Novel stationary-phase-upregulated protein of Porphyromonas gingivalis influences production of superoxide dismutase, thiol peroxidase and thioredoxin

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Porphyromonas gingivalis, an obligately anaerobic bacterium, is implicated as a major pathogen in the development and progression of chronic periodontitis. Although expression of several virulence factors of the bacterium has been found to be affected by environmental stress such as entrance into the stationary growth phase and heat, there is relatively little information on the mechanisms that may operate in the bacterium in response to environmental stress. In this study, a novel protein (UstA) was investigated that was initially identified following two-dimensional gel analysis. Expression of UstA was upregulated in stationary phase or by exposure to atmospheric oxygen. N-terminal sequencing and database analysis with the P. gingivalis genome sequence revealed that the UstA-encoding gene (ustA) was located upstream of a homologue of the usp gene encoding the universal stress protein on the chromosome. The ustA gene appeared to be transcribed in a monocistronic fashion, as revealed by primer extension and Northern blot analysis. To elucidate the role of UstA in the bacterium, chromosomal mutants carrying a disruption of the ustA gene were constructed. The ustA mutant grew slower than the wild-type parent strain in rich medium, resulting in a lower yield in stationary phase. Furthermore, in this mutant, expression levels of the P. gingivalis homologues of superoxide dismutase, thiol peroxidase and thioredoxin were markedly higher than those in the wild-type, especially in stationary phase. The ustA mutant was more resistant to diamide, a thiol-specific oxidant, than the wild-type. In addition, the ustA mutation suppressed hypersensitivities of the oxyR mutant to diamide, metronidazole and mitomycin C. These results suggest that UstA may play a significant role in oxidative stress responses in the bacterium.

INTRODUCTION

In their natural environment, bacteria are affected by constantly changing nutrient availability and by exposure to various forms of physical stress, including osmotic, oxidative and temperature shock. Exposure to starvation and stress leads to the reduction or cessation of bacterial growth, which results in a major switch in gene expression that allows their cells to cope with the new conditions. A quite simple and effective mechanism employed by bacteria that results in a major alteration in gene expression is the use of alternative sigma factors that alter RNA polymerase core specificity (Wick & Egli, 2004; Hengge-Aronis, 2000, 2002; Eisenstark et al., 1996).

Porphyromonas gingivalis is a Gram-negative anaerobic bacterium belonging to the phylum Bacteroidetes (Boone et al., 2001). This bacterium is one of the organisms that are strongly associated with chronic adult periodontitis and expresses numerous potential virulence factors, such as fimbrins, haemagglutinins, lipopolysaccharides and various proteases that are capable of hydrolysing collagen, immunoglobulins, iron-binding proteins and complement factors (Holt et al., 1999; Lamont & Jenkinson, 1998). To facilitate adaptation to life within the oral cavity, P. gingivalis must be capable of sensing and responding to the prevailing conditions.
environmental conditions, including variations in temperature, oxygen tension, pH, nutrient availability and the presence of other bacterial or host cells. Lu & McBride (1994) found that *P. gingivalis* homologues of DnaK and GroEL were upregulated when cells were shifted from 37 to 42 °C. These proteins were also induced by treatment with ethanol, but not by oxidative stress or change in pH. In addition, Shelburne et al. (2002) reported that *P. gingivalis* cells stressed *in vitro* by a 5 °C temperature increase showed a rapid rise in the mRNA associated with genes encoding the molecular chaperones (htpG, dnaK, groEL), superoxide dismutase *(sod)* and gingipain *(rgpA)*. Nevertheless, there is relatively little information on the mechanisms that may operate in this bacterium in response to entrance into the stationary growth phase.

In this study, we describe the identification of a gene whose product is characterized as a novel *P. gingivalis* protein with a molecular mass of 9 kDa and an isoelectric point of 4.5 that is upregulated in stationary phase (UstA) by using two-dimensional (2D) gel electrophoresis. Chemosomal mutants carrying a disruption of the *ustA* gene are constructed and analysed to gain insights to the physiological role of UstA.

### METHODS

#### Bacterial strains and plasmids.

Bacterial strains and plasmids used in the present study are listed in Table 1. Cells of *P. gingivalis* were grown anaerobically (10% CO₂, 10% H₂ and 80% N₂) in enriched brain heart infusion (BHI) broth and on enriched tryptic soy (TS) agar (Nakayama et al., 1995).

#### Chemicals.

Proteinase inhibitors Nα-p-tosyl-l-lysine chloromethyl ketone (TLCK) and iodoacetamide were purchased from Wako and leupeptin was from Peptide Institute. Hydrogen peroxide (H₂O₂), metronidazole and mitomycin C were obtained from Wako and t-butyl hydroperoxide (t-BOOH), cumene hydroperoxide (CM-OOH) and diamide were from Sigma.

#### Oxidative stress conditions.

Cells of *P. gingivalis* were grown anaerobically at 37 °C in enriched BHI broth. Exponential-phase (15 h after inoculation) or stationary-phase (48 h after inoculation) cultures were divided into two portions (40 ml each): one portion was left under anaerobic conditions and the other was incubated aerobically for 20 or 120 min with vigorous shaking at 150 r.p.m.

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

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Source and/or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>P. gingivalis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 33277</td>
<td>Wild-type</td>
<td>ATCC</td>
</tr>
<tr>
<td>KDP143</td>
<td>oxyR::tetQ</td>
<td>Ueshima et al. (2003)</td>
</tr>
<tr>
<td>KDP151</td>
<td>sod::lacZ, ermF, ermAM</td>
<td>This study</td>
</tr>
<tr>
<td>KDP301</td>
<td>ustA::ermF, ermAM</td>
<td>This study</td>
</tr>
<tr>
<td>KDP302</td>
<td>ustA::ermF, ermAM, fimA::tetQ</td>
<td>This study</td>
</tr>
<tr>
<td>KDP303</td>
<td>ustA::ermF, ermAM, fimA::tetQ, ustA*+</td>
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<tr>
<td>KDP304</td>
<td>fimA::tetQ</td>
<td>This study</td>
</tr>
<tr>
<td>KDP305</td>
<td>sod::lacZ, ermF, ermAM, ustA::tetQ</td>
<td>This study</td>
</tr>
<tr>
<td>KDP306</td>
<td>ustA::ermF, ermAM, oxyR::tetQ</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
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<tr>
<td>XL-1 Blue</td>
<td>General-purpose host strain for cloning</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>Host strain for expression vector pCR-Blunt II-TOPO</td>
<td>Novagen</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
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<tr>
<td>pGEM-T Easy</td>
<td>Ap*, plasmid vector for TA cloning</td>
<td>Promega</td>
</tr>
<tr>
<td>pCR-Blunt</td>
<td>Km*, plasmid vector for blunt-end cloning</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>II-TOPO</td>
<td></td>
<td></td>
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<tr>
<td>pKD355</td>
<td>Ap*, contains the ermF, ermAM DNA cassette between EcoRI and BamHI sites of pUC18</td>
<td>Ueshima et al. (2003)</td>
</tr>
<tr>
<td>pKD375</td>
<td>Ap*·Tc•*, contains the tetQ DNA cassette in pUC19</td>
<td>Shi et al. (1999)</td>
</tr>
<tr>
<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>
</tr>
<tr>
<td>pKD398</td>
<td>Ap*·Em*, sod::lacZ, containing a 5'-terminal region of <em>P. gingivalis</em> sod in pKD393</td>
<td>This study</td>
</tr>
<tr>
<td>pKD703</td>
<td>Ap*, contains 0.77 kb fimA-upstream and 0.78 kb fimA-downstream DNA fragments</td>
<td>Shoji et al. (2004)</td>
</tr>
<tr>
<td>pKD713</td>
<td>Ap*·Tc•*, contains the tetQ DNA cassette at BamHI site of pKD703</td>
<td>This study</td>
</tr>
<tr>
<td>pKD801</td>
<td>Ap*, contains the 3.2 kb PCR-amplified fragment (ustA region) in pGEM-T Easy</td>
<td>This study</td>
</tr>
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<td>pKD802</td>
<td>Ap*·Em*, contains the ermF, ermAM DNA cassette at BsiWI site within ustA of pKD801</td>
<td>This study</td>
</tr>
<tr>
<td>pKD803</td>
<td>Km*, contains the 0.6 kb PCR-amplified fragment (ustA region) in pCR-Blunt II-TOPO</td>
<td>This study</td>
</tr>
<tr>
<td>pKD804</td>
<td>Ap*·Tc•*, contains the 0.6 kb ustA region at EcoRI site of pKD713</td>
<td>This study</td>
</tr>
<tr>
<td>pKD805</td>
<td>Ap*·Tc•*, contains the tetQ DNA cassette at BsiWI site within ustA of pKD801</td>
<td>This study</td>
</tr>
</tbody>
</table>

*The tetQ gene is expressed only in *P. gingivalis* cells.*
2D gel electrophoresis. P. gingivalis cells were harvested and treated with 10% trichloroacetic acid. The resulting precipitates were washed several times with acetone and dried. Cells were then suspended in a lysing buffer (8 M urea, 4% CHAPS, 20 mM DTT and 40 mM Tris base). The lysates were sonicated and centrifuged at 22 000 g for 5 min. After centrifugation, the supernatant was subjected to 2D gel analysis. 2D gel electrophoresis was performed by using the MultiphorII system (Amersham Pharmacia) according to the manufacturer’s instructions. Briefly, 18 cm dehydrated isoelectric focusing strips with an immobilized pH gradient between pH 4 and 7 were rehydrated overnight in a rehydration solution [8 M urea, 20 mM DTT, 4% CHAPS and 0.00001% bromophenol blue (BBP) in 10 ml distilled water]. Then, each protein preparation at the same concentration was loaded on rehydrated gel strips and proteins were electrofocused at 20° C in four stages (500 V, 2 mA, 5 W for 1 min; 500 V, 2 mA, 5 W for 1 h; 3500 V, 2 mA, 5 W for 1-5 h; and 3500 V, 2 mA, 5 W for 6 h). Focused gel strips were equilibrated for 10 min in equilibration solution A (50 mM Tris/HCl, pH 8, 30% glycerol, 6 M urea, 2% SDS, 40 mM DTT) and for another 10 min in 10 ml equilibration solution B (50 mM Tris/HCl, pH 8, 30% glycerol, 6 M urea, 2% SDS, 0.00001% BBP, 135 mM iodoacetamide). For the second dimension electrophoresis, we used precast polyacrylamide gels with a linear polyacrylamide gradient from 12 to 14% with the appropriate precast buffer strips (Amersham Pharmacia). Gels were run at 1000 V, 20 mA and 40 W at 15° C for 45 min, after which the first dimension strip gels were removed. The gel was then run at 1000 V, 40 mA and 40 W at 15° C for 180 min. Proteins in the gels were stained with Coomassie brilliant blue (CBB) or electrophoretically transferred onto a PVDF membrane (Millipore) by the method of Matsudaira (1987).

Protein and DNA sequencing. After transfer of proteins onto the PVDF membrane, protein spots were cut from the membrane and subjected to protein sequencing using a model 470A gas-phase protein sequencer (Applied Biosystems). DNA sequencing was performed using plasmid templates and a dye-deoxy sequencing kit (Thermal cycler sequencing kit; Amersham Pharmacia) with a Long Ranger (FMC) polyacrylamide-urea gel and electrophoresed at 42°C for 1 h. The resulting products were loaded on a 5% polyacrylamide-urea gel and electrophoresed together with DNA samples that were obtained from a sequencing reaction of the corresponding double-stranded DNA with the same oligonucleotide primer.

Table 2. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5′-3′)</th>
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<tbody>
<tr>
<td>USTAFOR</td>
<td>CAGGTCTAAACGCGCTTCTGAGCAAC</td>
</tr>
<tr>
<td>USTAREV</td>
<td>TAGACGACTTCTTGGTTTCTTTACATTG</td>
</tr>
<tr>
<td>PE1</td>
<td>GAAATAGTTCGCTAGCGGTTCATCAGCAG</td>
</tr>
<tr>
<td>NH1</td>
<td>GATGACTGATGAAAGCTACCTCATTTC</td>
</tr>
<tr>
<td>NH2</td>
<td>CTGCCGATATCGATGTTCTGCTGCACCAGC</td>
</tr>
<tr>
<td>SH1</td>
<td>CTCTCCTACATTTATGTCATCT</td>
</tr>
<tr>
<td>SH2</td>
<td>GCAACTATTTTCGCGGCTGCAATTTC</td>
</tr>
<tr>
<td>CP1</td>
<td>GGTTACCAACAGCACAAGGTTTATC</td>
</tr>
<tr>
<td>CP2</td>
<td>GGCGGCCCTGTATGTCGCTGATTC</td>
</tr>
<tr>
<td>EX1</td>
<td>GGATATCTCAGCTTCTTGTCTATCAGTATC</td>
</tr>
<tr>
<td>EX2</td>
<td>GGATACGCAGAGGGGTCGATGCAGCG</td>
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Polynucleotide kinase (Takara) with [γ-32P]ATP (Amersham Pharmacia). Oligonucleotides including PE1 used in this study were purchased from Invitrogen and are listed in Table 2. RNA samples (50 μg) were incubated with 0-5 pmol radiolabelled oligonucleotide primer at 60°C for 60 min and then at 37°C for 90 min. After addition of dNTPs (0-5 mM each) and Superscript II RNase H- reverse transcriptase (200 U; Gibco Laboratories), the mixtures were incubated at 42°C for 1 h. The resulting products were loaded on a 5% LongRange (FMC) polyacrylamide-urea gel and electrophoresed together with DNA samples that were obtained from a sequencing reaction of the corresponding double-stranded DNA with the same oligonucleotide primer.

Northern blot analysis. Total RNA was extracted from P. gingivalis cells grown to the exponential phase (OD600=0-4) by using an RNeasy Protect Mini kit (Qiagen). RNA samples (20 μg) were electrophoresed in 1-0% agarose gel containing 2-2 M formaldehyde and then transferred to a nylon membrane (Pall Biodyne Plus membrane; Pall). The probe DNA (255 bp) was PCR-amplified from P. gingivalis chromosomal DNA with the oligonucleotides NH1 and NH2 (Table 2) as upstream and downstream primers, respectively. Random hexamer primers (Promega), dNTPs (except for dATP), KbcBEST DNA polymerase (Takara Biomedicals) and [α-32P]dATP were used for labelling the probe DNA. Hybridization was performed at 68°C and the final wash was at 50°C in 2 x SSC, 0-1% SDS.

Subcellular fractionation. P. gingivalis cells were harvested from 3000 ml full-grown culture by centrifugation at 10 000 g at 4°C for 30 min and resuspended in 100 ml PBS containing 0-1 mM TLCK, 0-1 mM leupeptin and 0-5 mM EDTA. The cells were disrupted in a French pressure cell at 100 MPa. Unbroken cells were removed by centrifugation at 1000 g for 10 min and the supernatant was subjected to ultracentrifugation at 100 000 g for 60 min. The precipitates were treated with 1% Triton X-100 in PBS containing 20 mM MgCl2 at 20°C for 30 min. The inner and outer membrane fractions were recovered as a supernatant and a pellet, respectively, by ultracentrifugation at 100 000 g at 4°C for 60 min (Murakami et al., 2002). Proteins were subjected to Tris-Tricine SDS-PAGE and transferred to PVDF membranes and immunoreacted with anti-UstA antiserum.

Preparation of anti-UstA antisera. A peptide derived from the amino acid sequence of UstA (G2–S7) with an N-terminal cystine residue, CgIVELDTTILERALS, which was conjugated to keyhole limpet haemocyanin, was purchased from Sigma Genosys. The conjugated peptide (250 μg) was mixed with Freund’s complete adjuvant and injected subcutaneously into a rabbit (Japan White) with two booster shots of a mixture of the conjugated peptide and Freund’s incomplete adjuvant. Animal care and experimental procedures were conducted in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee.

Construction of P. gingivalis ustA::erm mutants. The chromosomal DNA region including the ustA gene (3-3 kb) was PCR-amplified from the chromosomal DNA of P. gingivalis ATCC 33277 (referred to as strain 33277) using Advantage 2 DNA polymerase (BD Biosciences) with the oligonucleotides USTAFOR and USTAREV (Table 2) as upstream and downstream primers, respectively. The amplified DNA fragment was cloned into a T-vector (pGEM-T Easy; Promega), resulting in pKD801. The ermF–ermAM DNA cassette of pKD355 (Ushimura et al., 2003) was inserted into the BsW1 site within ustA of pKD801 to yield pKD802. pKD802 plasmid DNA was linearized by Nof digestion and introduced into cells of P. gingivalis 33277 and KDP143 (oxyR::tetQ) by electroporation as described previously (Nakayama et al., 1995), resulting in strains KDP301 (ustA::ermF ermAM) and KDP306 (ustA::ermF ermAM oxyR::tetQ), respectively.
To construct the usta+ complemented mutant, the whole usta gene region with its upstream and downstream flanking regions (0.6 kb) was PCR-amplified from the chromosomal DNA of P. gingivalis 33277 using Pyrobest DNA polymerase (Takara) with the oligonucleotides CP1 and CP2 (Table 2) as upstream and downstream primers, respectively. The amplified DNA fragment (559 bp) was cloned into pCR-Blunt II-TOPO (Invitrogen) according to the manufacturer’s recommendation. The resulting plasmid pKD803 was subjected to DNA sequencing. In addition, the tetQ DNA cassette of pKD375 (Shi et al., 1999) was inserted into the BamHI site of pKD703, a plasmid containing both flanking DNA regions of P. gingivalis fimA (Shoji et al., 2004), to yield pKD713. The whole usta region (0.6 kb) of pKD803 obtained by EcoRI digestion was inserted into the EcoRI site of pKD713 to yield pKD804. Plasmids pKD713 and pKD804 were linearized by BsiHI digestion and introduced into KDP301 (usta::ermF ermAM) by electroporation, resulting in KDP302 (usta::ermF ermAM fimA::tetQ) and KDP303 (usta::ermF ermAM fimA::tetQ ustA+), respectively, after 7 days incubation on enriched TS agar containing 1–0 μg tetracycline ml⁻¹. Correct gene replacement of these strains that had been generated by double crossover recombination events was verified by PCR and Southern blot analysis.

**Construction of P. gingivalis strains containing the sod–lacZ protein fusion gene.** To construct an sod–lacZ protein fusion gene, a DNA fragment comprising the 5′-terminal region of sod and its upstream region was PCR-amplified from the chromosomal DNA of P. gingivalis 33277 with primers EX1 and EX2 (Table 2). Primer EX1 can hybridize to the chromosomal DNA 0.92 kb upstream of the initiation codon of sod and generate a BamHI site at one end of the PCR product, while primer EX2 can hybridize to the chromosomal DNA within the sod gene and generate a BamHI site at the other end of the PCR product. The amplified DNA fragment was cloned into pGem-T Easy, sequenced, excised by digestion with BamHI and ligated to BamHI-digested DNA of the lacZ reporter suicide/integration plasmid pKD393 (Ushima et al., 2003). The correct orientation of the insert DNA was confirmed by sequencing. The resulting plasmid pKD398 DNA encoded a recombinant β-galactosidase fusions to the N-terminal 17 amino acids of P. gingivalis Fod. Circular plasmid DNA of pKD398 was then introduced into cells of P. gingivalis 33277 by electroporation to yield KDP151 (sod–lacZ). Moreover, the tetQ DNA cassette of pKD375 was inserted into the BsiWI site within ustA of pKD801 to yield pKD805. KDP151 was transformed to tetracycline resistance by electrotransformation with NotI-linearized pKD805 (usta::tetQ), resulting in KDP305 (haust–lacZ ustA+::tetQ). Correct DNA integration or replacement in KDP151 and KDP305 was confirmed by Southern hybridization.

**DNA probes and Southern blot hybridization.** A DNA fragment (523 bp) comprising the 3′-terminal region of ustA and its downstream region was PCR-amplified from the chromosomal DNA of P. gingivalis 33277 with the oligonucleotide primers SH1 and SH2 (Table 2). The ermF–ermAM DNA cassette (2.1 kb) obtained from pKD355 by BamHI and BglII double digestion was labelled with the AlkPhos Direct system for chemiluminescence (Amersham Pharmacia). Southern blotting was performed by using a nylon membrane and developed with CDP-star detection reagent (Amersham Pharmacia).

**Assays for superoxide dismutase (SOD) and β-galactosidase activities.** P. gingivalis cells were harvested, suspended in 0.1 M phosphate buffer (pH 7.5) containing 0.1 mM TLCK and 0.1 mM leupeptin and sonicated. Extracts were assayed for SOD activity by the cytochrome c-xanthine oxidase method (McCord & Fridovich, 1969). For β-galactosidase assays, P. gingivalis cells were suspended in buffer Z (Miller, 1972) and subjected to the β-galactosidase assays described in Miller (1972). All assays were performed in duplicate and repeated at least three times.

**RESULTS**

**A novel protein upregulated in stationary phase**

Protein expression of cells of P. gingivalis 33277 in exponential- and stationary-phase cultures was investigated with 2D gel electrophoresis. Several protein spots were found to increase or decrease in stationary phase. The present study was focused on a protein with a molecular mass of 9 kDa and an isoelectric point of 4–5, the amount of which was markedly increased in stationary phase (Fig. 1). We named it UstA (upregulated in stationary phase protein A). The N-terminal amino acid sequence of UstA was determined, resulting in the sequence XXTITFNELXR. The sequence was subjected to BLAST search analysis using the P. gingivalis W83 genome database at the TIGR website (http://tigrblast.tigr.org/cmr-blast/), which indicated that the amino acid sequence TITFNLXR was encoded by the genome region between nucleotides 281385 and 281365 (Nelson et al., 2003). This genome region is located between open reading frames (ORFs) PG0246 and PG0248. The nucleotide sequence of this region of the W83 genome revealed that an ORF could extend upstream from the N terminus of PG0246 and that the N terminus of the new ORF, including the whole PG0246 ORF, started with the sequence MKFTTTFNLRR (Fig. 2a, b), suggesting an error in ORF assignment in this region of the W83 genome database. The UstA-encoding gene (usta) region of P. gingivalis 33277 genomic DNA was PCR-amplified using primers CP1 and CP2, which hybridized to regions upstream and downstream of ustA, respectively, and the amplified DNA was sequenced. The nucleotide sequence of the ustA gene of 33277 was the same as that of W83 except that the R74 codons of ustA of strains 33277 and W83 were AGA and AGG, respectively (Fig. 2b). The deduced molecular mass and isoelectric point of UstA were 9194–89 and 4.74, respectively, consistent with those of UstA obtained by the 2D gel analysis.

To determine whether other bacteria have a UstA homologue or not, we performed a BLAST search analysis using the amino acid sequence of UstA. Homologues were found in the genomes of Bacteroides thetaiotaomicron and Bacteroides fragilis (Xu et al., 2003; Tang et al., 1999). The
amino acid sequence of *P. gingivalis* UstA had 70% sequence identity to the homologue from *B. thetaiotaomicron* and 67% identity to that from *B. fragilis* (Fig. 3a). An ORF encoding a putative universal stress protein (Usp) was located immediately downstream of *P. gingivalis* ustA and its homologous genes in *B. thetaiotaomicron* and *B. fragilis* (Figs 2a and 3b). The BatI operon, related to bacteroides aerotolerance, was located immediately

Fig. 1. 2D gel analysis of *P. gingivalis* cell extracts. Crude protein extracts of *P. gingivalis* 33277 cells in exponential phase (a) or in stationary phase (b) were subjected to 2D gel analysis. The arrow in (b) indicates UstA.

Fig. 2. Location and nucleotide sequence of *ustA*. (a) Location of *ustA* on the *P. gingivalis* chromosome. Arrows show the ORF of each gene; PG numbers are ORF numbers of *P. gingivalis* W83 at TIGR. Regions hybridizing to primer oligonucleotides (Table 2) are indicated. (b) Nucleotide sequence and deduced amino acid sequence of the *ustA* gene region of *P. gingivalis* 33277. The bold, underlined A indicates the start site of the *ustA* mRNA. The underlined nucleotide sequence shows the region hybridizing to the 30-mer oligonucleotide PE1 used for primer extension. Putative −35 and −10 regions of the *ustA* promoter, determined according to Jackson et al. (2000) and Bayley et al. (2000), are indicated.
upstream of the ustA homologues in B. thetaiotaomicron and B. fragilis; however, the Batl operon was separated from the ustA locus in the P. gingivalis genome (Xu et al., 2003; Tang et al., 1999; Nelson et al., 2003).

Transcriptional analysis of the ustA locus
To determine the start site and length of the ustA mRNA, we performed primer extension and Northern blot analysis (Fig. 4a, b). The ustA mRNA started with an A residue 49 bases upstream of the initiation codon of ustA and was about 400 bases in length, indicating that ustA might be transcribed in a monocistronic fashion (Figs 2b and 4). To determine whether ustA expression was changed under aerobic conditions, an exponential-phase culture of strain 33277 was exposed to atmospheric oxygen for 20 and 120 min. The amount of ustA mRNA was increased markedly after 20 min exposure to atmospheric oxygen, indicating that ustA might be an oxidative-stress-responsive gene.

Subcellular localization of the UstA protein
To determine the subcellular localization of the UstA protein, we fractionated cells of P. gingivalis 33277 into cytoplasm/periplasm, inner membrane and outer membrane fractions. These fractions were subjected to SDS-PAGE and immunoblot analyses using anti-UstA antiserum. Anti-UstA antiserum reacted with a protein with a molecular mass of 9-0 kDa in the cytoplasm/periplasm fraction, whereas no reaction was seen in the inner membrane or outer membrane fractions (Fig. 5). Since the UstA protein had no signal peptide sequence at the N terminus, UstA is likely to be a cytoplasmic protein.
Construction of ustA and ustA fimA::ustA+ mutants of P. gingivalis

To analyse the biological significance of UstA in P. gingivalis cells, a UstA-deficient mutant was constructed. The ustA gene DNA, disrupted by insertion of the ermF–ermAM DNA cassette, was introduced into cells of P. gingivalis 33277 by electroporation (Fig. 6a). A number of erythromycin-resistant colonies were obtained. Southern blot hybridization and immunoblot analyses revealed the correct construction of the UstA-deficient mutant (Fig. 6b). In addition, we constructed a derivative of the shuttle vector plasmid pYKP028 (Kumagai et al., 2003) containing the wild-type ustA gene for a complementation test and introduced the ustA+ plasmid into ustA-mutant cells; however, we could not obtain a ustA mutant that stably harboured the plasmid, probably because of instability of the shuttle vector in the mutant. Thus, we attempted to introduce wild-type ustA gene DNA into the chromosome of the ustA mutant. A ustA+ complemented strain was constructed by introduction of the wild-type ustA gene into the fimA locus of the ustA mutant. Immunoblot analysis using anti-UstA antiserum revealed that the ustA fimA::ustA+ mutant expressed the UstA protein as well as the wild-type strain 33277 (Fig. 6c).

Growth of the ustA mutant

First, we analysed growth of the ustA mutant in enriched BHI broth under anaerobic conditions. The ustA mutant was found to grow more slowly than the wild-type parent strain in exponential phase and the final yield of the ustA mutant was markedly less than that of the wild-type parent.
(Fig. 7). Growth of the ustA fimA::ustA + strain was almost the same as that of the wild-type parent, indicating that the growth retardation observed in the ustA mutant was caused by loss of UstA.

2D gel electrophoresis analysis of protein expression of the ustA mutant

From the retardation in growth in the ustA mutant, we attempted to compare the protein expression of the ustA mutant with those of the wild-type parent and the ustA fimA::ustA + strain. Proteins were prepared from these strains grown anaerobically in exponential and stationary phases, which were then analysed by 2D gel electrophoresis. In stationary phase, we observed three protein spots of the ustA mutant that were markedly increased compared with those of the wild-type parent and ustA fimA::ustA + strains (Fig. 8). Next, we determined N-terminal amino acid sequences of the three proteins, resulting in MTGE-

Expression of the sod gene of the ustA mutant

Among these proteins, Sod of P. gingivalis has been well characterized (Amano et al., 1990, 1992, 1994; Nakayama, 1990, 1994; Lynch & Kuramitsu, 1999). In our previous studies (Nakayama, 1990, 1994), we cloned the sod gene from P. gingivalis 33277, constructed an sod mutant and found that the sod mutant showed a rapid loss of viability on exposure to atmospheric oxygen. In addition, SOD activity was increased when P. gingivalis cells were exposed to atmospheric oxygen (Nakayama, 1994). We determined the SOD activity of the ustA mutant in exponential or stationary phase with or without exposure to atmospheric oxygen (Table 3). In exponential phase, SOD activities of the ustA mutant under anaerobic and aerobic conditions were 2-7 and 2-9 times higher than those of the wild-type parent, respectively, which was consistent with the result of the Sod protein spots in 2D gel. To confirm that UstA affects expression of the sod gene, we constructed sod +:: lacZ fusion strains and compared the β-galactosidase activity of the sod +:: lacZ ustA mutant with that of the sod +:: lacZ ustA + strain (Fig. 9). β-Galactosidase activity of the sod +:: lacZ ustA mutant was 1-9 and 2-4 times higher than that of the sod +:: lacZ ustA + strain under anaerobic and aerobic (120 min aeration) conditions, respectively.

Sensitivity of the ustA mutant to several peroxides and DNA-damaging agents

Since Sod, Tpx and Trx were increased in the ustA mutant, we attempted to determine the sensitivity of the ustA mutant to several peroxides and DNA-damaging agents (Fig. 10). The oxyR mutant that was used as a control strain showed hypersensitivity to H2O2, t-BOOH, CM-OOH, diamide and metronidazole, whereas the ustA mutant was more resistant to diamide and there were no statistically significant differences in sensitivity to other chemicals between the ustA mutant and the wild-type parent. Interestingly, the ustA oxyR double mutant was more resistant to diamide, metronidazole and mitomycin C than the oxyR single mutant.

DISCUSSION

In bacteria such as Escherichia coli, stationary-phase responses are mainly controlled by a sigma subunit of RNA polymerase, σ38 (RpoS). Many of the more than 50 σ38-controlled genes confer stress tolerance, including xthA, dps, katG, katE, gor and sodC (Loewen et al., 1998; Hengge-Aronis, 2000, 2002). σ38 is very similar to the vegetative sigma subunit σ70 (RpoD) with respect to structure and molecular function (Hengge-Aronis, 2002). According to the gene identification using the P. gingivalis genome sequence database (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spt?database=pgp), the P. gingivalis genome possesses one RpoD homologue (PG0594), one RpoN homologue (PG1105) and six sigma factors belonging to the ECF subfamily. Since the putative molecular mass of the P. gingivalis RpoD homologue is 33 kDa, it appears to be more similar to E. coli RpoS than to E. coli RpoD with respect to molecular size; however, there is no RpoD homologue with a molecular mass of 70 kDa in P. gingivalis. This suggests that stationary-phase responses in P. gingivalis may not be controlled by a special sigma subunit.
Fig. 8. 2D gel analysis of the ustA mutant. Crude protein extracts of *P. gingivalis* 33277 (column 1), KDP301 (column 2) and KDP303 (column 3) cultured under anaerobic or aerobic (120 min exposure to air) conditions were subjected to 2D gel analysis. The areas including the Sod (a), Tpx (b) and Trx (c) protein spots are magnified.
like *E. coli* RpoS. The *P. gingivalis* genome sequence database was constructed using strain W83, which is different from strain 33277 used in this study; however, Chen et al. (2004) have recently reported that the chromosomes of the two strains are very similar, with approximately 93% of the predicted genes in common, suggesting that it may be unlikely that strain 33277 possesses another RpoD/RpoS homologue.

This study demonstrates that a 9 kDa protein of *P. gingivalis*, the gene of which is located upstream of uspA, is upregulated in stationary phase, and we propose to call it UstA. UstA may be involved in oxidative stress responses, since expression of redox proteins such as Sod, Tpx and Trx is increased in the ustA mutant. UstA homologues were found in only the genus *Bacteroides* in addition to the genus *Porphyromonas*, implying that UstA may be present in rather restricted bacterial groups and may regulate oxidative stress responses in response to environmental conditions.

### Table 3. SOD activity

SOD activities are given in U (mg protein)^{-1}.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Exponential phase</th>
<th>Stationary phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anaerobic</td>
<td>Aerobic</td>
</tr>
<tr>
<td></td>
<td>(120 min)</td>
<td></td>
</tr>
<tr>
<td>33277</td>
<td>3.9 ± 0.3</td>
<td>8.3 ± 0.2</td>
</tr>
<tr>
<td>KDP301</td>
<td>25.1 ± 2.7</td>
<td>76.2 ± 3.8</td>
</tr>
<tr>
<td>KDP303</td>
<td>5.7 ± 1.8</td>
<td>13.1 ± 7.9</td>
</tr>
</tbody>
</table>

![Time-course of induction of β-galactosidase activity in sod-lacZ fusion strains.](image)

*Fig. 9.* Time-course of induction of β-galactosidase activity in sod-lacZ fusion strains. *P. gingivalis* KDP151 (sod-lacZ) (●) and KDP305 (sod-lacZ ustA) (○) were grown anaerobically in enriched BHI broth at 37 °C. At an *A*_{600} of 0.3, the cultures were shifted to aerobic conditions. Samples were withdrawn after the indicated times and the activity of β-galactosidase was determined by the method of Miller (1972). Calculations were performed by using established formulae and enzyme activities are expressed in Miller units (Miller, 1972).

![Sensitivity of *P. gingivalis* cells to peroxides and DNA-damaging agents.](image)

*Fig. 10.* Sensitivity of *P. gingivalis* cells to peroxides and DNA-damaging agents. (a) Sensitivity of *P. gingivalis* cells to H_{2}O_{2}, t-BOOH, CM-OOH, metronidazole and mitomycin C. (b) Sensitivity of *P. gingivalis* cells to diamide. *P. gingivalis* 33277 (wild-type), KDP143 (oxyR::tet), KDP301 (ustA::erm), KDP303 (ustA::erm fimA::tet ustA^{+}) and KDP306 (ustA::erm oxyR::tet) were grown anaerobically in enriched BHI broth for 48 h. The cells were spread on enriched TS agar plates and a paper disc containing H_{2}O_{2}, t-BOOH, CM-OOH, diamide, metronidazole or mitomycin C was placed at the centre of the plate, followed by anaerobic incubation at 37 °C for 4 days. The diameters of the clear zones next to the discs were measured (in mm). The data shown are means ± SD of triplicate experiments. MET, Metronidazole; MMC, mitomycin C. *, P < 0.05; **, P < 0.01.
In these bacterial species, *P. gingivalis*, *B. fragilis* and *B. thetaiotaomicron*, the *ustA* gene is located downstream of *ustA* in the same orientation (Nelson et al., 2003; Tang et al., 1999; Xu et al., 2003). The production of Usp in various bacteria was found to be stimulated by a large variety of conditions, such as stationary phase, starvation of carbon, nitrogen, phosphate, sulphate and amino acids and exposure to heat, oxidants, metals, uncouplers, polymyxin, cycloserine, ethanol, antibiotics and other stimulants (Gustavsson et al., 2002; Kvint et al., 2003). Primer extension and Northern blot hybridization analyses revealed that *ustA* was transcribed in a monocistronic fashion. We have not found so far that expression of these two genes is coordinately regulated; however, they may be related to each other with respect to stress responses. Further studies are contemplated to explore the interaction of UstA with Usp.

The *ustA* mutant was more resistant to diamide than the wild-type parent strain, which can be explained by overexpression of Tpx and Trx in the *ustA* mutant, since Tpx interacting with Trx functions as a thiol-specific antioxidant (Cha et al., 1995, 1996). In contrast, sensitivities of the *ustA* single mutant to H$_2$O$_2$, t-BOOH, CM-OOH, mito-ustA were also seen in sensitivity to diamide, oxidants, metals, uncouplers, polymyxin, metronidazole, and suppression of these two genes is co-

The suppressive effects of the *ustA* mutation might be partially explained by the increase of Sod, Tpx or Trx in the *ustA* mutant, but participation of other redox proteins or DNA-repair enzymes might not be ruled out.

2D gel analysis revealed that expression of Sod, Tpx and Trx was increased in the *ustA* mutant. Using the *sod*-*lacZ* protein fusion strain, we found that the β-galactosidase activity in the *ustA* mutant was about twice that of the wild-type under anaerobic conditions and was 2-4-fold higher 2 h after exposure to atmospheric oxygen, consistent with the result that the SOD activity of the *ustA* mutant was higher than those of the wild-type parent and the *ustA* complemented strain; however, the increase of β-galactosidase activity cannot account for the whole increase in SOD activity. This apparent difference might be explained by possible instability of the chimeric β-galactosidase encoded by the *sod*-*lacZ* protein fusion gene and/or duplication of the *sod* promoter DNA region in the *sod*-*lacZ* protein fusion strain. The mechanisms of effects of UstA on expression of Tpx and Trx also remain to be determined.

In conclusion, we found a novel 9 kDa protein named UstA in *P. gingivalis* that was induced in stationary phase or oxidative conditions. Sod, Tpx and Trx were upregulated in the *ustA* insertional mutant. The *ustA* mutation conferred resistance to diamide on *P. gingivalis* wild-type cells and suppressed hypersensitivities of the *oxyR* mutant to diamide, metronidazole and mitomycin C. These results suggest that UstA plays a significant role in oxidative stress responses in *P. gingivalis*.

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