OxyR inactivation reduces the growth rate and oxidative stress defense in *Capnocytophaga ochracea*

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Abstract

Objective: The human oral cavity harbors several bacteria. Among them, *Capnocytophaga ochracea*, a facultative anaerobe, is responsible for the early phase of dental plaque formation. In this phase, the tooth surface or tissue is exposed to various oxidative stresses. For colonization in the dental plaque phase, a response by hydrogen peroxide (H_2O_2)-sensing transcriptional regulators, such as OxyR, may be necessary. However, to date, no study has elucidated the role of OxyR protein in *C. ochracea*.

Methods: Insertional mutagenesis was used to create an *oxyR* mutant, and gene expression was evaluated by reverse transcription-polymerase chain reaction and quantitative real-time reverse transcription-polymerase chain reaction. Bacterial growth curves were generated by turbidity measurement, and the sensitivity of the *oxyR* mutant to H_2O_2 was assessed using the disc diffusion assay. Finally, a two-compartment system was used to assess biofilm formation.

Results: The *oxyR* mutant grew slower than the wild-type under anaerobic conditions. The agar diffusion assay revealed that the *oxyR* mutant had increased sensitivity to H_2O_2. The transcript levels of oxidative stress defense genes, *sod*, *ahpC*, and *trx*, were lower in the *oxyR* mutant than in the wild-type strain. The turbidity of *C. ochracea*, simultaneously co-cultured with *Streptococcus gordonii*, was lower than that observed under conditions of homotypic growth. Moreover, the percentage decrease in growth of the *oxyR* mutant was significantly higher than that of the wild-type.

Conclusions: These results show that OxyR in *C. ochracea* regulates adequate *in vitro* growth and escapes oxidative stress.

Keywords

Capnocytophaga ochracea, OxyR, *in vitro* growth, hydrogen peroxide, *Streptococcus gordonii*, polymicrobial community

1

[1] Abbreviations: TSA, tryptic soy agar; TSB, tryptic soy broth

1. Introduction

The microbiota of the oral cavity is highly complex and consists of more than 700 different types of bacteria [1]. Dental plaque biofilm is a polymicrobial biofilm that is organized on the surface of the tooth. A shift in the composition of the microbiome, called dysbiosis, possibly leads to dental caries and periodontal disease [2]. During dental plaque formation, bacteria must overcome environmental stress and competition with other species to proliferate. Initial colonizers of the dental plaque, such as *Streptococcus gordonii* and *Streptococcus sanguinis*, release hydrogen peroxide (H_2O_2) to compete with the bacteria around them [3,4]. Bacteria in the subgingival plaque can trigger the host immune system, characterized by the release of reactive oxygen species (ROS) by polymorphonuclear neutrophils and macrophages [5]. These findings indicate that oxygen stress is a major obstacle that must be overcome for growth in the dental plaque.

Capnocytophaga ochracea is a gram-negative, rod-shaped bacterium with gliding motility. It is frequently isolated from chronic periodontal lesions [6,7] and is occasionally detected in septicemia and abscesses [8,9]. During dental plaque formation, *C. ochracea* is an early colonizer [10] and has the ability to adhere to the acquired pellicle and co-aggregate with *S. sanguinis* [11,12]. *Capnocytophaga* species have a type IX secretion system (T9SS), which is involved in gliding motility and biofilm formation [13]. *Capnocytophaga gingivalis* reportedly transports some non-motile bacterial species more efficiently than others via its gliding activity and helps shape the spatial organization of a polymicrobial community [14]. Reportedly, *C. ochracea* co-aggregates with *Fusobacterium nucleatum,* which acts as a bridge between the early colonizers and late colonizers, including periodontopathic bacteria such as *Porphyromonas gingivalis* [10,15]; further, secretion of

C. ochracea increases the biofilm formation by *F. nucleatum* [16]. Network analysis of the subgingival dental plaque bacteria revealed that *C. ochracea* is linked to a cluster of bacteria of pathogenic genera, including *Porphyromonas*, and commensal genera, including *Streptococcus* [17]. These reports suggest that *C. ochracea* plays an important role in the colonization by periodontopathic bacteria in biofilms consisting of early colonizers; however, to the best of our knowledge, there have been no reports on the oxygen stress defense mechanism of *C. ochracea*.

OxyR is a well-known peroxide-sensing transcription activator. Several antioxidant ROS scavengers, such as superoxide dismutase (SOD), alkyl hydroperoxide reductase (Ahp), thioredoxin (Trx), glutaredoxin (Grx), and catalase, are present in OxyR regulons [18,19]. OxyR has been characterized in dental plaques caused by gram-negative bacteria, such as *P. gingivalis, Prevotella intermedia*, *Tannerella forsythia*, and *Actinobacillus actinomycetemcomitans* [5,20-22]. OxyR is reportedly involved in protecting the bacteria from oxygen stress and in the transcriptional regulation of the fimbrillin gene, biofilm formation, and resistance to the complement system (a component of the innate immunity) [20,21,23]. Therefore, in this study, we aimed to identify the role of OxyR in *C. ochracea* in oxidative stress escape and biofilm formation using an *oxyR* mutant strain. Here, we searched for an *oxyR* homolog for the whole genome sequence of *C. ochracea* ATCC 27872 and characterized the role of *oxyR* in *C. ochracea*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. *Capnocytophaga ochracea* ATCC 27872 (wild-type) and the *oxyR* mutant were cultured anaerobically (10% CO_2, 10% H_2, and 80% N_2) at 37 °C on tryptic soy agar (TSA; Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 5 μg/mL hemin (Sigma-Aldrich, St. Louis, MO, USA), 0.5 μg/mL menadione (Nacalai Tesque, Kyoto, Japan), and 5% defibrinated horse blood (Nippon Bio-Test Laboratories, Tokyo, Japan) [13,24]. For the liquid cultures, *C. ochracea* cells were grown in tryptic soy broth (TSB; Becton Dickinson) supplemented with 5 μg/mL hemin and 0.5 μg/mL menadione [13,24]. For selecting and maintaining the *oxyR* mutant, erythromycin (15 μg/mL) was added to either the medium or the agar plate [13,24]. We cultured *S. gordonii* DL1 anaerobically at 37 °C in TSB or Mitis-salivarius agar plates.

2.2. Screening of oxyR and *construction of the C. ochracea oxyR mutant*

The sequence of the *oxyR* homolog in *C. ochracea* was searched using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the OxyR sequence of *P. gingivalis*. A comparison of the OxyR sequence of *C. ochracea* and that of other species was performed

using GENETYX-MAC version 21 (GENETYX, Tokyo, Japan). The *oxyR* mutant from *C. ochracea* was generated as follows: genomic DNA was isolated from *C. ochracea* ATCC 27872 using the Gentra Puregene Yeast/Bact. Kit (Qiagen, Tokyo, Japan) by following the manufacturer's instructions. The flanking region of *oxyR* was amplified from the genomic DNA of *C. ochracea* ATCC 27872 using Takara Ex Taq DNA polymerase (Takara Bio, Otsu, Japan). The upstream regions of *oxyR* were amplified using the primer pair Cap-OxyR-U5 and Cap-OxyR-U3, and the downstream regions of *oxyR* were amplified using the primer pair Cap-OxyR-D5 and Cap-OxyR-D3 (Table 2). Each upstream or downstream fragment was cloned into the pGEM-T-Easy vector (Promega, Tokyo, Japan), resulting in pCOoxyR1 and pCOoxyR2. Next, the *Bam*HI-*Sac*I fragment was extracted from pCOoxyR2 and ligated into the *Bam*HI-*Sac*I site of pCOoxyR1 to yield pCO001. The *ermF* cassette fused with a promoter was inserted into pCO001 using an In-Fusion HD cloning kit (Takara Bio, Otsu, Japan) per the manufacturer's protocol. The *ermF* gene was amplified with pKD355 [25] using KOD-Plus Neo (Toyobo, Osaka, Japan) and the primer sets pKD355-ermF-inF-5-1 and pKD355-ermF-inF-3-1. The pCO001 plasmid was then digested with *Bam*HI. The amplified *ermF* DNA, *Bam*HI-digested pCO001, and the In-Fusion reaction buffer were mixed and incubated at 50 °C for 15 min. The mixture was then transformed into *Escherichia coli* DH5α, and the plasmid pCO002 was extracted. This

plasmid was linearized by *Not*I digestion and introduced into *C. ochracea* ATCC 27872 by electroporation as previously described [13,24], resulting in COTY001. As shown in Fig. 1A, 1C, and 4, the isogenic mutants were confirmed by polymerase chain reaction (PCR), conventional reverse transcription-PCR (RT-PCR), or quantitative real-time reverse transcription-PCR (qRT-PCR).

2.3. RNA isolation from C. ochracea

Capnocytophaga ochracea cells were allowed to grow to an optical density $(OD)_{660}$ of 0.3–0.4, after which the total RNA was extracted from the cells using TRIzol reagent (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. DNase treatment was performed using the TURBO DNA-free™ kit (Thermo Fisher Scientific) at 37 °C for 15 min, and PCR was performed to confirm the deletion of the detectable genomic DNA in the RNA samples. The RNA sample concentration was determined using a Quantus fluorometer (Promega) with a QuantiFluor RNA system (Promega) and the quality was checked by analyzing the integrity of RNA using a Tape Station (Agilent Technologies, CA, USA). RNA was reverse transcribed using ReverTra Ace (Toyobo) with a random hexamer primer, following the manufacturer's instructions, to synthesize cDNA.

2.4. RT-PCR

RT-PCR was carried out to confirm the co-transcription of *oxyR* and the flanking genes

using cDNA. The primers used here are listed in Table 2. Each reaction mixture contained 1 μL of cDNA mixed with 0.25 μL of Takara *Ex Taq* DNA polymerase (2.5 units), 5.0 μL of Takara *Ex Taq* Buffer, 4.0 μL of dNTP mix (2.5 mM each), 2.5 μL of 10 μM gene-specific primer pairs (Table 2), and 34.75 μL of RNase-free water. PCR was performed using a Takara PCR Thermal Cycler Dice (Takara Bio). The PCR cycling conditions were as follows: one cycle at 94 °C for 2 min, followed by 25 cycles at 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 min. The PCR products were visualized on an agarose gel by staining with Midori Green Direct (Nippon Genetics, Tokyo, Japan).

2.5. Analysis of growth rate of C. ochracea in liquid culture

The growth rate of *C. ochracea* strains in liquid culture was evaluated as described previously [13,24]. Briefly, *C. ochracea* strains were anaerobically cultured for 4 days at 37 °C on blood agar plates and inoculated into TSB. After 24 h, each culture was diluted with fresh TSB to an OD_{660} of 0.1. The diluted samples were incubated anaerobically at 37 °C for 3 days. Bacterial growth was monitored by measuring the OD at 660 nm using a spectrophotometer (UV-2550; Shimadzu, Kyoto, Japan). Data are represented as mean ± standard deviation (SD) of 10 experiments.

2.6. Agar diffusion assay

The susceptibility of *C. ochracea* strains to H_2O_2 was investigated as previously described [26,27]. *Capnocytophaga ochracea* strains were cultured anaerobically in TSB, and 1 mL

aliquots of stationary phase culture were spread on TSA plates. Excess bacterial suspension was removed, and the surfaces of the agar plates were dried. Thereafter, 6-mm paper disks containing various concentrations of H_2O_2 were placed on the agar surface. Inhibition zones were measured after anaerobic incubation at 37 °C for 7 days. Experiments were performed in triplicate with three biologically independent replicates.

2.7. qRT-PCR

To investigate the gene expression in the *oxyR* mutant, qRT-PCR was carried out on an ABI StepOne Plus Real-Time PCR System (Thermo Fisher Scientific). Each reaction mixture contained 1 μL of cDNA mixed with 10 μL of TaqMan Fast Universal PCR Master Mix (Thermo Fisher Scientific), 1 μL of gene-specific primer and probe mixture (Table 2), and 8 μL of RNase-free water. The PCR cycling conditions were as follows: 95 °C for 20 s, followed by 40 cycles at 95 °C for 1 s and 60 °C for 20 s. The experiments were performed in triplicate with three biologically independent replicates. Gene expression levels were normalized to the level of the housekeeping transcript 16s rRNA gene. The primers used here are listed in Table 2.

2.8. Effect of co-culture of C. ochracea and S. gordonii on the growth of C. ochracea

To investigate the role of *oxyR* against oxygen stress from *S. gordonii,* the growth of *C. ochracea* under co-culture with *S. gordonii* was evaluated. A two-compartment separated co-culture system was used according to the method described by Okuda et al. [16].

Briefly, *C. ochracea* strains were anaerobically cultured for 4 days at 37 °C on TSB blood agar plates. A pre-culture was prepared anaerobically by inoculating fresh colonies from the blood agar plates into TSB. Thereafter, *S. gordonii* was anaerobically cultured for 2 days at 37 °C on Mitis-salivarius agar plates (Becton Dickinson). A pre-culture was prepared anaerobically by inoculating fresh colonies from the Mitis-salivarius agar plates into TSB. After 24 h, each culture was diluted with fresh TSB to an OD_{660} of 0.1. First, 1 mL of *C. ochracea* strain was placed in each well of a polystyrene 12-well plate (Dow Corning, Midland, MI, USA). An insert cup (Corning) was then placed in each well and designated as the upper well. Next, 1 mL of *S. gordonii* was poured into the upper well. After anaerobic incubation at 37 °C for 3 days, the upper wells were removed. The cells were then harvested with a cell scraper, and the turbidity of the whole cell culture was measured using a spectrophotometer (UV-2550). The data are represented as mean ± SD of eight experiments.

2.9. Statistical analysis

The one-way analysis of variance (ANOVA) and Dunnett's or Kruskal–Wallis multiple comparison tests were used to compare the data for the wild-type *C. ochracea* and the *oxyR* mutant using GraphPad Prism (GraphPad Software 8.0). The level of significance was set at $p < 0.05$.

3. Results

3.1. Construction of an oxyR mutant by gene-directed mutagenesis in C. ochracea ATCC 27872

The BLAST results revealed a possible *oxyR* homolog (*Coch_0002*) of the *C. ochracea* ATCC 27872 genome. Residues 91–291 of the deduced amino acid sequence from *Coch_0002* was similar to the C-terminal substrate-binding domain of the LysR-type transcriptional regulator OxyR (cd08421). The similarity of *Coch_0002* to OxyR in other species belonging to Bacteroidetes is shown in Fig. S1. The sequence identities between *C. ochracea* and *Capnocytophaga sputigena*, *Capnocytophaga canimorsus*, *P. gingivalis, P. intermedia*, and *T. forsythia* were 97%, 72%, 29%, 36%, and 33%, respectively. Cysteines of the catalytic region were conserved in all species (red asterisk).

To elucidate the role of OxyR, we constructed an *oxyR (Coch_0002)* mutant in which *oxyR* was replaced by *ermF* via homologous recombination, and the mutant obtained was confirmed by PCR using *oxyR*-specific and *ermF*-specific primers (Fig. 1A, 1C [5]).

In the *C. ochracea* genome, the region between Coch_0001 and the upstream ends of *oxyR* consists of nine nucleotides and the region between the downstream ends of *oxyR* and Coch_0003 consists of 56 nucleotides (Fig. 1B). Based on these facts, we speculated that *Coch_0001*, *oxyR*, and *Coch_0003* may form an operon and that the expression of the *Coch_0003* downstream gene of *oxyR* may be reduced by the polar effect due to insertional inactivation of *oxyR*. To verify this possibility, we assessed the transcription of *Coch_0001*, *oxyR*, and *Coch_0003* by conventional RT-PCR using gene-specific primers (Fig. 1B). As shown in Fig. 1C (2) and (3), the PCR products were generated using primers CO-

Coch0001-5 and Cap-OxyR-check-3 (1176 bp) or primers Cap-OxyR-check-5 and CO-Coch0003-3 (1245 bp), indicating that *Coch_0001*, *oxyR* (*Coch_0002*), and *Coch_0003* formed an operon. The expression of *Coch_0001* and *Coch_0003* was similar in both the wild-type and *oxyR* mutant strains, indicating that the polar effect of *ermF* insertion could be ruled out (Fig. 1C [4] and [6]).

3.2. Growth of the oxyR mutant

We analyzed the growth of the *oxyR* mutant in TSB under anaerobic conditions (Fig. 2). Inactivated *oxyR* strain grew more slowly compared with wild-type in the exponential phase, and the turbidity of the *oxyR* mutant culture in the stationary phase was reduced to approximately half of that of the wild-type ATCC 27872.

3.3. Sensitivity of the oxyR mutant to H_2O_2

Next, we determined the sensitivity of the *oxyR* mutant using an agar diffusion assay. The *oxyR* mutant showed larger zones than the wild-type strain at both 6% and 15% H_2O_2 concentration (Fig. 3).

3.4. Transcriptional analysis of the oxyR mutant under anaerobic conditions

We performed qRT-PCR to confirm that OxyR affects the expression of genes related to

oxidative stress defense, such as *sod*, *ahpC*, and *trx*, in *C. ochracea*. The transcript levels of *sod* and *ahpC* were considerably lower in the *oxyR* mutant than in the wild-type strain (Fig. 4). The transcript level of *trx* in the *oxyR* mutant decreased to 50% (Fig. 4).

3.5. Effect of co-culture with S. gordonii on the growth of C. ochracea

We then examined the role of *oxyR* using a system of co-culture of *C. ochracea* with *S. gordonii,* which produces H_2O_2, using a two-compartment separated co-culture system. In the presence of *S. gordonii*, the growth of the wild-type strain was reduced by approximately 25%, whereas that of the *oxyR* mutant was reduced by approximately 80%, that is, the reduction in growth of the *oxyR* mutant upon co-culture with *S. gordonii* was significantly higher than that of the wild-type strain (Fig. 5). This result indicated that *oxyR* might play an important role in growth competition against *S. gordonii*.

4. Discussion

Sequence comparison of *Coch_0002* and *oxyR* of *P. gingivalis*, *P. intermedia*, and *T. forsythia* revealed that the amino acid sequence identity of *Coch_0002* was approximately 30% and the cysteine residues in the catalytic domain of *oxyR* were conserved in *Coch_0002.* The residues 91–291 of *Coch_0002* were similar to the C-terminal substrate-binding domain of the LysR-type transcriptional regulator OxyR (cd08421). These data suggest that *Coch_0002* possibly acts as an *oxyR.* In addition, this study showed that the sensitivity of the *oxyR* mutant to H_2O_2 significantly increased compared with the wild-

type. Although the RT-PCR analysis showed that the RNA of these genes was polycistronic, the transcript level of *Coch_0003* between the wild-type and *oxyR* mutant strains was similar. These results indicate that *oxyR* may be involved in the response against oxidative stress in *C. ochracea.*

OxyR is involved in oxygen stress defense against periodontal pathogens, such as *P. gingivalis* [5,19,21,22,28]. The qRT-PCR analysis results showed a significant reduction in the expression of *sod* and *ahpC* in the mutant strain. Ohara et al. reported that recombinant *P. gingivalis* OxyR directly binds to the *sod* and *ahpC* promoter regions [19]. Honma et al. showed that recombinant *T. forsythia* OxyR directly binds to the *sodF* promoter region [21]. Naito et al. showed that *P. intermedia* OxyR mainly regulates the transcription of *ahpCF* [22]; this suggests that the response against oxidative stress was caused by the regulation of transcriptional expression of *sod* and *ahpC* by OxyR. The study also showed that the transcriptional expression of *trx* in the *oxyR* mutant decreased to 50%. Honma et al. showed that the expression of *trx* and *ahpC* significantly decreased in the *T. forsythia oxyR* mutant compared to that in the wild-type strain [21]. However, whether recombinant *C. ochracea* OxyR directly binds to the promoter regions of *sod*, *ahpC*, and *trx* warrants further investigation.

Next, we found that the *oxyR* mutant had a slower growth rate than the wild-type strain. Wen et al. revealed that the inactivation of OxyR in *Haemophilus parasuis* considerably impairs growth [29]. In addition, Honma et al. reported that OxyR of *T. forsythia* is important for auto-aggregation and a mixed biofilm formation with *F. nucleatum* [21]. We postulate three reasons for the phenomenon observed. First, the *oxyR* mutant strain adapts poorly to the changes in the surrounding environment. We consider the amount of air as a

candidate for environmental change. Second, the *oxyR* mutant strain has an inferior metabolic capacity to the wild-type strain. Finally, differences were observed in the outer surface of the *oxyR* mutant compared with those on the surface of the wild-type. For example, there may be changes in adhesion factors or proteins related to nutrient uptake that are expressed on the outer membrane. Further comprehensive expression analyses of *oxyR* mutant using RNA sequencing are required to clarify these points.

A significant reduction in the growth of *C. ochracea oxyR* mutant co-cultured with *S. gordonii* was observed in comparison with the growth of wild-type *C. ochracea* strain co-cultured with *S. gordonii*. This result indicated that OxyR in *C. ochracea* plays an important role in the environment in which H_2O_2 is produced by *S. gordonii*. *Capnocytophaga ochracea* settles on the tooth surface in the early phase of dental plaque formation [30]. At this time point, many early colonizing bacteria, such as oral streptococci, are already present, and among them, *S. gordonii* and *S. sanguinis* produce H_2O_2 and interfere with the colonization of other oral bacteria [31]. Therefore, the response to the oxygen stress created by the competitor species is an important factor determining colonization in the early phase of dental plaque formation. Zhu et al. reported that *A. actinomycetemcomitans*, a facultative anaerobic bacterium, shelters *P. gingivalis* from the H_2O_2 produced by *S. sanguinis* [32]. Meuric et al. reported that the *Capnocytophaga* and *Streptococcus* are majorly present in healthy subgingival biofilms; *Streptococcus* is also present in chronic periodontitis biofilms [33]. In lesions of periodontitis disease, the genus *Capnocytophaga* is detected more frequently than *P. gingivalis* [34]. Furthermore, *P. gingivalis* and *S. gordonii* interact and exhibit increased fitness *in vivo* [35]. It is possible that *C. ochracea* shelters *P. gingivalis* from excess H_2O_2 present in the environment.

5. Conclusions

Our findings indicate that OxyR is necessary for *C. ochracea* to cope with oxidative stress and is also important for co-existence with *S. gordonii*. These results show that OxyR in *C. ochracea* regulates adequate *in vitro* growth and escapes oxidative stress.

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the findings reported in this paper.

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Yuichiro Kikuchi: Conceptualization, Investigation, Visualization, Writing- Original Draft, Writing- Review & Editing. Kazuko Okamoto-Shibayama: Writing- original draft preparation. Eitoyo Kokubu: Writing- original draft preparation. Kazuyuki Ishihara: Conceptualization, Visualization, Writing- Original Draft, Writing- Review & Editing, Supervision, Project administration, Funding acquisition.

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

Strain or plasmid	Description	Reference or source
***Escherichia coli* strain**		
DH5α	General-purpose host strain	Thermo Fisher Scientific
Capnocytophaga ochracea		
ATCC 27872	Wild-type	American Type Cell Culture
COTY001	*oxyR::ermF*, Em^r	This study
Streptococcus gordonii		
DL1	Wild-type	Laboratory stock
Plasmids		
pGEM-T Easy	Ap^r, plasmid vector for TA cloning	Promega
pKD355	Ap^r, containing the *ermF ermAM* DNA cassette	[25]
pCOoxyR1	Ap^r, containing the 956 bp PCR-amplified fragment	This study
pCOoxyR2	Ap^r, containing the 1031 bp PCR-amplified fragment	This study
pCO001	Ap^r, containing the 2.0 kb PCR-amplified fragment	This study
pCO002	Ap^r Em^r, containing the *ermF* DNA cassette within	This study

Abbreviations: PCR, polymerase chain reaction

Table 2. Primers and probes used in this study.

Primer/probe	Nucleotide sequence (5′-3′)
For the construction of the *oxyR* mutant	
Cap-OxyR-U5	GCGACTTGTGGAGCAAAGAG
Cap-OxyR-U3	GGGATCCCTATCGACGGATTGTGCAGAG
Cap-OxyR-D5	GGGATCCCGTAGAAAGACCGTTATACTATGAGCCT
Cap-OxyR-D3	ATAATTACTTGTTTCCAGCGGTTT
Cap-OxyR-check-5	ACAAAAGGGGTATATAGGAGGTGAG
Cap-OxyR-check-3	AAAGTCATTCCTAAGCCTTCGTTAG
pKD355-ermF-inF-5-1	CCGTCGATAGGGATCCATAAGTTGAACTCAAGAAG
pKD355-ermF-inF-3-1	TCTTTCTACGGGATCCCTACGAAGGATGAAATTTT
For RT-PCR	
CO-16SrRNA-5	CTTATGGGTTGTAAACTGCTTTTGT
CO-16SrRNA-3	TGGGATACTTATCACTTTCGCTTAG
CO-Coch0001-5	TGAGTATGGTACCCTAATGGAAAAA
CO-Coch0001-3	TCCTATTTCTTCAAGCACTTCACTT

CO-Coch0003-5	TTTCATTTGCGTCTTGTAGTTTTAC
CO-Coch0003-3	ATAATTACTTGTTTCCAGCGGTTTT

For qRT-PCR

CO-16SrRNA-5	CCCTTACATCTTGGGCTACAC
CO-16SrRNA-probe	ACGTGCTACAATGGCCGTTACAGA
CO-16SrRNA-3	CCGAACTGTGACCGTCTTTAT
CO-OxyR-5	AGATAGAGGACGGGAGTTATGA
CO-OxyR-probe	CCCTCTGCACAATCCGTCGATAGT
CO-OxyR-3	AGGCTCATAGTATAACGGTCTTTC
CO-Sod-5	AGATAACCCGCTAATGCCTAAC
CO-Sod-probe	AGCGTGTTCCCACACATCTAATCCC
CO-Sod-3	AGGACGACGGTTCTGATAGT
CO-AhpC-5	GTACACTTTGCTTGGCTCAATAC
CO-AhpC-probe	CAACCGCAACCTCGCTCGTATTT
CO-AhpC-3	TCACGAGAGTCGAGAATACCT
CO-trx-5	TAGAAGGAATGGTGTGGCATAA
CO-trx-probe	AAAGGCGCAGGGATACTCGTTACC

CO-trx-3	AAGTTTCCTTCCGGGTCTATG

Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; qRT-PCR, quantitative real-time reverse transcription-PCR

Figure legends

Fig. 1. Physical and transcriptional organization of the *oxyR* region in the *Capnocytophaga ochracea* ATCC 27872 chromosome.

A. Polymerase chain reaction (PCR) confirmation of the *oxyR* mutant. *oxyR* (lanes 1–4) and *ermF* (lanes 5–8). Lane 1, 100 bp ladder marker; lane 2, ATCC 27872 PCR product; lane 3, *oxyR* mutant PCR product; lane 4, targeting vector PCR product; lane 5, 1 kb ladder marker; lane 6, ATCC 27872 PCR product; lane 7, *oxyR* mutant PCR product; and lane 8, targeting vector PCR product. **B.** Diagram of the chromosomal structure of *Coch_0001*, *oxyR*, *Coch_0003,* and *Coch_0004*. Black arrows indicate the direction of gene transcription. The insertion site of *ermF* is shown and the primer pairs used for conventional reverse transcription-PCR (RT-PCR) analysis are mentioned. Primers: 1. CO-Coch0001-5, 2. CO-Coch0001-3, 3. Cap-OxyR-check-5, 4. Cap-OxyR-check-3, 5. CO-Coch0003-5, and 6. CO-Coch0003-3. **C.** Conventional RT-PCR analysis results of *C. ochracea* wild-type and *oxyR* mutant. Lane 1, 100 bp ladder marker; lane 2, ATCC 27872 RT-PCR product; lane 3, ATCC 27872 RT-PCR product without reverse transcriptase (control); lane 4, *oxyR* mutant RT-PCR product; lane 5, *oxyR* mutant RT-PCR product without reverse transcriptase (control); and lane 6, ATCC 27872 genomic DNA PCR product.

Fig. 2. Growth of *Capnocytophaga ochracea* ATCC 27872 and *oxyR* mutant. Growth curve of ATCC 27872 (blue line) and the *oxyR* mutant (red line) in tryptic soy broth. The strains were grown anaerobically at 37 °C for 3 days. The culture turbidity was monitored

by measuring the optical density of the cultures at 660 nm.

Fig. 3. Sensitivity of *Capnocytophaga ochracea* strains to hydrogen peroxide (H_2O_2). *Capnocytophaga ochracea* was grown anaerobically in tryptic soy broth and was spread on tryptic soy agar plates. Paper disks containing H_2O_2 were placed on the plate, followed by an anaerobic culture at 37 °C for 7 days. The diameters of the inhibition zones were measured. Data are expressed as mean ± standard deviation (SD) of nine independent experiments. *** $p < 0.001$.

Fig. 4. Expression of the oxidative stress defense genes in *Capnocytophaga ochracea* strains. *Capnocytophaga ochracea* strains were grown anaerobically in tryptic soy broth and were cultivated to an optical density (OD_{660}) of 0.6–0.8 on enriched tryptic soy agar plates. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using *sod*-, *ahpC*-, and *trx*-specific primers or probes. Data are expressed as mean ± standard deviation (SD) of nine independent experiments. *** $p < 0.001$.

Fig. 5. Effect of co-culture with *Streptococcus gordonii* on the growth of *Capnocytophaga ochracea*. Following 24 h of growth in anaerobic conditions, *C. ochracea* strains inoculated in the lower well were cultured anaerobically at 37 °C with *S. gordonii* in the upper well using a two-compartment system. The culture turbidity was monitored by measuring the optical density of the cultures at 660 nm. Data are expressed

as mean ± standard deviation (SD) of nine independent experiments. Significant differences (*** $p < 0.001$) were found in all combinations of blue and red bars.

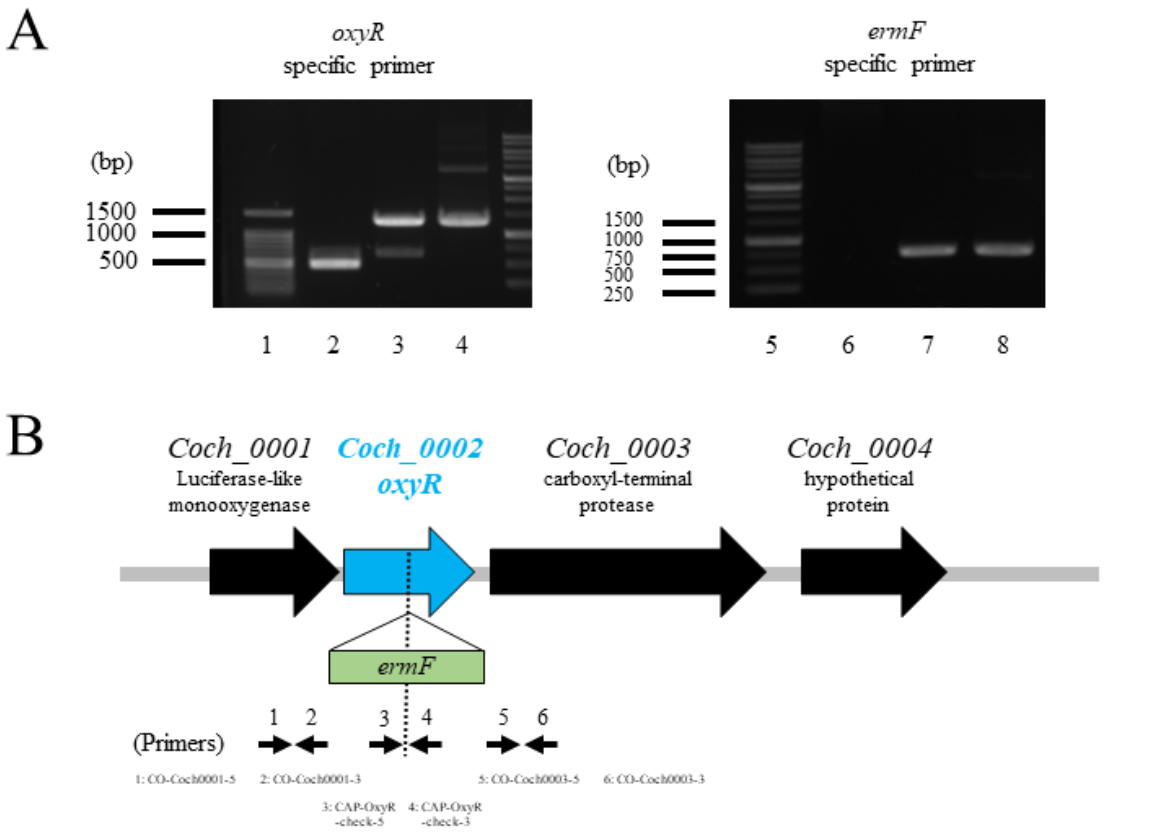

A
oxyR
specific primer
ermF
specific primer
(bp)
1500
1000
500
(bp)
1500
1000
750
500
250
1 2 3 4
5 6 7 8
B
Coch_0001
Luciferase-like
monooxygenase
Coch_0002
oxyR
Coch_0003
carboxyl-terminal
protease
Coch_0004
hypothetical
protein
ermF
(Primers)
1 2 3 4 5 6
1: CO-Coch0001-5
2: CO-Coch0001-3
3: CAP-OxyR -check-5
4: CAP-OxyR -check-3
5: CO-Coch0003-5
6: CO-Coch0003-3
C
RT RT
(bp) (+) (-) (+) (-)
1500
1000
500
CO-16SrRNA-5
-CO-16SrRNA-3
(1)
Primer 1
- Primer 4
(2)
Primer 3
- Primer 6
(3)
1 2 3 4 5 6
RT RT
(bp) (+) (-) (+) (-)
(4)
Coch_0001
(5)
oxyR
(6)
Coch_0003
1 2 3 4 5 6

Figure 1

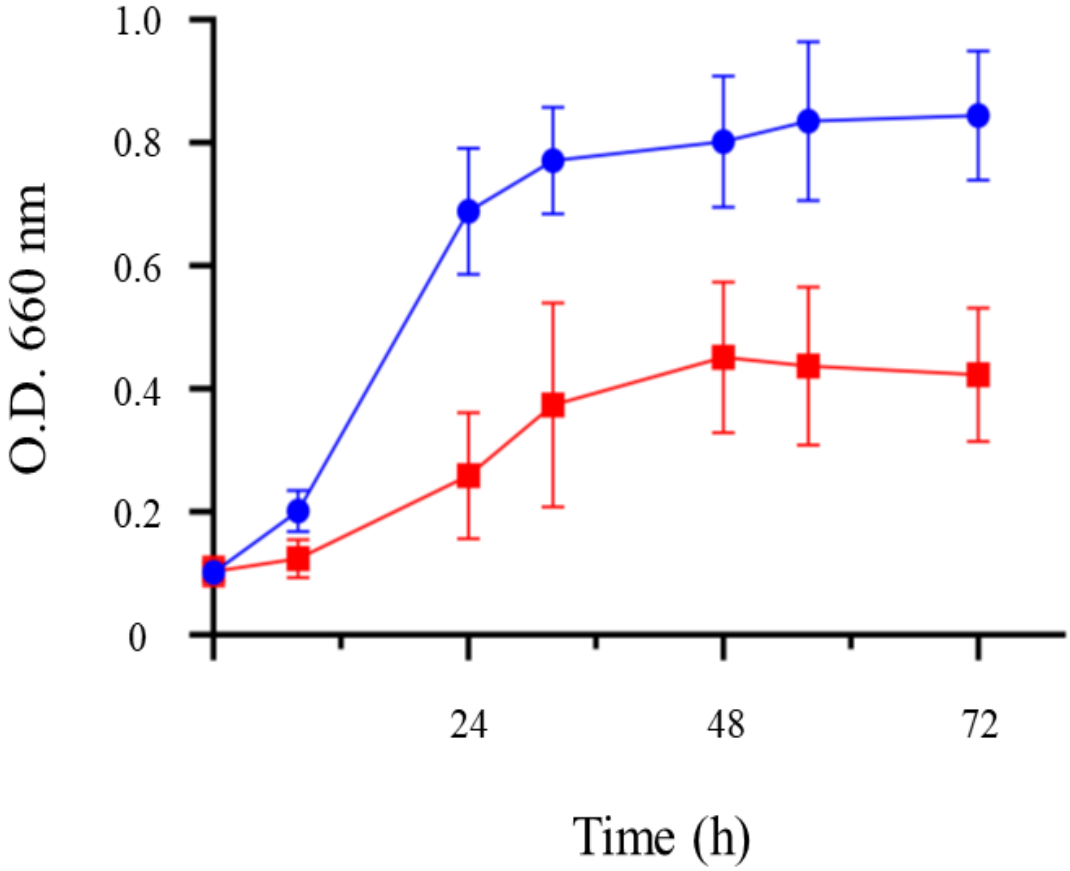
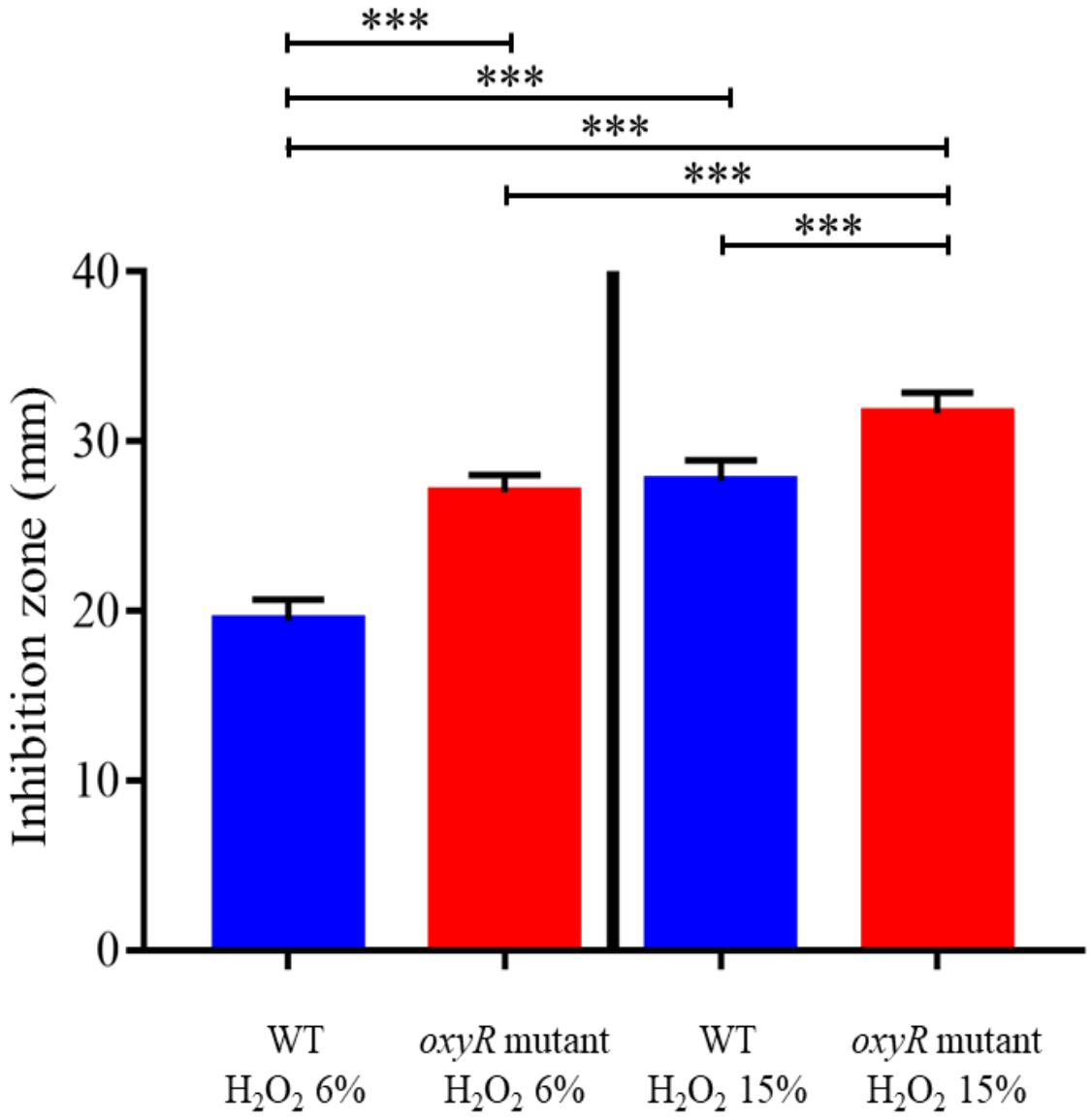


Figure 2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Inhibition zone (mm)
40
30
20
10
0
WT
H2O2 6%
oxyR mutant
H2O2 6%
WT
H2O2 15%
oxyR mutant
H2O2 15%

Figure 3

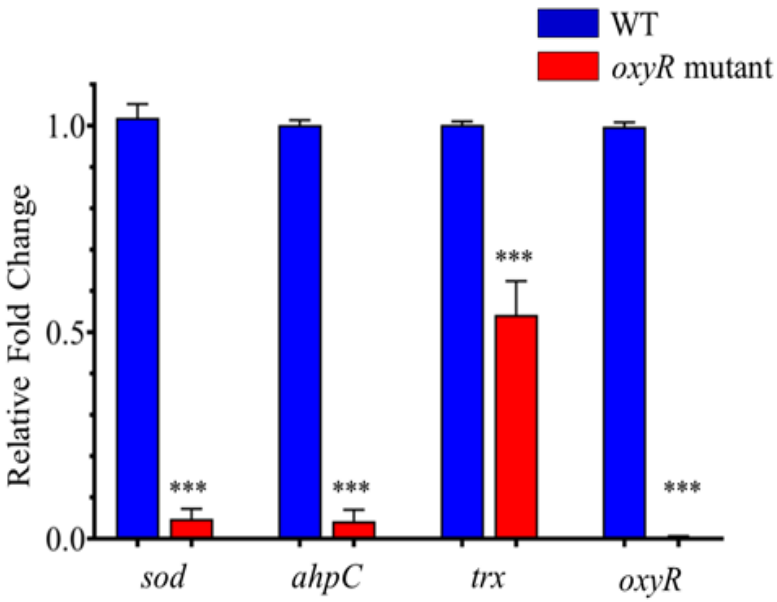
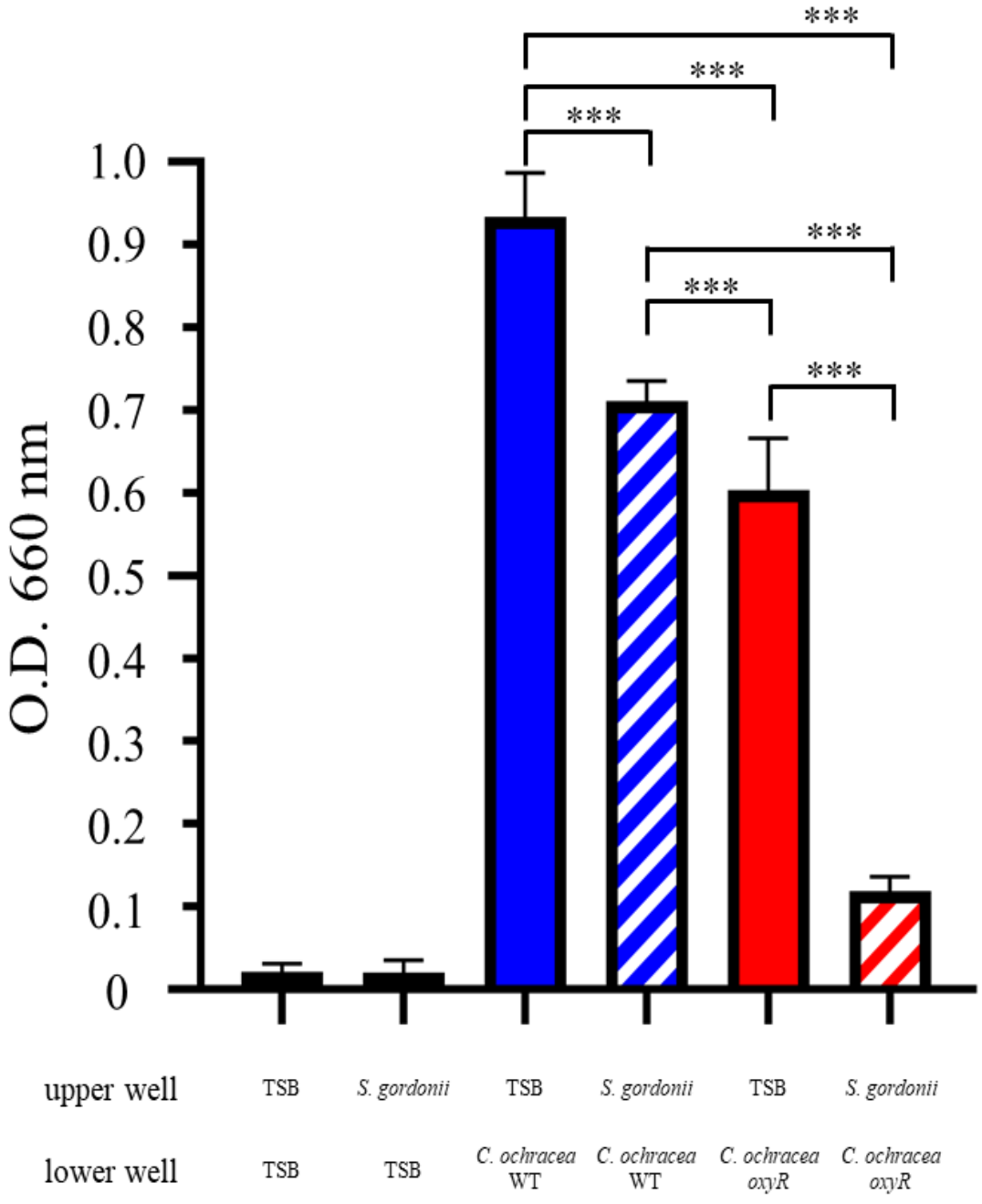

WT
oxyR mutant
Relative Fold Change
1.0
0.5
0.0
***
***
***
***
sod
ahpC
trx
oxyR

Figure 4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1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O.D. 660 nm
upper well
TSB
S. gordonii
TSB
S. gordonii
TSB
S. gordonii
lower well
TSB
TSB
C. ochracea WT
C. ochracea WT
C. ochracea oxyR
C. ochracea oxyR

Figure 5