

The OxyR homologue in *Tannerella forsythia* regulates expression of oxidative stress responses and biofilm formation

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Tannerella forsythia is an anaerobic periodontal pathogen that encounters constant oxidative stress in the human oral cavity due to exposure to air and reactive oxidative species from coexisting dental plaque bacteria as well as leukocytes. In this study, we sought to characterize a *T. forsythia* ORF with close similarity to bacterial oxidative stress response sensor protein OxyR. To analyse the role of this OxyR homologue, a gene deletion mutant was constructed and characterized. Aerotolerance, survival after hydrogen peroxide challenge and transcription levels of known bacterial antioxidant genes were then determined. Since an association between oxidative stress and biofilm formation has been observed in bacterial systems, we also investigated the role of the OxyR protein in biofilm development by *T. forsythia*. Our results showed that aerotolerance, sensitivity to peroxide challenge and the expression of oxidative stress response genes were significantly reduced in the mutant as compared with the wild-type strain. Moreover, the results of biofilm analyses showed that, as compared with the wild-type strain, the *oxyR* mutant showed significantly less autoaggregation and a reduced ability to form mixed biofilms with *Fusobacterium nucleatum*. In conclusion, a gene annotated in the *T. forsythia* genome as an *oxyR* homologue was characterized. Our studies showed that the *oxyR* homologue in *T. forsythia* constitutively activates antioxidant genes involved in resistance to peroxides as well as oxygen stress (aerotolerance). In addition, the *oxyR* deletion attenuates biofilm formation in *T. forsythia*.

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INTRODUCTION

Tannerella forsythia is recognized as one of the microbial pathogens implicated in the development of periodontal disease, a bacterially induced inflammation of tooth-supporting tissue and bone (Grossi *et al.*, 1994, 1995; Tanner *et al.*, 1998; Tran *et al.*, 2001). *T. forsythia* with *Porphyromonas gingivalis* and *Treponema denticola*, known as the red-complex, in dental plaque biofilms, are associated with an increased risk of periodontitis development (Socransky *et al.*, 1998). *T. forsythia* is an obligate anaerobe that is expected to encounter constant oxidative stress in the oral cavity due to reactive oxidative species produced by other bacteria (mainly oral streptococci) as well as host leukocytes (primarily neutrophils and macrophages). Therefore, to colonize, form biofilms and survive in the oral cavity, the ability of the bacteria to tolerate oxygen and peroxides is critical. Currently, knowledge of the oxidative stress defence mechanisms in *T. forsythia* is lacking. In this regard, while aerobes such as *Escherichia coli*

contain two independent regulatory systems dependent on the SoxR/SoxS and the OxyR systems for responding to superoxide and peroxide challenges, respectively (Zheng & Storz, 2000), obligate anaerobes such as *P. gingivalis* express a single OxyR protein to respond to both types of challenge, from peroxide or air (Diaz *et al.*, 2006).

OxyR is a redox-sensitive protein in the LysR family of DNA-binding transcription modulators, and regulates the expression of genes important in defence against oxidative stress in Gram-negative organisms (Zheng & Storz, 2000). The genome of *T. forsythia* (available at <http://www.oralgen.lanl.gov/> under the previously designated organism name *T. forsythensis*) does not contain genes encoding homologues of the *soxR/soxS* system. However, putative genes encoding an OxyR homologue and enzymes such as SodF (superoxide dismutase), Dps (a non-specific DNA-binding protein), rubrerythrin and AhpC (alkylhydroperoxide reductase subunit C) involved in antioxidant responses have been identified in the *T. forsythia* genome. The expression of these antioxidant response genes has been shown to be OxyR-dependent in the Gram-negative

Abbreviations: CSLM, confocal scanning laser microscopy; qRT-PCR, real-time quantitative RT-PCR; rOxyR, recombinant OxyR.

anaerobe *P. gingivalis* (Amano *et al.*, 1990; Diaz *et al.*, 2004; Ohara *et al.*, 2006). Furthermore, a catalase gene homologue is absent in *T. forsythia*.

Recent studies have also implicated the oxidative stress response sensor OxyR in biofilm development in microbes including *E. coli* (Reisner *et al.*, 2003), *Haemophilus influenzae* (Murphy *et al.*, 2005), *Pseudomonas aeruginosa* (Sauer *et al.*, 2002), *Neisseria gonorrhoeae* (Seib *et al.*, 2007), *Campylobacter jejuni* (Sampathkumar *et al.*, 2006), *Streptococcus mutans* (Wen *et al.*, 2005) and *Candida albicans* (Murillo *et al.*, 2005). For example, in *E. coli*, OxyR activation promotes biofilm formation by inducing Ag43 adhesin expression (Danese *et al.*, 2000), and in *Serratia marcescens*, biofilm formation is severely impaired in *oxyR*-defective mutants (Shanks *et al.*, 2007). While the mechanisms that relate oxidative stress defences and biofilm formation are poorly understood at present, it is thought that the upregulation of oxidative stress-induced biofilm formation in the host may provide bacteria with a defence strategy. In addition to the role of OxyR in defence against oxidative stress and biofilm formation, this protein has been reported to regulate virulence factor expression in human pathogenic species such as *Ps. aeruginosa* (Lau *et al.*, 2005), *Ser. marcescens* (Shanks *et al.*, 2007), *E. coli* (Johnson *et al.*, 2006), *N. gonorrhoeae* (Seib *et al.*, 2007) and *P. gingivalis* (Wu *et al.*, 2008).

In this study we evaluated the role of an OxyR homologue in *T. forsythia* with respect to its role in defence against oxidative stress and in biofilm formation.

METHODS

Bacterial strains and culture conditions. *T. forsythia* strains were grown anaerobically (5% CO₂, 10% H₂, 85% N₂) in BF broth or on BF agar plates (Honma *et al.*, 2007) with or without appropriate antibiotics. *P. gingivalis* strains were grown anaerobically (85% N₂, 10% H₂, 5% CO₂) at 37 °C in Trypticase soy broth (TSB) or on TSB blood agar plates, supplemented with yeast extract (1 mg ml⁻¹), haemin (5 µg ml⁻¹) and menadione (1 µg ml⁻¹). *Fusobacterium nucleatum* ATCC 25586 was also maintained anaerobically in TSB or on agar plates (Becton Dickinson). *E. coli* strains were grown in Luria-Bertani (LB) medium aerobically at 37 °C. *E. coli* strain DH5 α (Invitrogen) was used as a host for cloning and plasmid purification. *E. coli* strain BL21 (DE3) (EMD Bioscience) was used as a host for expression and purification of the His-tagged OxyR recombinant protein.

Construction of the isogenic *T. forsythia oxyR* homologue inactivated mutant (TFM104). *T. forsythia* gene sequences were retrieved from the Oral Pathogen Sequence Database (Oralgen), Los Alamos National Laboratory, Los Alamos, NM, USA, under the previous name of the organism, *T. forsythensis* (<http://www.oralgen.lanl.gov/>), and gene designations correspond to identification (ID) numbers deposited in the database. An *oxyR*-inactivated insertional mutant was constructed by a previously described allelic replacement strategy (Honma *et al.*, 2007). For this purpose, a DNA fragment containing the *ermF* gene flanked by upstream and downstream DNA regions of TF0104 (*oxyR* homologue) was electroporated into *T. forsythia* ATCC 43037 cells, and transformants were selected on agar-erythromycin plates. The sequences of oligonucleotides used in this

study are shown in Table 1. First, a DNA fragment containing TF0104 with flanking sequences was amplified by PCR using primers #1 and #2 from *T. forsythia* 43037 genomic DNA. This PCR product was then used as template to amplify the upstream (1784 bp) and downstream (1125 bp) fragments of TF0104 with primer sets #1 and #3 and #2 and #5, respectively. The *ermF* fragment (797 bp) was amplified from pVA2198 (Fletcher *et al.*, 1995) with primers #4 and #6. Primers #3, #4, #5 and #6 contained overlap sequences for *ermF* and TF0104 to allow generation of fusion fragments by a PCR overlap strategy. A fusion DNA fragment (3708 bp) containing *ermF* flanked by TF0104 DNA sequences was generated by an overlap PCR strategy using primers #1 and #2 (Horton *et al.*, 1993). Gel-purified PCR fragments of TF0104 flanking regions (1784 and 1125 bp) and *ermF* (797 bp) served as template for overlap PCR. The fusion product was transformed into *T. forsythia* 43037 by electroporation, as previously described (Honma *et al.*, 2007). Transformants were plated onto BF agar plates containing 5 µg erythromycin ml⁻¹ and incubated anaerobically at 37 °C for 14 days. Following incubation, 19 erythromycin-resistant colonies were isolated, which were then screened by PCR and Southern blotting. One of the transformants, named TFM104, which was confirmed to have an *oxyR* deletion, was used for further analyses.

Response of the *T. forsythia oxyR* mutant to oxidative stress. In order to estimate the sensitivity of *T. forsythia* strains to hydrogen peroxide (H₂O₂) and air, the following experiments were performed. H₂O₂ sensitivity was estimated by a modification of a previously described protocol (Chen *et al.*, 2006). Briefly, *T. forsythia* strains were grown to mid-exponential phase (OD₆₀₀ 0.5) in BF broth and harvested by centrifugation, washed twice with PBS and adjusted to OD₆₀₀ 1.0. *T. forsythia* strains were then exposed anaerobically to a twofold diluted series of H₂O₂ (0 µM to 10 mM) for 20 min at 37 °C. The treated bacterial cells were recovered and inoculated into BF broth. Bacterial cell survivability was expressed as OD₆₀₀ values of inocula after 48 h incubation.

The aerotolerance assays were carried out as follows. *T. forsythia* strains were grown to mid-exponential phase (OD₆₀₀ 0.5) in BF broth, harvested by centrifugation, and adjusted to OD₆₀₀ 1.0 after washing twice with PBS. Adjusted *T. forsythia* strains were diluted 1 to 10 with BF broth, and exposed to air for different time periods with shaking at room temperature. Following aerobic incubation, *T. forsythia* cells were harvested by centrifugation, resuspended in BF broth, and incubated anaerobically at 37 °C. Aerotolerance of *T. forsythia* strains was expressed as OD₆₀₀ values of inocula after 48 h incubation. Both H₂O₂ survival and the aerotolerance assay were repeated three times.

Estimation of mRNA expression of OxyR-regulated genes. The relative expression of a select group of genes known to be regulated by the OxyR regulator in bacteria was evaluated by real-time quantitative RT-PCR (qRT-PCR). Briefly, total RNA was prepared from bacterial cells harvested from 2 ml cultures (OD₆₀₀ 0.4) for each wild-type and mutant strain, using the RiboPure Total RNA isolation kit with DNase I treatment (Ambion). In some experiments, catalase (5 µg ml⁻¹; Sigma Aldrich) was added to BF broth 4 h before inoculation to remove any oxidative factors present in broth, as suggested by Diaz *et al.* (2006). Total RNA samples were isolated from *T. forsythia* strains, which were exposed to 0.1 mM H₂O₂ for 30 min. The cDNA was synthesized by utilizing SuperScript III reverse transcriptase (Invitrogen) with random hexamers (Promega) according to the manufacturers' recommendations. Real-time PCR was performed on cDNA samples after adjusting the A₂₆₀ to 0.1 with gene-specific primers (Table 1) using iQ SYBR green Supermix (Bio-Rad) and an iCycler thermal cycler equipped with the MyiQ real-time PCR detection system as per the manufacturer's recommendations. The 16S rRNA levels were used for normalization and the relative expression levels of each transcript were then calculated using the

Table 1. Primers used in this study

Primer	Use of primer and remarks	Sequence (5'–3')
Mutant construction		
#1	PCR primer for <i>oxyR</i>	ACCAATCAATGTCGAGGAACG
#2	PCR primer for <i>oxyR</i>	GGATACACTTTACTACGGCAACTCA
#3	PCR overlap primer (<i>ermF</i> : lower case type)	gtcctgaaaaatttcacctctgTGAAGCAACGCTGTATTTTATACTTG
#4	PCR overlap primer (<i>ermF</i> : lower case type)	CAAGTATAAAATACAGCGTTGCTTCaagaaggatgaattttcaggagc
#5	PCR overlap primer (<i>ermF</i> : lower case type)	cgggcaattctttttgtcatCTCTGTACCATTTATTGATAAAATTGATGC
#6	PCR overlap primer (<i>ermF</i> : lower case type)	GCATCAATTTTATCAATAAATGGTACAGAGatgacaaaaaagaattgcccg
qRT-PCR		
Tf 16S-F	16S rRNA	GGGTGAGTAACGCGTATGTAACCT
Tf 16S-R		ACCCATCCGCAACCAATAAA
dps-F	Tf0105; Dps family DNA-binding protein	CAACCGTCAAGTGTATTATGC
dps-R		TCAGATACTCCGAGAAGCG
bcp-F	TF0245; bacterioferritin comigratory protein	GTATTTCTATCCCAAAGACAACAC
bcp-R		CAGGGTTCGTATCCGTATCG
trx-F	TF2739; thioredoxin	CGGAGTGGAAAGTATGAAGG
trx-R		AGCGTTGGAAGACTGTTG
ahpC	TF0383; alkyl hydroperoxide reductase subunit C	CGTATCTTTGTGTCGAAATTG
ahpC-R		GGAATTTGCGAATTAATAATCGG
trxB-F	TF0375; thioredoxin reductase	CGAAAAAGTGCCTGTTTGA
trxB-R		ACGCTGCTTTTTCATGTCT
prx-F	TF1518; peroxiredoxin	GGTGGTTGGTGCCTTATTG
prx-R		TCGTTGTTGAGGTCGGAGAG
trxA-F	TF2761; thioredoxin	TCGGAATGGAAATACTTAGGTGAC
trxA-R		ACGGGATGGAACGGATGC
trxM-F	TF2732; thioredoxin M	GGCATTAAACAGTAACAGAC
trxM-R		GTATTCGAAACCACCTC
fur-F	TF2532; ferric-uptake regulator	ATCCGTCATCAATTCACC
fur-R		ATCCCGTAAACATAGAGC
sod-F	TF2927; <i>sodF</i> , Fe-Mn	GCCAGCAGACAATTGAATATCA
sod-R		TTCGGCAAGTTTGCCCTTA
oxy-F	TF0104; OxyR homologue	ACCGGCATTTTGCCAAAG
oxy-R		ATTGATATACGACCCCTCGC
rub-F	TF0103; rubredoxin	TGTGATTACATTTACGATCCGGTA
rub-R		CGACCGGTTCAAAATCATCT
rOxyR		
TF104F	<i>NdeI</i> site underlined	CGCCATATGACGATTTCAGCAGTTAGAATATATTATTGC
TF104R	<i>BamHI</i> site underlined	GCGCGGATCCCTAGAGGTCTACCTTAATCGTATGACG
Mobility shift assay		
Tf-sod-F	PCR; <i>sodF</i> promoter fragment	TCACTTGAAGTCATGCAAATCC
Tf-sod-R		AGGGAAGCTTTGGTGTTC
Tf-dps-F	PCR; <i>dps</i> promoter fragment	GACAAGCTTCTGCCGCTTT
Tf-dps-R		TGTACGGCACTGTTCACTTCTT
Shuttle vector construction		
oxyRCow-Bam	<i>BamHI</i> site underlined	CGCGGATCCGCGTCTCGCGCAAGGTCTTCA
oxyRCow-Sal	<i>SalI</i> site underlined	ACGCGTGCAGTCATATGTCAGCTGTTCTTTATATCAGGG

– $\Delta\Delta C_t$ method described for the MyIQ real-time PCR detection system (Bio-Rad).

Expression and purification of recombinant OxyR. The putative OxyR-encoding ORF TF0104 was PCR-amplified from genomic DNA using primers TF104F (engineered *NdeI* site) and TF104R (engineered *BamHI* site). The PCR fragment was digested with *NdeI* and *BamHI* restriction enzymes and cloned into the pET15b expression vector at the same sites. The recombinant plasmid clone with the correct in-frame insertion sequence, designated pET15-OxyR, allowing expres-

sion of OxyR as a fusion protein with a C-terminal polyhistidine tag, was transformed into *E. coli* BL21 (DE3). A clone transformed with pET15-OxyR was grown in LB medium containing 50 μg ampicillin ml^{-1} at 37 °C. When the OD_{600} reached 0.4, IPTG was added to 0.4 mM final concentration and cultures were incubated for an additional 4 h with vigorous shaking. The cells were harvested by centrifugation at 5000 g for 30 min, suspended in PBS and subjected to cell disruption with ultrasonication. The cell lysates were centrifuged at 10 000 g for 30 min to separate the soluble fraction from the insoluble fraction containing bacterial inclusion bodies. The

insoluble fraction was found to be enriched in recombinant OxyR protein, as judged by SDS-PAGE analysis. The recombinant protein present in inclusion bodies was then solubilized in 8 M urea and affinity-purified on a PrepEase Ni-TED Column (USB Biochemicals) under denaturing conditions according to the manufacturer's instructions. The eluted recombinant OxyR (rOxyR) protein was dialysed against a series of buffers with stepwise decreases in the urea concentration to allow proper protein folding [10 mM Tris/HCl (pH 7.6) and 1 % (v/v) glycerol, plus urea at 6, 4, 2 and 1 M]. Finally, the protein solution was dialysed against the storage buffer containing 10 mM Tris/HCl (pH 7.6), 1 % (v/v) glycerol, 100 mM KCl, 10 mM MgCl₂ and 1 mM EDTA.

Electrophoretic mobility shift assay. DNA probes containing the promoter regions of the *sodF* (TF2927) and *dps* (TF0105) genes were PCR-amplified from genomic DNA with primer sets Tf-sod-F and Tf-sod-R, and Tf-dps-F and Tf-dps-R, respectively; this corresponds to nucleotides -132 to +79 (211 bp fragment) and -201 to +47 (248 bp fragment) relative to the start codons of the *sodF* and *dps* genes, respectively. The DNA probes were end-labelled with [γ -³²P]ATP (Perkin-Elmer) by T4 polynucleotide kinase (Promega) and purified with a QIAquick PCR purification kit (Qiagen). The standard protein-DNA binding reaction mixture contained 1 μ Ci (37 kBq; 1 ng) probe DNA and 4 μ g purified rOxyR in binding buffer [10 mM Tris/HCl (pH 7.0), 50 mM KCl, 1 mM DTT, 100 μ g BSA ml⁻¹, 100 μ g poly-(di-dC) ml⁻¹ and 5 % (v/v) glycerol]. For competitive assays, 50-fold unlabelled DNA fragments were used. After incubation for 20 min at room temperature, protein-DNA complexes were separated from the unbound probe on 5 % native polyacrylamide gels in running buffer [22 mM Tris base, 22 mM boric acid and 0.5 mM EDTA (pH 8.0)] at 220 V for 2 h. The gels were then dried and analysed by autoradiography.

TF0104 complementation in a *P. gingivalis oxyR* mutant.

Complementation of the TFM0104 mutant with *oxyR* was not possible due to the lack of a genetic complementation system for *T. forsythia*. Thus, we attempted heterocomplementation in another obligate oral anaerobe, *P. gingivalis*. For this purpose, we complemented a *P. gingivalis oxyR* deletion mutant PgOxyRE (gift from Hua Xie, Meharry Medical College) (Wu *et al.*, 2008) with the *T. forsythia* TF0104 gene. To construct this complemented mutant, a DNA fragment containing TF0104 with up- and downstream flanking regions was amplified with primers oxyRCow-Bam and oxyRCow-Sal containing restriction sites *Bam*HI and *Sal*I, respectively (Table 1). The PCR fragment was digested with *Bam*HI and *Sal*I and cloned into the *Bacteroides/E. coli* shuttle vector pT-Cow (Gardner *et al.*, 1996) at the *Bam*HI and *Sal*I sites located within the *tetC* gene. Purified recombinant plasmid with correct restriction profile and insertion sequences was transferred into a conjugal donor strain *E. coli* pRK231. This was followed by conjugation of *E. coli* pRK231 harbouring the recombinant plasmid with PgOxyRE (Gen⁺, Em⁺) overnight, anaerobically as described previously (Honma *et al.*, 2001). Transconjugants were selected on BF agar plates supplemented with gentamicin (100 μ g ml⁻¹), erythromycin (5 μ g ml⁻¹) and tetracycline (1 μ g ml⁻¹) to select for the plasmid-resident *tetQ* gene. Transconjugants appeared after 10 days incubation and were confirmed by PCR and Southern blot analysis. The recombinant insert from plasmid DNA isolated from the complemented strain was sequenced with primers oxy-F and oxy-R and shown to be 100 % identical to wild-type *T. forsythia oxyR*.

Biofilm formation and aggregation assay. In order to investigate the role of OxyR in *T. forsythia* biofilm formation, the effect of *oxyR* deletion on the development of single and multi-species biofilms was determined by performing biofilm assays as described previously (Honma *et al.*, 2007). Briefly, bacterial cultures of *T. forsythia* and *F. nucleatum* were grown in half-strength growth medium to OD₆₀₀

0.05. Cells were then dispensed (0.5 ml per well) in triplicate wells of 24-well culture plates and incubated anaerobically. For mixed biofilms, each bacterial culture was adjusted to OD₆₀₀ 0.05, dispensed into the wells and incubated as above. After incubation for 2 days, planktonic cells were aspirated and the wells were washed three times with PBS followed by staining of biofilms with crystal violet (0.1 % crystal violet for 15 min). Bound dye was solubilized in 50 % acetic acid and A₅₉₅ was read. Total biofilm was calculated by normalizing dye binding (A₅₉₅) to total bacterial growth (biofilm cells + planktonic cells) determined in parallel identical wells by measuring A₅₉₅.

The aggregation abilities of *T. forsythia* strains with or without *F. nucleatum* were evaluated by a modification of the protocol of Levesque *et al.* (2003). Briefly, bacterial cells were harvested at late-exponential phase, washed with coaggregation buffer [1 mM Tris (pH 7.5), 150 mM NaCl, 0.1 mM CaCl₂ and 0.1 mM MgCl₂] and adjusted to OD₆₆₀ 1.0. To quantify the time-course of coaggregation, OD₆₆₀ readings were taken at 0, 15, 30, 60, 90 and 120 min and the percentage of coaggregation calculated using the following equation: 100 - [(OD₆₆₀ at each time point/OD₆₆₀ at 0 min) × 100].

Confocal scanning laser microscopy (CSLM) analysis. For structural analysis of the biofilms and to obtain a better understanding of the bacterial interactions, CSLM was performed with a Bio-Rad MRC1024 confocal scanning laser (Kr/Ar) microscope attached to a Nikon Diaphot microscope and an APO Plan × 601.4 numerical aperture objective available in the Confocal Microscopy and 3-D Imaging Facility, School of Medicine and Biomedical Sciences, University at Buffalo, as previously described (Honma *et al.*, 2007; Sharma *et al.*, 2005). Briefly, biofilms were formed in two-chamber polystyrene tissue-culture vessels (BD Falcon) as described above and stained with the Bacterial Live-Dead viability kit (Molecular Probes) according to the manufacturer's recommendations. The staining allowed differentiation of live (green) and dead (red) bacteria and identification of *F. nucleatum* and *T. forsythia* due to their distinct bacterial morphology (*F. nucleatum* as long rods vs small fusiform *T. forsythia* cells).

Statistical analysis. Strain comparisons for biofilm formation, aggregation and gene expression in real-time PCR were analysed using Student's *t* test.

RESULTS

OxyR homologue TF0104 contributes to survivability against air and peroxides

OxyR is a redox-sensitive protein regulator that belongs to the LysR family of DNA-binding transcription modulators. An OxyR homologue has been identified as ORF TF0104 in the *T. forsythia* genome deposited at the Oragen database (<http://www.oralgen.lanl.gov/> under *T. forsythensis*). The TF0104-encoded OxyR homologue shows 60 % similarity (42 % identity) with the redox-sensitive transcriptional activator of *Bacteroides thetaiotaomicron* VPI-5482, 60 % similarity (40 % identity) with the redox-sensitive transcriptional activator OxyR of *Bacteroides fragilis* NCTC 9343, 58 % similarity (37 % identity) with the redox-sensitive transcriptional activator OxyR of *P. gingivalis* and 54 % similarity (28 % identity) with the OxyR protein of *E. coli*. The cysteine residues at positions 199 and 208, present in the prototype *E. coli* OxyR protein and important for redox activity, are conserved in the TF0104-encoded OxyR homologue. To confirm the identity and role of OxyR-like

homologue TF0104 in *T. forsythia*, a TF0104-defective mutant was constructed and characterized. The chromosomal location of the *ermF* ORF was confirmed by Southern blotting as well as by sequencing of the chromosomal region at the integration site by DNA sequencing of PCR-generated fragments (data not shown). The double-crossover allelic exchange strategy utilized in this study resulted in replacement of the TF0104 sequence from the translational start to stop codons with that of the *ermF* gene. Therefore, *ermF* is expressed from the *oxyR* promoter and the upstream and downstream regions (putative transcriptional terminators) are kept intact, eliminating the risk of polar effects in the mutant. Furthermore, the *oxyR* gene in *T. forsythia* appears to be a monocistronic transcription unit. It is flanked upstream by the *dps* gene in an opposite transcriptional orientation. The intergenic region between the *dps* and *oxyR* genes is 129 nt long and the sequence analysis of this region in the mutant showed that this region was not affected by the *ermF* insertion (data not shown). Similarly, downstream from *oxyR* is a putative rubredoxin-encoding gene with a 277 nt long intergenic region, which was also confirmed by sequence analysis to be intact in the mutant. Thus, taken together, in all probability the insertion of the *erm* cassette did not cause any polar effects on flanking genes. One such mutant with correct integration was named TFM104 and was further characterized. Mutant TFM104 was compared to the parental strain with respect to the functions of OxyR in oxidative stress defence responses. The results of the H₂O₂ sensitivity assay showed that the maximum H₂O₂ concentration for growth of the mutant TFM104 was 0.25 mM as compared to 1 mM for *T. forsythia* wild-type (Fig. 1a). Moreover, while both *T. forsythia* 43037 and TFM104 showed sensitivity, TFM104 was significantly more sensitive to aerobic stress than 43037 after 2 h incubation (Fig. 1b). These results suggested that TF0104 plays an important role in resistance to oxidative stress.

Expression of oxidative response genes in *T. forsythia* is OxyR-dependent but does not increase in response to H₂O₂

The OxyR protein plays an important role in the expression of antioxidant-related genes in the majority of bacteria examined, including the oral Gram-negative obligate anaerobe *P. gingivalis* (Diaz *et al.*, 2006). According to a DNA microarray analysis, expression of the antioxidant response genes encoding alkyl hydroperoxide reductase C subunit (*ahpC*), F subunit (*ahpF*), Dps family protein (*dps*), superoxide dismutase (*sod*) and ferritin is dependent upon the expression of OxyR in *P. gingivalis* (Diaz *et al.*, 2006). In this study we analysed and compared the expression of *T. forsythia* homologue antioxidant-related genes (Table 2) between *T. forsythia* 43037 and *oxyR*-inactivated mutant TFM104 by qRT-PCR. The mRNA expression levels of *T. forsythia* antioxidant-related genes were downregulated in the *oxyR* mutant TFM104 as compared to the parental strain.

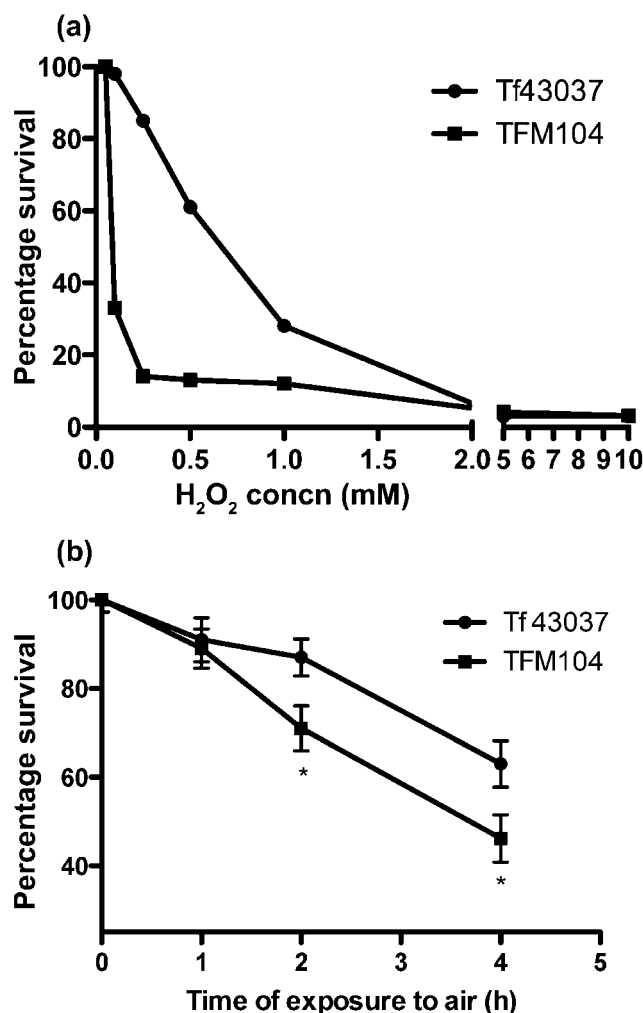


Fig. 1. Sensitivity to oxidative stress. (a) Resistance to H₂O₂ was determined by exposing mid-exponential phase cells to serial dilutions of H₂O₂ anaerobically for 20 min. Percentage survival for each peroxide concentration was then calculated following normalization with untreated controls under similar conditions (b). To determine aerotolerance, cells harvested at the mid-exponential phase were exposed to air for the indicated times. Percentage survival was then determined for each time point normalized to the initial time 0, as described in the text; **P*<0.05.

The mRNA expression levels of the above-mentioned antioxidant genes were also estimated under H₂O₂-stimulated conditions. The expression levels of these antioxidant genes did not change significantly in response to H₂O₂ treatment; no significant fold-changes (*P*>0.05) in the expression levels of the genes were observed when comparison was made between peroxide-treated and untreated wild-type cells under anaerobic growth by qRT-PCR, as described above. The *dps* gene has been shown to play a role in the protection of *E. coli* against peroxide stress, and its expression is regulated by OxyR (Altuvia *et al.*, 1994). Dps is also significantly upregulated

Table 2. Genes with decreased levels of expression in the *oxyR* mutant TFM104 compared with *T. forsythia* 43037 wild-type (during anaerobic growth)

Gene*	Description	Fold decrease†	P value
TF0105 <i>dps</i>	DNA-binding stress protein, Dps family	21.15	0.013
TF0245 <i>bcp</i>	Bacterioferritin comigratory protein	7.28	0.00010
TF2739 <i>trx</i>	Thioredoxin	4.90	0.00001
TF0383 <i>ahpC</i>	Alkyl hydroperoxide reductase, subunit C	4.53	0.00056
TF2927 <i>sodF/sodB</i>	Superoxide dismutase, Fe-Mn	4.46	0.00353
TF0103	Rubredoxin	2.81	0.03356
TF0375 <i>trxB/ahpF</i>	Thioredoxin reductase	2.75	0.00550
TF1518 <i>prx</i>	Peroxiredoxin	2.53	0.00132
TF2761 <i>trxA</i>	Thioredoxin	2.36	0.00781
TF2732	Thioredoxin M	2.29	0.00187
TF2532 <i>fur</i>	Ferric-uptake regulator	2.08	0.00068

*Gene identification corresponds to that of the Oragen database.

†Only genes exhibiting a greater than twofold decrease in transcription are shown.

several fold in *E. coli* and *B. fragilis* after H₂O₂ exposure compared with untreated cultures (Rocha & Smith, 1997). On the other hand, H₂O₂ does not have any significant effect on the expression of *dps* in the anaerobe *P. gingivalis* (Diaz *et al.*, 2006). Since the *dps* transcript is detected in wild-type cultures grown anaerobically without H₂O₂ treatment, and it does not increase after treatment with H₂O₂, it is assumed that OxyR is in a constitutively active state in *P. gingivalis*. Our findings also suggest that *T. forsythia* OxyR is at its maximum level of activation during anaerobic growth, as addition of peroxide had no effect on the expression of *oxyR*-regulated genes. To rule out the possibility that peroxides are present in broth and constitutively keep OxyR in an active state, *T. forsythia* was grown in BF broth pretreated with catalase, as described by Diaz *et al.* (2006). Under these conditions, we did not find any changes in the expression of antioxidant genes before and after H₂O₂ treatment, similar to what was observed with non-catalase-treated broth (data not shown). However, the fact that under anaerobic growth the expression levels of *dps*, *ahpC* and *sodF* were significantly reduced in the *oxyR* mutant as compared with levels in the wild-type strain suggests that OxyR is necessary for the expression of these antioxidant genes under anaerobic growth.

rOxyR binds *dps* and *sodF* promoter regions

We next sought to determine whether OxyR binds to the *T. forsythia* *dps* and *sodF* genes, which are regulated by OxyR, utilizing gel mobility shift assays. Our results showed that purified 6His-tagged rOxyR protein encoded by TF0104 at a concentration of at least 10 nM caused a mobility shift of DNA fragments containing these target promoters (Fig. 2a). Weaker than expected shifts due to rOxyR binding to respective promoters were probably due to the following

reasons. The rOxyR protein utilized for gel shift assays was obtained following renaturation of urea-solubilized protein by stepwise dialysis; this probably resulted in the recovery of only a fraction of the total protein in a conformationally active state. Furthermore, the promoter regions of antioxidant genes in *T. forsythia*, including *dps* and *sodF*, indicated the presence of putative OxyR-binding sequences when compared with *P. gingivalis* OxyR-regulated promoters as well as with consensus OxyR-binding sequences found in *E. coli* (Fig. 2c).

Restoration of OxyR function by heterocomplementation with the putative *T. forsythia* *oxyR*

Currently, genetic systems for gene complementation in *T. forsythia* are unavailable. Therefore, we attempted genetic complementation of a *P. gingivalis* *oxyR* deletion mutant (PgOxyRE) with the TF0104 gene carried on a shuttle plasmid. By utilizing this strategy, a heterocomplemented strain designated Pg0270EC was obtained, which was then compared to PgOxyRE in aerotolerance assays. The results (Fig. 3) showed that complementation with TF0104 significantly restored the loss of OxyR function in the *P. gingivalis* *oxyR* mutant; the TF0104-complemented strain survived significantly better under oxygen stress than the *oxyR* deletion mutant or the pT-Cow-complemented strains. These results further demonstrated that TF0104 encodes a functional OxyR protein. Interestingly, several attempts to complement the *T. forsythia* *oxyR* mutant TFM104 were unsuccessful. We believe that these results are due to the fastidious and stringent dependence of *T. forsythia* on anaerobic environments for growth, and also to the fact that a brief exposure to air stress during conjugal mating with *E. coli* might be lethal to a mutant already defective in the expression of its oxidative stress response sensor.

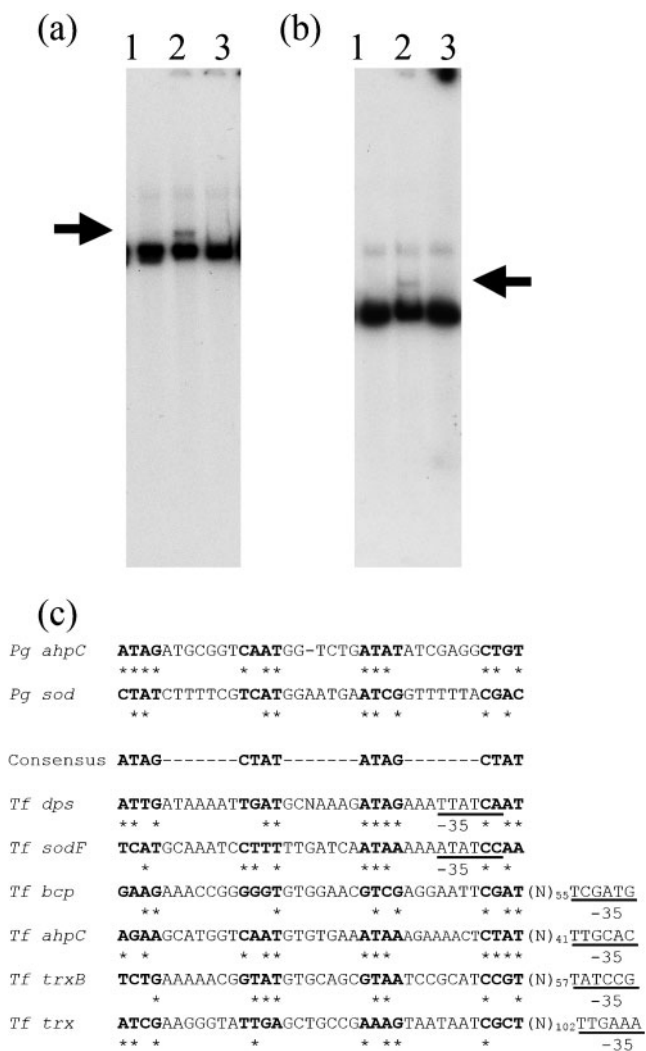


Fig. 2. Electrophoretic mobility shift analysis of the promoter region DNA of the *T. forsythia* *dps* and *sod* genes with rOxyR. Mobility shift assays were performed with purified rOxyR (1 μ g) and 2 ng 32 P-labelled *dps* (a) and *sod* (b) gene promoter DNA fragments. Lanes: 1, labelled promoter; 2, labelled promoter with rOxyR; 3, same as lane 2 with corresponding unlabelled promoter DNA as competitor. Arrows indicate the complex of probe DNA and rOxyR. (c) Comparison of the putative OxyR-binding sequences within the *T. forsythia* (*Tf*) antioxidant genes analysed in the present study with *P. gingivalis* (*Pg*) *ahpC* and *sod* gene OxyR-binding motifs. The consensus sequence shown is the *E. coli* OxyR-binding motif. Putative -35 boxes are underlined.

T. forsythia *oxyR* contributes to biofilm formation with *F. nucleatum*

We have previously shown that *T. forsythia* 43037 forms synergistic biofilms with *F. nucleatum* (Honma *et al.*, 2007; Sharma *et al.*, 2005). In this study, we tested the ability of TFM104 to form synergistic biofilms with *F. nucleatum*. Our results showed that TFM104 and *F. nucleatum* form weaker mixed biofilms than that of *T. forsythia* 43037 and *F.*

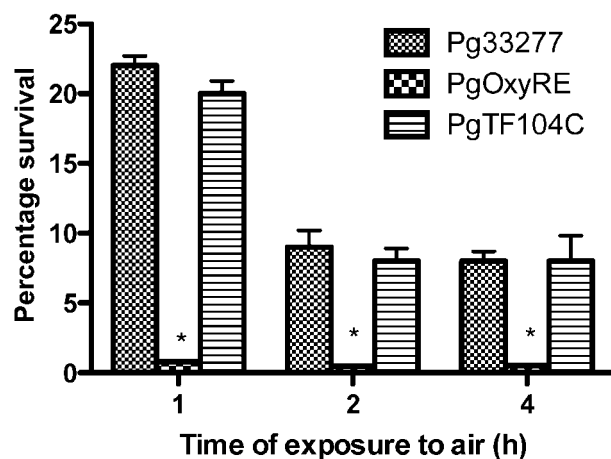


Fig. 3. Heterocomplementation with TF0104 restores the OxyR phenotype in the *P. gingivalis* mutant. Survival of *P. gingivalis* *oxyR* mutant (Pg0270E), complemented strain PgTF104C and *P. gingivalis* strain 33277 was compared following exposure to air for the indicated times. Percentage survival was then determined for each time point normalized to the initial time 0. While survival of Pg0270E was significantly reduced, as expected ($P < 0.05$ compared with the wild-type), survival of the TF104-complemented strain was similar to that of the wild-type strain.

nucleatum (Fig. 4). To determine whether surface adhesion expression was affected in TFM104, resulting in reduced biofilm-forming ability, aggregation assays were performed. As per the aggregation assay results, the autoaggregation activity of TFM104 was found to be lower than that of the wild-type *T. forsythia* strain (Fig. 5). *T. forsythia* biofilm morphology was further analysed by CSLM (Fig. 6). In agreement with the results of autoaggregation assays, CSLM analysis showed that wild-type *T. forsythia* cells formed small autoaggregated microcolonies, whereas the *oxyR* mutant TFM104 cells remained uniformly dispersed with minimum to no microcolony formation. When viewed in the *x-z* plane, microcolonies of the wild-type strain extended away from the attached surface and were taller than microcolonies of the mutant strain TFM104 (Fig. 6a, b). Moreover, the mixed biofilms of wild-type *T. forsythia* and *F. nucleatum* were thicker than those of the TFM104 mutant and *F. nucleatum*, 2.88 and 1.64 μ m, respectively, under *x-z* digital view (Fig. 6c, d). Within the mixed biofilms, *F. nucleatum* cells were observed as long rods and *T. forsythia* was observed as fusiform. More than 99% of total bacterial cells were live (stained green). A qualitative estimation also indicated that a higher proportion of the wild-type *T. forsythia* cells were present in mixed biofilms than mutant TFM104 cells in the respective mixed biofilms.

DISCUSSION

This report describes the identification and functional characterization of an *oxyR* homologue encoded by the

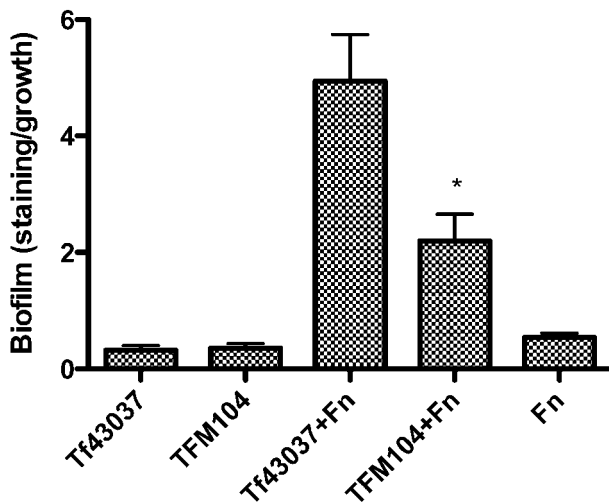


Fig. 4. Analysis of biofilm activity of *T. forsythia* strains. Monospecies or mixed-species biofilms were formed in polystyrene wells and biofilm formation was calculated as crystal violet staining (A_{595}) normalized to total bacterial growth (OD_{600}). Tf43037, monospecies wild-type *T. forsythia* biofilm; TFM104, monospecies mutant TFM104 biofilm; Tf43037+Fn, mixed *T. forsythia* and *F. nucleatum* biofilm; TFM104+Fn, mixed *F. nucleatum* and TFM104 biofilm; Fn, monospecies *F. nucleatum* biofilm. Data points represent mean \pm SD of triplicate samples. Data are representative of three independent experiments. * $P < 0.01$ compared to mixed *T. forsythia* wild-type and *F. nucleatum* biofilm.

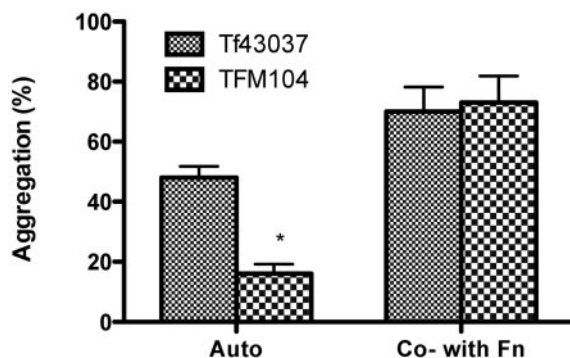


Fig. 5. Aggregation activity of *T. forsythia* strains. For autoaggregation activity, *T. forsythia* strains were harvested at mid-exponential phase, washed in aggregation buffer and adjusted to OD_{660} 1.0, and incubated at 37 °C in glass cuvettes. Aggregation activity was calculated based on absorbance of the cells after 30 min incubation by utilizing the equation given in Methods. For coaggregation activity, *T. forsythia* cells were mixed with *F. nucleatum* cells prewashed in aggregation buffer and adjusted to OD_{660} 1.0. Data points represent mean \pm SD of triplicate samples. Data are representative of three independent experiments. * $P < 0.05$.

ORF TF0104 in *T. forsythia*. The OxyR-like proteins are redox sensors that belong to the LysR family of DNA-binding transcription modulators and control the expression of a number of antioxidant genes in bacteria. In aerobic organisms such as *E. coli*, the OxyR protein is activated in response to peroxide stress through formation of disulfide bonds and regulates antioxidant genes involved in defence against peroxide stress (Zheng & Storz, 2000). While OxyR is mainly responsible for tolerance to peroxide stress in aerobic *E. coli* and the facultative anaerobe *B. fragilis*, it appears to play an essential role against peroxide as well as oxygen (air) stress in obligate anaerobes such as *P. gingivalis* (Diaz *et al.*, 2006). The aerotolerance of the facultative anaerobe *B. fragilis* depends on alternative mechanisms that are not OxyR-dependent (Sund *et al.*, 2006; Tang *et al.*, 1999). To date, except for the role of OxyR in *P. gingivalis*, this protein has not been well studied in Gram-negative obligate anaerobes. Thus, the role of the OxyR regulator in *T. forsythia* was examined by characterizing a deletion mutant of the *oxyR* homologue. Our results showed that, in comparison with the wild-type *T. forsythia* strain 43037, an *oxyR* deletion mutant TFM104 was defective in its ability to resist H_2O_2 and aerobic stress. The mutant showed significantly reduced expression of antioxidant gene-encoding enzymes involved in the detoxification of H_2O_2 in comparison with the parental strain under anaerobic growth conditions. Since *T. forsythia* lacks catalase activity (Hudspeth *et al.*, 1999), bacterioferritin comigratory protein (BCP; an alkyl hydroperoxide reductase C family protein) (Jeong *et al.*, 2000) and AhpC may compensate for the absence of catalase activity in peroxide detoxification. Superoxide dismutase activity has been shown to be important in the obligate anaerobe *P. gingivalis* for protection against aerobic exposure (Ohara *et al.*, 2006). In addition, the Dps protein with ferritin-like activity has the capacity to attenuate the production of reactive oxygen species (Chiancone *et al.*, 2004). Dps assists in controlling iron pools that can lead to hydroxyl radical production via the Fenton reaction. Taken together, we predicted that OxyR-dependent expression of antioxidant homologue genes should be critical for protection against oxidative stress in *T. forsythia*. Interestingly, the levels of oxidative stress-induced genes did not increase in response to peroxide stress, indicating a constitutively activated stage of OxyR in this bacterium. This is analogous to the situation in another Gram-negative obligate anaerobe, *P. gingivalis* (Diaz *et al.*, 2006). It is hypothesized that a constitutively activated state of OxyR can benefit obligate anaerobes living in the oral cavity by protecting them against constant challenge from atmospheric oxygen and antibacterial oxidative responses of host leukocytes. The mechanisms underlying constitutive activation of OxyR in *P. gingivalis* and *T. forsythia* have yet to be determined. However, it is likely that OxyR proteins in anaerobes have evolved due to amino acid substitutions that induce conformational changes favouring disulfide bond formation and locking OxyR in an active state. This notion is supported by the fact that

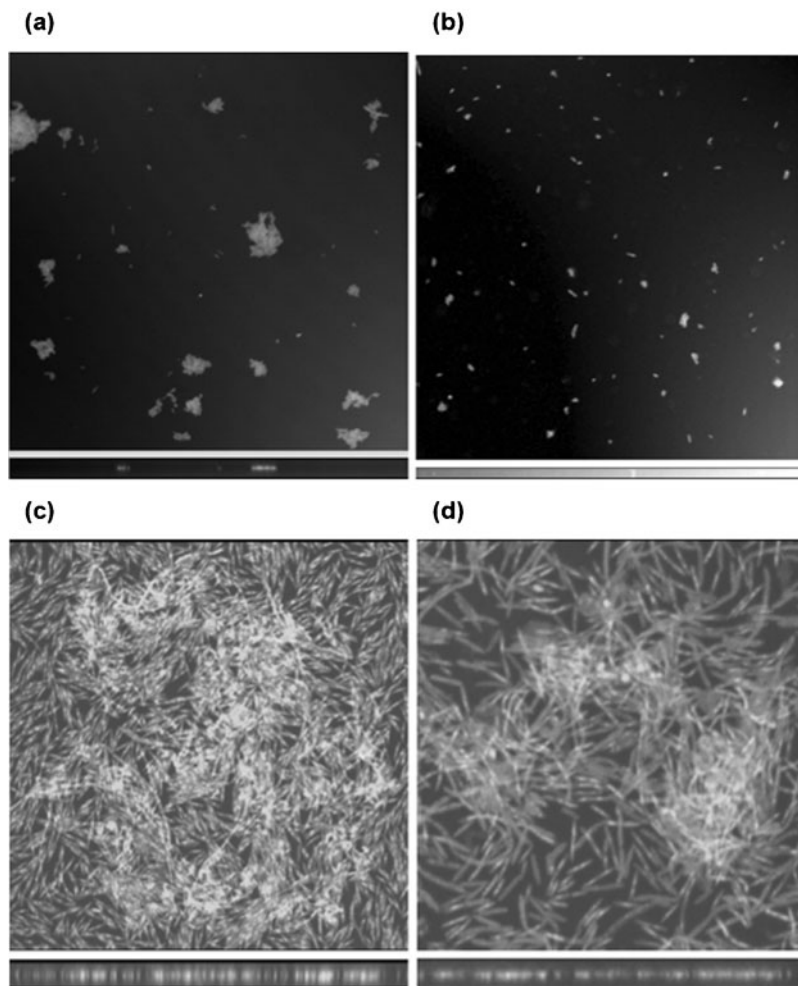


Fig. 6. CSLM images of autoaggregation and mixed biofilms. (a, b) Autoaggregation. Monospecies cultures were incubated for 3 h in two-well culture dishes and surface-attached bacterial cells were stained with live-dead bacterial stain as described in the text [(a) *T. forsythia* wild-type; (b) mutant TFM104]. (c, d) Mixed biofilms. *T. forsythia* wild-type and *F. nucleatum* (c) or TFM104 mutant and *F. nucleatum* (d) were coincubated in two-well polystyrene tissue-culture vessels for 36 h and stained with a bacterial live-dead staining kit as described in the text. *x-z* reconstruction (biofilm thickness) for each condition is shown below the *x-y* image: 2.88 μm (c) and 1.68 μm (d).

Bacteroides mutants have been isolated with constitutively active OxyR conformational states. Single amino acid substitutions in the OxyR protein in these strains have been shown to cause constitutive activation (Rocha *et al.*, 2000).

In this study we also showed that the deletion of *oxyR* results in attenuation of mixed biofilm formation of *T. forsythia* with *F. nucleatum*. We have previously shown that *T. forsythia* forms weak biofilms alone but forms synergistic biofilms in mixed cultures with *F. nucleatum* (Sharma *et al.*, 2005). Here we showed that the OxyR mutant TFM104 was significantly attenuated in its ability to form mixed biofilms with *F. nucleatum*. In this regard, links between biofilm formation and oxidative stress defences have been observed in several microbes, including *E. coli* (Reisner *et al.*, 2003), *H. influenzae* (Murphy *et al.*, 2005), *Ps. aeruginosa* (Sauer *et al.*, 2002), *C. jejuni* (Sampathkumar *et al.*, 2006), *Strep. mutans* (Blumer *et al.*, 2005), *Can. albicans* (Murillo *et al.*, 2005), *Ser. marcescens* (Shanks *et al.*, 2007) and *P. gingivalis* (Wu *et al.*, 2008). To our knowledge, *F. nucleatum* is not known to increase oxidative stress, which could explain the reduction in synergistic biofilm formation by the *oxyR* mutant TFM104 with *F. nucleatum* in mixed cultures. In fact, the growth of *F.*

nucleatum has been shown to maintain low redox potentials [$E(h) = -274$ mV] with no detectable oxygen in the medium and to support the growth of anaerobic *P. gingivalis* (Diaz *et al.*, 2000). An alternative explanation for the reduced biofilm phenotype of TFM104 compared with the parental strain might be differences in the expression of surface components, which could affect biofilm formation. For example, in *E. coli*, OxyR regulates the expression of a surface adhesin, Ag43 (Danese *et al.*, 2000). Ag43 promotes biofilm formation via its strong cell-to-cell aggregation characteristics (autoaggregation). Interestingly, it is the reduced form of OxyR which then acts as a repressor for derepression of the Ag43 encoding gene upon exposure to oxidative stress, leading to Ag43 expression (Schembri *et al.*, 2003). It is believed that Ag43-mediated cell aggregation and biofilm formation confer protection against H_2O_2 killing (Schembri *et al.*, 2003). In *Ser. marcescens*, OxyR regulates biofilm formation through induction of fimbriae production (Shanks *et al.*, 2007). In *P. gingivalis*, OxyR acts as a repressor of the surface adhesin FimA involved in biofilm formation, and therefore an *oxyR* deletion mutant forms increased biofilms compared with its parental strain (Wu *et al.*, 2008). Our results presented here with respect

to *T. forsythia* show that in comparison with the parental strain, an *oxyR* mutant, TFM104, is significantly reduced in its ability to autoaggregate and form mixed biofilms with *F. nucleatum*. The coaggregation ability of the mutant with *F. nucleatum* did not differ significantly from that of the parental strain, indicating that the adhesins involved in autoaggregation and coaggregation may be different. With regard to the effect of autoaggregation in biofilm development, in a previous study we also observed that the ability of *T. forsythia* to form autoaggregated microcolonies is important in biofilm development (Honma *et al.*, 2007). We showed that the surface exopolysaccharide-deficient mutants with greater surface hydrophobicity than the wild-type strain make aggregated microcolonies and subsequently better biofilms than the wild-type strain. While the underlying mechanisms linking oxidative stress defences and biofilm formation are not well understood, the OxyR regulation of biofilm formation in *T. forsythia* is expected to promote bacterial adaptation in the oxidative environment of the oral cavity. Taken together, our findings provide evidence for a role for the OxyR pathways in antioxidant responses and suggest that OxyR also affects biofilm formation in *T. forsythia*.

In summary, TF0104 encodes an OxyR homologue in *T. forsythia* that acts as a positive regulator of the expression of oxidative defence responses and also affects biofilm formation. Thus, *T. forsythia* OxyR likely plays roles in defence against oxidative stress mediated by neutrophils and other bacteria, as well as in biofilm development, critical to the ability of the bacterium to persist in the oral cavity.

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