilis sod, respectively (Fig. 3b). It is notable that the space between the second and the third elements of the putative OxyR-binding sequence near the ahpC promoter was 6 bp, whereas the consensus space was 7 bp. The putative OxyR-binding sequences were present immediately upstream of -35 boxes in the promoter regions of ahpC and sod of P. gingivalis. However, there was a 14 bp space between the OxyR-binding sequence and the -35 box in the promoter region of B. fragilis sod (Fig. 3b).

OxyR-controlled induction of the sod'–lacZ and ahpC'–lacZ fusions

To confirm whether expression of the sod and ahpC genes is induced by the oxidative stress in an OxyR-dependent fashion, we constructed plasmids (pKD398 and pKD395) containing sod'–lacZ and ahpC'–lacZ protein fusion genes, respectively, using the lacZ reporter suicide/integration plasmid with the promoter and 5' regions of sod and ahpC. Plasmids pKD398 and pKD395 were introduced into P. gingivalis ATCC 33277 (wild-type) to yield KDP151 (sod'+ sod'–lacZ) and KDP147 (ahpC'+ ahpC'–lacZ), respectively. An oxyR::tetQ mutation was then introduced into KDP151 and KDP147, resulting in KDP152 (oxyR::tetQ sod'+ sod'–lacZ) and KDP 149 (oxyR::tetQ ahpC'+ ahpC'–lacZ), respectively. Exposure to atmospheric oxygen did not enhance the increase under aerobic conditions. On the other hand, such an increase was not observed in the oxyR background, indicating that the oxidative induction of the sod gene expression was controlled by OxyR (Fig. 4a). Exposure to atmospheric oxygen also caused an increase of the ahpC expression in the wild-type strain; however, such an increase was not observed in the oxyR background, indicating that the oxidative induction of the ahpC gene expression was also controlled by OxyR (Fig. 4b). Expression of ahpC under anaerobic conditions was lower in the oxyR background than in the wild-type background, compared to that of sod (Fig. 4), which was consistent with the result of primer extension.

Fig. 3. (a) Nucleotide sequences of the promoter regions of P. gingivalis ahpC and sod, and B. fragilis sod. The transcriptional start sites are in bold and underlined. Putative -10 and -35 boxes are in bold letters; DNA sequences that correspond to the OxyR-binding motif are in italic and underlined. (b) Comparison of the putative OxyR-binding sequences of P. gingivalis ahpC, P. gingivalis sod and B. fragilis sod. The consensus sequence of the OxyR-binding motif of E. coli is given in the first line. The sequences that correspond to the OxyR-binding motif are underlined and the nucleotides identical to those in the consensus OxyR-binding motif are indicated by asterisks. Putative -35 boxes are in bold.
Northern blot analysis of \textit{ahpC}

\textit{ahpF} was located downstream of \textit{ahpC}. To determine the relationship of expression of the two genes, total RNAs were prepared from \textit{P. gingivalis} cells and Northern blot hybridization was carried out using an \textit{ahpC} DNA probe. As shown in Fig. 5, two fragments (0.5 and 2.4 kb) in total RNA from the wild-type strain hybridized with the \textit{ahpC} DNA probe. An \textit{ahpF} DNA probe hybridized to the 2.4 kb fragment, but not to the 0.5 kb fragment (data not shown). Since the 0.5 kb fragment had the capacity to encode AhpC alone but the 2.4 kb fragment could encode both AhpC and AhpF, these results implied that a transcriptional terminator might be located between \textit{ahpC} and \textit{ahpF}. In contrast, no fragment in the total RNA from the \textit{oxyR} mutant hybridized with the \textit{ahpC} DNA probe, suggesting that the \textit{ahpC} expression is fully controlled by OxyR.

\textbf{Fig. 4.} Induction of $\beta$-galactosidase activity in the \textit{sod}--\textit{lacZ} fusion and the \textit{ahpC}--\textit{lacZ} fusion strains. Cells were anaerobically grown in enriched BHI medium at 37 °C. At an OD$_{600}$ of 0.3, the cells were exposed to atmospheric oxygen with (open symbols) or without (solid symbols) hydrogen peroxide. Samples were withdrawn after the indicated times, and the activity of $\beta$-galactosidase was determined by the method of Miller (1972). (a) \textit{P. gingivalis} KDP151 (\textit{sod}+ \textit{sod}--\textit{lacZ}) (circles), KDP152 (\textit{sod}+ \textit{sod}--\textit{lacZ oxyR::Tc}) (triangles) and ATCC 33277 (wild-type) (squares). (b) \textit{P. gingivalis} KDP147 (\textit{ahpC}+ \textit{ahpC}--\textit{lacZ}) (circles) and KDP149 (\textit{ahpC}+ \textit{ahpC}--\textit{lacZ oxyR::Tc}) (triangles).

\textbf{Fig. 5.} Northern hybridization analysis of \textit{P. gingivalis} \textit{ahpC} transcripts. Total RNA was electrophoresed, blotted, and hybridized to the \textit{ahpC} probes (see Methods). (a) Stained with ethidium bromide; (b) autoradiography. The positions of transcripts are indicated by arrows (A and B). Lanes: 1, an anaerobic culture of \textit{P. gingivalis} ATCC 33277 (wild-type); 2, an aerobic culture of ATCC 33277 with hydrogen peroxide; 3, an anaerobic culture of KDP143 (\textit{oxyR}); 4, an aerobic culture of KDP143 with hydrogen peroxide. (c) Gene organization in the vicinity of the \textit{ahpC} locus of \textit{P. gingivalis} W 83. Arrows A and B indicate the \textit{ahpC} transcripts shown in (b).
Sensitivity of the *P. gingivalis* oxyR mutant to atmospheric oxygen

In order to determine whether OxyR contributes to aero-tolerance of *P. gingivalis*, oxyR mutant cells that had been cultured anaerobically in enriched BHI medium overnight were diluted twofold with fresh enriched BHI medium and then incubated aerobically with vigorous shaking. Samples were withdrawn at intervals and plated, after dilution in enriched BHI medium, on enriched TS plates. The plates were incubated anaerobically at 37 °C for 7 days. ○, ATCC 33277 (wild-type); ▲, KDP143 (oxyR).

**Fig. 6.** Sensitivity of *P. gingivalis* oxyR mutant to atmospheric oxygen. *P. gingivalis* cells that had been cultured anaerobically in enriched BHI medium overnight were diluted twofold with fresh enriched BHI medium and then incubated aerobically with vigorous shaking. Samples were withdrawn at intervals and plated, after dilution in enriched BHI medium, on enriched TS plates. The plates were incubated anaerobically at 37 °C for 7 days. ○, ATCC 33277 (wild-type); ▲, KDP143 (oxyR).

**DISCUSSION**

Bacteria adapt to environments with reactive oxygen species by increasing the expression of detoxification enzymes and proteins such as SOD and catalase, and by upregulating the functions to repair DNA damage caused by oxidative stress. These responses are coordinated by transcription factors that regulate target genes in response to oxidative stress. Among the transcription factors, OxyR and SoxR have been well characterized. The OxyR protein shares homology with the LysR family of bacterial regulators (Christman *et al.*, 1989; Mongkolsuk & Helmann, 2002). OxyR possesses a helix–turn–helix DNA-binding motif in its N-terminal domain and forms a tetramer (Storz *et al.*, 1990). The initial reaction of OxyR with hydrogen peroxide takes place at Cys-199, resulting in the formation of Cys-sulfenic acid. This unstable Cys-sulfenic acid is postulated to react with Cys-208 to yield a disulfide bond. It has been proposed that formation of the Cys-199 sulfenic acid derivative of OxyR is sufficient to explain the *in vivo* OxyR regulation in response to peroxide stress (Kim *et al.*, 2002). On the other hand,

**r-OxyR binds to the upstream regions of ahpC and sod**

The upstream region of the *ahpC* and *sod* genes from *P. gingivalis* and that of the *sod* gene from *B. fragilis* contained the consensus OxyR-binding sequence. We attempted to obtain evidence of binding of OxyR to the upstream region of these genes. Purified His$_6$-tagged r-OxyR protein at a concentration of at least 10 nM caused a mobility shift of DNA fragments containing these target promoters, indicating direct binding of OxyR (Fig. 7). A DNA fragment harbouring the *P. gingivalis* oxyR promoter was not shifted by r-OxyR (data not shown).

**Fig. 7.** Electrophoretic mobility shift analysis of the promoter region DNA of the *P. gingivalis* *ahpC*, *P. gingivalis* *sod* and *B. fragilis* *sod* genes with r-OxyR. Mobility shift assays were performed with various amounts of purified r-OxyR and 2 ng $^{32}$P-labelled *P. gingivalis* *ahpC* (a), *P. gingivalis* *sod* (b), and *B. fragilis* *sod* (c) promoter DNA fragments, and various amounts of the corresponding unlabelled DNA fragments (Comp.). The amounts of r-OxyR and of unlabelled DNA fragments are shown. Arrowheads and asterisks indicate the complex of probe DNA and r-OxyR and the free probe DNA, respectively.
SoxR shares homology with the mercury-dependent MerR regulator of *E. coli* (Amabile-Cuevas & Demple, 1991; Wu & Weiss, 1991). It also contains a helix–turn–helix DNA-binding motif in the N-terminal domain and forms a homodimer. The homodimer of SoxR contains two redox-active [2Fe–2S] clusters. SoxR becomes activated to stimulate the transcription of its target gene *soxS* when the [2Fe–2S] clusters are oxidized by superoxide radical or nitric oxide. The redox potential of the [2Fe–2S] cluster is reported to be −285 mV (Ding et al., 1996; Gaud & Weiss, 1995).

A number of *sod* genes in bacteria are regulated by SoxR, which appears to be reasonable because the target molecule for SOD is the superoxide radical, which activates the transcription regulator SoxR. In *E. coli*, two cytoplasmic SODs have been identified, Mn-SOD and Fe-SOD. *sodA*, encoding Mn-SOD, is inducible in response to superoxide-generating agents and its induction is regulated by SoxR, whereas *sodB*, encoding Fe-SOD, is constitutively expressed independent of oxidative stress; however, these two genes are regulated by iron uptake/storage regulation systems including Fur and RyhB (Compan & Touati, 1993; Dubrac & Touati, 2002; Masse & Gottesman, 2002).

In this study, we demonstrated that the *P. gingivalis* *sod* gene was inducible by oxidative stresses and this induction was regulated by the redox-sensing transcription factor OxyR. Inspection of the *P. gingivalis* genome proved the absence of *soxRS* homologues in the organism. Is it effective for OxyR to regulate the *sod* gene in *P. gingivalis*? Although OxyR is primarily identified as a sensor for hydrogen peroxide, it can also respond to disulfide stress resulting from defects in the systems that function to maintain an intracellular reducing environment. In *E. coli* wild-type cells, the thiol–disulfide redox potential of the cytoplasm (−280 mV) is 95 mV lower than the redox potential of OxyR (−185 mV), indicating that OxyR is usually in a reduced state (Zheng et al., 1998). Aslund et al. (1999) found, however, that *E. coli* mutant strains with defects in both the glutathione- and thioredoxin-dependent thiol reduction systems, such as a gorR *txrA* mutant, show constitutive activation of OxyR without oxidative stresses, probably because the degree of thermodynamic barrier for OxyR may be smaller in the mutant strains than in the wild-type strain. They also mentioned that in *E. coli* wild-type cells, the high thermodynamic barrier can be overcome by the high reactivity of OxyR with hydrogen peroxide; however, the oxidized protein is in a metastable state. We found that a *P. gingivalis* *sod* mutant is extremely sensitive to atmospheric oxygen (Nakayama, 1994), whereas a *P. gingivalis* *dps* mutant is insensitive to it (Ueshima et al., 2003), indicating that exposure to atmospheric oxygen may primarily produce superoxide radical and increase the cytoplasmic redox potential in *P. gingivalis*. The redox potential in the *P. gingivalis* cytoplasm may be more easily affected by extracellular redox environments than that in the *E. coli* cytoplasm, enabling activation of OxyR to yield induction of the *sod* gene. Instability of cytoplasmic redox status in *P. gingivalis* may account for its anaerobicism.

The *sod* gene of *B. fragilis*, like that of *P. gingivalis*, is inducible by oxidative stress (Gregory, 1985). However, *B. fragilis* *sod* may not be regulated by OxyR since *sod* gene expression was at a basal level in anaerobic conditions and was inducible on exposure to atmospheric oxygen in the constitutively active oxyR (Con) strain (Smalley et al., 2002). Our findings that the putative OxyR-binding sequence was present in the upstream region of *B. fragilis* *sod* and that r-OxyR could bind to this region are apparently inconsistent with the findings of Smalley et al. (2002). We speculate that OxyR has the potential to bind to the OxyR-binding motif in the *B. fragilis* *sod* promoter region but cannot regulate this gene because of the unusual length of space (14 bp) between the OxyR-binding motif and the −35 box. This OxyR-binding sequence might be a vestige of an ancestral *sod* gene in *B. fragilis* which might have been originally regulated by OxyR. Although *B. fragilis* belongs to the same order as *P. gingivalis*, its genome size is about twice that of *P. gingivalis*. Therefore, *B. fragilis* may have acquired a novel regulatory mechanism for controlling *sod* expression independent of OxyR.

The present study revealed that *P. gingivalis* *ahpC* has two transcripts with the same start site but different lengths; one (0.5 kb) has the capacity to encode AhpC alone and the other (2.4 kb) has the capacity to encode both AhpC and AhpF. The two transcripts are fully controlled by OxyR because the oxyR mutant showed no *ahpC* or *ahpCF* transcripts. Full control of *ahpCF* expression by OxyR is also observed in *B. fragilis* (Rocha et al., 2000). In contrast, *P. gingivalis* *sod* has two transcripts with different start sites; one (T1) is regulated by OxyR and the other (T2) is independent of OxyR regulation. This finding implies that regulation of *P. gingivalis* *sod* expression may be more complex than that of *ahpCF*.

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