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Alkyl hydroperoxide peroxidase subunit C (*ahpC*) protects against organic peroxides but does not affect the virulence of *Porphyromonas gingivalis* W83

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The cloned *Porphyromonas gingivalis* alkyl hydroperoxide reductase (*ahpC*) gene complemented an *ahpC* defect in *Escherichia coli*. To study the role of *ahpC* in protecting against oxidative stress in *P. gingivalis* a 1.8 kb fragment containing the *ahpC* gene was amplified from the chromosome of *P. gingivalis* W83. This gene was insertionally inactivated using the *ermF-ermAM* antibiotic resistance cassette and used to create a *ahpC*-deficient mutant by allelic exchange. One mutant strain, designated FLL141, demonstrated no change in the growth rate, black pigmentation, beta-hemolysis or level of proteolytic activity compared to the parent strain. Although *P. gingivalis* FLL141 was more sensitive to hydrogen peroxide than the parent strain, there was no change in its virulence potential in the mouse model compared to the wild-type strain. These findings suggest that the *ahpC* gene plays a role in peroxide resistance in *P. gingivalis* but does not contribute significantly to virulence.

Key words: *ahpC*; alkyl hydroperoxide reductase; oxidative stress; *Porphyromonas gingivalis*; virulence

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Porphyromonas gingivalis, a black-pigmented, gram-negative anaerobe, has been implicated as an important etiologic agent in adult periodontitis. This organism possesses several putative virulence factors (e.g. hydrolytic enzymes, fimbriae, hemagglutinin, capsule, and lipopolysaccharide) that can directly affect the periodontium or elicit host functions that result in destruction typical of advanced periodontitis (15, 21, 25). In addition, colonization of the inflammatory microenvironment of the periodontal pocket by this organism would require an ability to overcome oxidative stress resulting from bactericidal metabolites generated from neutrophils (2) and occasional exposure to air (4). Reactive oxygen metabolites such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxide radical (OH^-) are major components of bactericidal activity of polymorphonuclear leukocytes (10, 35). The toxic oxygen metabolites can be neutralized by superoxide dismutase (SOD), catalase, and peroxidase, all of which are generally expressed by aerobic and many anaerobic bacteria (6, 33). Although *P. gingivalis* is oxygen tolerant (4) and expresses SOD activity (11, 24, 25), it is lacking catalase activity (3, 11, 24, 25).

Protection against organic peroxides in bacteria is facilitated by the peroxide-scavenging enzyme ahp (alkyl hydroperoxide reductase) (33). This enzyme consists of two components, a 22 kDa protein (*ahpC*) with peroxidase activity and a 57 kDa flavoprotein (*ahpF*) (38). *ahpC* has been shown to act as a specific alkyl hydroperoxide-scavenging enzyme for protection against oxygen radical damage (16). The *ahpC* homolog in *P. gingivalis* was identified from the *P. gingivalis* genome project (27). This gene is approximately 63% identical to the *ahpC* gene in *Bacteroides fragilis,* which also demonstrates a peroxide-scavenging function (34). Using the cloned *ahpC* gene, a mutant defective in that gene was constructed by allelic exchange. This strain,

designated *P. gingivalis* FLL141, exhibited increased sensitivity to hydrogen peroxide. However, in the mouse model of virulence, there was no change in the pathogenic potential of the *ahpC*-defective mutant compared to the wild-type strain. These results suggest that the *ahpC* homolog in *P. gingivalis* may play an important role in protecting this organism against toxic hydroperoxides but does not significantly affect its virulence.

Materials and methods

Bacterial strains and culture conditions

P. gingivalis was grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) supplemented with hemin (5 μg/ml), and cysteine (0.1%). *Escherichia coli* strains were grown in Luria-Bertani (LB) broth. Unless otherwise stated all cultures were incubated at 37°C. *P. gingivalis* was maintained in an anaerobic chamber (Coy Manufacturing, Ann Arbor, MI) in 10% H_2, 10% CO_2, and 80% N_2. Growth rates for *P. gingivalis* strains were determined spectrophotometrically (optical density at 600 nm). Hemolysis and pigmentation were determined by plating *P. gingivalis* on Brucella Blood agar (Anaerobe Systems, Morgan Hill, CA) which was incubated for 7–10 days.

DNA isolation and analysis

P. gingivalis chromosomal DNA was prepared by the method of Marmur (19). For plasmid DNA analysis, DNA extraction was performed following the alkaline lysis procedure of Birnboim & Doly (7). For large scale preparation, plasmids were purified using the Qiagen plasmid midi kit as per the manufacturer's instructions (Qiagen, Valencia, CA). DNA was digested by restriction enzymes as specified by the manufacturer (Roche Applied Science, Indianapolis, IN). DNA fragments were separated by electrophoresis (0.7% agarose; TAE buffer [0.4 M Tris-acetate, 0.001 M EDTA, pH 8.0]) and purified using the Geneclean II kit according to the manufacturer's recommendations (Qbiogene, Carlsbad, CA).

DNA sequencing

Nucleotide sequences were determined by the dideoxy-chain termination method (12) at the DNA core facility of Loma Linda University (Loma Linda, CA). Oligonucleotide primers used in sequencing reactions were obtained from Invitrogen (Invitrogen Corp., Carlsbad, CA). Nucleotide sequences were analyzed using the Sequencer software package (Gene Codes Corporation, Ann Arbor, MI).

Table 1. Oligonucleotide primers used in this study

Primers	Oligonucleotide sequence	Characteristics
P1	5' GTGAGCTAAATTCATGGC 3'	5' primer upstream of *ahpC* gene
P2	5' ATGCTGACCTCATAGGGA 3'	3' primer downstream of *ahpC* gene
P3	5' ATGACTCCTATCCTGAACACCG 3'	5' primer from ATP of *ahpC* gene
P4	5' TCAATGCTCGGTTTCAGTGT 3'	3' primer from end of *ahpC* gene
P5	5' AGTCGGTGCCTACGCTAG 3'	3' primer from end of *ahpF* gene
P6	5' AAATCAGGTTCTCGATCCCGA 3'	5' primer specific for an intragenic region of *ahpF* gene

Polymerase chain reaction analysis of RNase treated chromosomal DNA from *P. gingivalis*

The polymerase chain reaction (PCR) amplification was performed with the Perkin Elmer Cetus DNA Thermal Cycler (Perkin-Elmer Corporation, Norwalk, CT). The primers used in this study (Table 1) were synthesized at the Nucleic Acid Core Facility at Loma Linda University. The reaction mixture (50 μl), containing 1 μl of template DNA (0.5 μg), 1 μM of each primer, 0.2 mM of dNTPs in 1X Expand High Fidelity System buffer, was denatured for 2 min at 94°C, then 1.73 U of Expand High Fidelity System enzyme was added (Roche Applied Science). The PCR reaction consisted of 30 cycles with a temperature profile of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. The final extension was performed at 72°C for 7 min. The PCR amplified DNA was then identified by 1% agarose gel electrophoresis.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of DNAse treated RNA extracted from *P. gingivalis*

Total RNA was extracted from *P. gingivalis* W83 and *P. gingivalis* FLL141 grown to mid-log phase using the Totally RNA kit (Ambion, Austin, TX). The reaction mixture (50 μl) contained 1 μg of template RNA, 1 μM of each primer, 0.2 mM of dNTPs, 1 mM of magnesium sulfate, 1 unit of reverse transcriptase/platinum Taq DNA polymerase (Invitrogen) for first strand and second strand cDNA synthesis. *Taq* polymerase alone was used for control reactions. The reverse transcription reaction was performed at 50°C for 44 min and then stopped by raising the temperature to 94°C for 2 min. PCR amplification was performed with a Perkin-Elmer Cetus DNA Thermal Cycler. The amplification consisted of 30 cycles with a temperature profile of 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min. The final products were analyzed by 1% agarose gel electrophoresis.

Cloning and expression of *P. gingivalis ahpC* gene

The 0.5 kb *ahpC* gene was amplified by PCR from *P. gingivalis* chromosomal DNA using primers P1 and P2 (Table 1). The *ahpC* gene was TOPO® TA cloned into the pTrcHis2 expression vector (Invitrogen) and transformed into *E. coli* TOP10 cells. The transformed *E. coli* cells were shaken at 37°C for 1 h and 50 μl aliquots were plated on LB agar plates containing ampicillin (100 μg/ml) and incubated overnight at 37°C. Plasmid DNA was extracted from ampicillin-resistant colonies and digested with *EcoRI* (New England Biolabs, Beverly, MA) to confirm the orientation of the *ahpC* gene. One plasmid with the *ahpC* gene in the correct orientation was chosen and designated pFLL302.1. The expression of the *ahpC* gene was then induced for 3 h using 1 mM IPTG (isopropyl-beta-D-galactopyranoside). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) was then performed with a 4–12% Bis Tris separating gel (Invitrogen). The samples were electrophoresed at constant voltage of 200 V for 45 min and stained with Simply blue stain (Invitrogen) for 1 h, destained in distilled water for 30 min and then visualized.

Complementation of *E. coli ahpC*-defective mutant

E. coli J1370 (36), an *ahpC*-defective mutant, was transformed with the plasmid pFLL302.1 carrying the *P. gingivalis ahpC* homolog. Transformed *E. coli* cells were plated on LB agar plates with ampicillin (100 μg/ml) and grown overnight at 37°C. One ampicillin-resistant transformant carrying pFLL302.1 was compared with *E. coli* strains J1370 and MG1655 (36), the wild-type strain (generously donated by Dr. Jim Imlay, University of Illinois, Urbana, IL). All strains were grown overnight in LB broth. The overnight cultures, 100 μl of each, were sub-

cultured into 100 ml LB broth and grown to mid log phase (optical density [OD] 0.4). Aliquots of 100 µl of each of the cultures were then plated onto a LB agar with and without 1 mM IPTG. A 3% H_2O_2 solution of 10 µl was then placed on blank 6 mm antibiotic disks and placed in the center of the plate. Blank disks with distilled water were used as controls. The plates were then incubated at 37°C overnight and the zones of inhibition were measured. All experiments were performed in triplicate.

Mutagenesis of the cloned *P. gingivalis ahpC* homolog

A 1.8 kb fragment containing the *ahpC* gene and flanking regions was amplified by PCR using P1 and P2 primers (Table 1). The 1.8 kb fragment was digested with *AccI* and *EcoRV*, purified using Geneclean II kit (Qbiogene, Carlsbad, CA) and ligated into the *AccI-Sma I* site of pBluescript KS + (Stratagene, La Jolla, CA). The recombinant plasmid carrying the cloned *ahpC* gene and flanking DNA was digested with *MscI* and dephosphorylated. pVA2198 containing the 2.1 kb *ermF-ermAM* cassette was digested with *SacI and BamHI* and treated with Klenow (Roche, Indianapolis, IN). The blunt ended *ermF-ermAM* antibiotic cassette was ligated into the *MscI* site of pFLL139 (Fig. 1). The recombinant plasmid pFLL140 was used as a donor to electrophorese *P. gingivalis* W83.

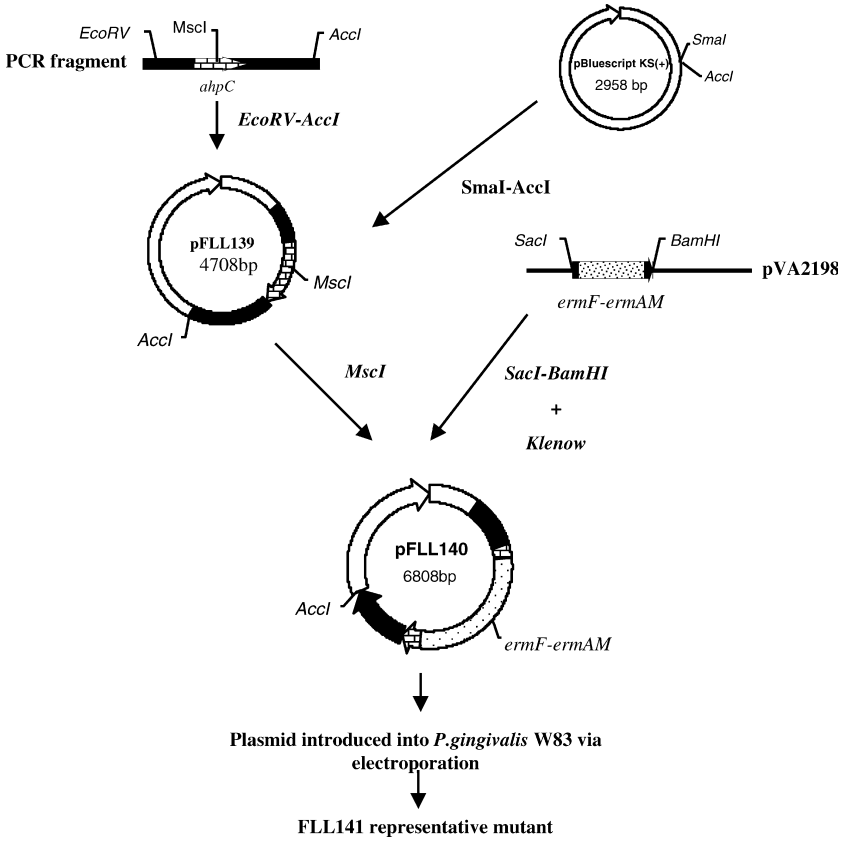

Fig. 1. Construction of an alkyl hydroperoxide reductase mutant by allelic exchange. The *P. gingivalis ahpC* homolog was amplified by PCR from the chromosome of strain W83. pFLL140 contained part of the *ahpC* gene interrupted by the *ermF-ermAM* cassette. This plasmid was introduced in *P. gingivalis* W83 by electroporation. A reciprocal recombination event between areas of homology on the target cell's chromosome and regions flanking the *ermF-ermAM* cassette of pFLL140 replaced the wild-type *ahpC* gene with a fragment containing the *ermF-ermAM* cassette. *P. gingivalis* cells were incubated about 12 h post electrophoration and plated on supplemented BHI with clindamycin (0.5 µg/ml) for 7–10 days at 37°C.

Electroporation of *P. gingivalis*

Electroporation of cells was performed as previously reported (14). Briefly, 1 ml of an actively growing culture of *P. gingivalis* was used to inoculate 10 ml BHI broth supplemented with hemin and vitamin K, which then was incubated overnight at 37°C. Warmed medium (37°C), 70 ml, was then inoculated with 3 ml of the overnight culture and incubated for an additional 4 h. The cells were harvested by centrifugation at 2600 × *g* for 7 min at 4°C and washed in 70 ml of electroporation buffer (10% glycerol and 1 mM $MgCl_2$ filter sterilized and stored at 4°C), and the pellet suspended in 0.5 ml of electroporation buffer. A 100 µl aliquot of cells plus 1 µg of DNA was placed in a sterile electrode cuvette (0.2 cm gap). The cells were pulsed with a Bio-Rad gene pulser (Bio-Rad, Hercules, CA) for 4.2 ms at 2500 V (12.5 kV/cm) and then incubated on ice for 3 min. The cell suspension was added to 0.5 ml of BHI broth supplemented with hemin and vitamin K and incubated for approximately 16 h. A 100 µl sample was plated on solid medium containing clindamycin (5 µg/ml) and incubated anaerobically at 37°C for 7–10 days.

Sensitivity testing

P. gingivalis W83 and *P. gingivalis* FLL141, the *ahpC* defective isogenic mutant strain, were tested for sensitivity to hydrogen peroxide and cumene hydrogen peroxide. *P. gingivalis* W83 and FLL141 were grown to early log phase (OD_{600} of 0.2) in BHI broth supplemented with hemin and vitamin K. H_2O_2 or cumene hydrogen peroxide at concentrations of 0.1 mM, 0.25 mM, 0.5 mM or 1 mM was then added to the cell cultures and further incubated for 16 h. At 4-h intervals, the optical density (600 nm) of the cells was determined. Cell cultures without peroxides were used as controls.

Proteolytic assay

Preparations of whole cell culture media were made as previously reported (1). The presence of Arg-X and Lys-X activity was determined using a microplate reader (Bio-Rad Laboratories) according to the methods of Potempa et al. (29).

Virulence testing

P. gingivalis strain W83 and the isogenic mutant strain FLL141 were tested for invasiveness in a murine model as previously described (14). Briefly, 1 ml of an actively growing culture of *P. gingivalis* was used to inoculate 15 ml of BHI broth supplemented with hemin (1 µg/ml), vitamin K (1 µg/ml), and cystine (0.5 µg/ml) and incubated overnight at 37°C. To 85 ml

Table 2. Complementation of *E. coli ahpC* mutant with *P. gingivalis ahpC*

E. coli strains	Diameter of disk inhibition zones (cm)	
	3% hydrogen peroxide + IPTG	– IPTG
MG1655 (wild-type)	0.6	0.6
J1370 (*ahpCF*::kan)	1.7	1.6
J1370 + *P. gingivalis ahpC*	0.7	1.4

of prewarmed BHI (37°C) was added the 15 ml of overnight culture, incubated for 6 h and then used to inoculate 900 ml of prewarmed BHI broth and further incubated for 24 h at 37°C. The cells were centrifuged and washed in sterile phosphate-buffered saline (PBS) and adjusted to the desired concentration in PBS. All mice were challenged by subcutaneous injections of 0.1 ml of bacterial suspension at two sites on the dorsal surface. Mice were then examined daily to assess their general health status, as well as the presence and location of lesions. Weights were determined for all surviving mice. These experiments were performed under the authorization of an institutionally approved protocol (18).

Results

Complementation of *E. coli ahpC*-defective mutant with *P. gingivalis ahpC* homolog

Chromosomal DNA from *P. gingivalis* W83 was subject to PCR analysis using primers (P3 and P4; Table 1) that would amplify the 0.5 kb *ahpC* gene. This purified DNA fragment was inserted into pTrcHis2 under control of the *trc* (9, 23) promoter, confirmed by nucleotide sequencing analysis and designated pFLL302.1. *E. coli* J1370, an *ahpC*-defective mutant transformed with the expression plasmid pFLL302.1 (*ahpC* homolog), was exposed to hydrogen peroxide (Table 2). *E. coli* J1370 with the plasmid pFLL302.1 showed a similar level of sensitivity to hydrogen peroxide as the wild-type strain (*E. coli* MG1655) in the presence of IPTG. In contrast, in the absence of IPTG, *E. coli* J1370 carrying pFLL302.1 demonstrated a similar sensitivity to hydrogen peroxide as J1370 (Table 2). To further confirm whether the *P. gingivalis* AhpC was expressed in *E. coli*, a cell lysate of *E. coli* J1370 carrying pFLL302.1 was analyzed by SDS-PAGE. As shown in Fig. 2 (lane 1), the predicted 22 kDa protein was observed to be up-regulated in the presence of IPTG. Collectively, these data indicate that the *P. gingivalis ahpC* homolog can complement an *E. coli* AhpC-defective mutant and may function similarly to *E. coli ahpC* in detoxifying hydrogen peroxide.

Inactivation of the *ahpC* gene in *P. gingivalis* W83 by allelic exchange mutagenesis

An isogenic *P. gingivalis* W83 mutant defective in the *ahpC* gene was constructed by allelic exchange mutagenesis as depicted in Fig. 1. The recombinant plasmid pFLL140, which carries the *ermF-ermAM* cassette in the unique *Msc*I site of the *ahpC* gene, was used as a donor to electroporate *P. gingivalis* W83. Because the plasmid cannot replicate in *P. gingivalis*, we predicted that two double crossover events between the regions flanking the *erm* marker and the wild-type gene on the chromosome would result in replacement of a segment of the wild-type gene, with the fragment conferring clindamycin resistance. Following electroporation and plating on selective media, we detected 50 clindamycin-resistant colonies after 6 days of incubation. Randomly chosen colonies were further plated on Brucella blood agar (Anaerobic Systems Inc., San Jose, CA) to determine any pleiotropic phenotypic effects of the clindamycin-resistant mutants. Similar to the wild-type strain, all of the clindamycin-resistant mutants displayed a β-hemolytic and black-pigmented phenotype. Chromosomal DNA from three randomly chosen colonies and the wild-type W83 strain was subjected to PCR using primers for the 1.8 kb *ahpC* gene fragment. If the *ahpC* gene was interrupted by the *ermF-ermAM* cassette, a 3.9 kb fragment was expected to be amplified. As shown in Fig. 3, the expected 3.9 kb fragment and 1.8 kb fragment was observed in the three clindamycin-resistant strains and the wild-type W83, respectively. Taken together these results suggest the insertional inactivation of the chromosomal *ahpC* gene with the 2.1 kb *ermF-ermAM* antibiotic cassette. One strain from the three *ahpC*-defective mutants, designated *P. gingivalis* FLL141, was randomly chosen for further studies.

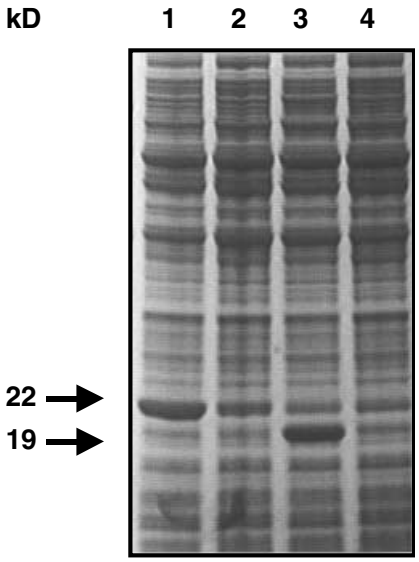

Fig. 2. Expression of *P. gingivalis ahpC* gene in *E. coli. E. coli* TOP10 cells carrying the recombinant plasmids were grown to mid log phase and induced with 1 mM IPTG for 3 h. Samples were separated by SDS PAGE and stained with Simply Blue®. Lane 1: *E. coli* carrying plasmid pFLL302.1 (*ahpC* homolog) induced with 1 mM IPTG for 3 h. Lane 2: *E. coli* carrying plasmid pFLL302.1 in the absence of IPTG. Lane 3: *E. coli* carrying plasmid pFLL300.1 expressing unrelated protein (bacterioferritin comigratory protein) induced with 1mM IPTG. Lane 4: *E. coli* carrying plasmid pFLL300.1 in the absence of IPTG. Each lane contains 20 μg of protein.

RT-PCR analysis of *P. gingivalis* FLL141 and W83

To further confirm the inactivation of the *ahpC* gene in *P. gingivalis* FLL141 and to determine whether the *ahpC* and *aphF* genes were part of the same transcriptional

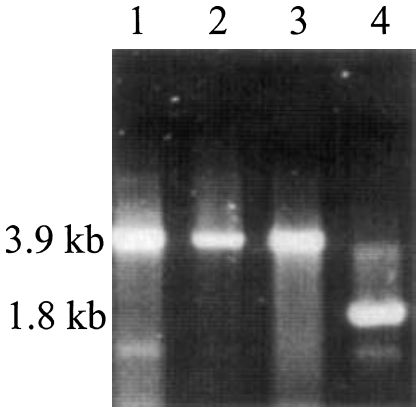

Fig. 3. PCR analysis of allelic exchange mutants of *P. gingivalis* carrying the *ahpC* gene inactivated with the ermF-ermAM cassette. Oligonucleotide primers specific for the *ahpC* gene (P1 and P2; Table 1) were used to amplify that gene from total cellular DNA from *P. gingivalis*. Lane 1: *P. gingivalis* FLL141 (*ahpC*::*ermF-ermAM*). Lane 2: *P. gingivalis* FLL141.1 (*ahpC*::*ermF-ermAM*). Lane 3: *P. gingivalis* FLL141.2 (*ahpC*::*ermF-ermAM*). Lane 4: *P. gingivalis* W83 (wild-type).

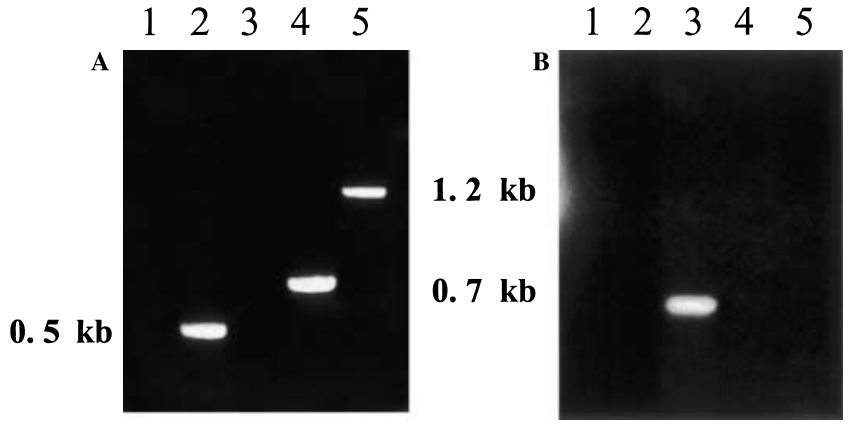

Fig. 4. RT-PCR analysis of RNA extracted from *P. gingivalis*. Total RNA was extracted from W83 (Panel A) or FLL141 (Panel B) grown to mid-log phase using the Totally RNA kit (Ambion, Austin TX). *Panel A.* Lane 1: intragenic primers (P3 and P4, Table 1) for *ahpC* minus reverse transcriptase (negative control). Lane 2: intragenic primers (P3 and P4; Table 1) for *ahpC* plus reverse transcriptase. Lane 3: intragenic primers (P6 and P5, Table 1) for *ahpF* minus reverse transcriptase (negative control). Lane 4: intragenic primers (P6 and P5; Table 1) for *ahpF* plus reverse transcriptase. Lane 5: 5′ primer (P3; Table 1) from *ahpC* and 3′ primer (P5; Table 1) from *ahpF*. *Panel B.* Lanes 1: intragenic primers (P3 and P4, Table 1) for *ahpC* plus reverse transcriptase. Lane 2: intragenic primers (P3 and P4; Table 1) for *ahpC* minus reverse transcriptase (negative control). Lane 3: intragenic primers (P6 and P5; Table 1) for *ahpF* plus reverse transcriptase. Lane 4: intragenic primers (P6 and P5; Table 1) for *ahpF* minus reverse transcriptase (negative control). Lane 5: 5′ primer (P3; Table 1) from *ahpC* and 3′ primer (P5; Table 1) from *ahpF*. All lanes contain 5 μl of the amplified mixture.

unit, total RNA was isolated from the wild-type W83 and the *ahpC*-defective mutant FLL141 grown to mid log phase. Specific intragenic oligonucleotide primers for the *ahpC* and *ahpF* genes (Table 1) were used in RT-PCR analysis. Since primers specific for the *ahpC* and *ahpF* genes would yield 0.5 kb and 0.7 kb fragments, respectively, a 0.5 kb fragment should be amplified in the wild-type strain but should be missing in *P. gingivalis* FLL141. Furthermore, if both genes are part of the same transcriptional unit, a 1.2 kb fragment should be amplified in the wild-type strain which should be absent in the mutant strain. As shown in Fig. 4, fragments of 0.5 kb and 0.7 kb in size were amplified in the wild-type strain using *ahpC* and *ahpF* specific primers, respectively. A 1.2 kb fragment was amplified in the wild-type when the *ahpC*-specific 5′ primer and the *ahpF*-specific-3′ primer were used. In contrast to these results, only a 0.7 kb fragment using the *ahpF*-specific primers was amplified in the *ahpC*-defective mutant FLL141. No amplified fragments were observed for either the wild-type strain or the mutant when reverse transcriptase was absent in the reaction mix (Fig. 4). This confirms the inactivation of the *ahpC* gene in *P. gingivalis* FLL141 and may suggest cotranscription of the *ahpC* and *ahpF* genes in *P. gingivalis*.

Proteolytic activity of *P. gingivalis* FLL141

Strains of *P. gingivalis* W83 and *P. gingivalis* FLL141 were assayed for proteolytic activity using α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) and Z-lysine *ρ*-nitroanilide. In late exponential growth phase cultures, both arginine-X and lysine-X proteolytic activities of *P. gingivalis* FLL141 were 99.8% of the activity compared to the wild-type W83. These data suggest that under the same physiological conditions, the proteolytic profile for *P. gingivalis* W83 and *P. gingivalis* FLL141 was unaltered.

Sensitivity of *P. gingivalis* FLL141 to organic peroxides

P. gingivalis W83 and the isogenic mutant *P. gingivalis* FLL141 were assessed for sensitivity to peroxides. Hydrogen peroxide and cumene hydroperoxide were prepared at various concentrations in BHI. In contrast to the parent strain, *P. gingivalis* FLL141 demonstrated an 8-fold greater sensitivity to hydrogen peroxide at a concentration of 0.5 mM (Fig. 5). The threshold for a difference in sensitivity to hydrogen peroxide between the *ahpC*-defective mutant and the wild-type was reached at 0.5 mM. Similarly, both strains showed the same sensitivity to cumene

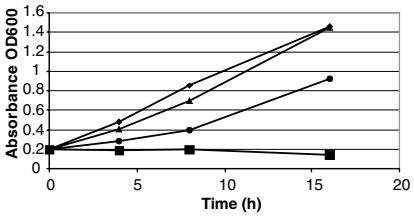

Fig. 5. Sensitivity of *P. gingivalis ahpC* mutant to hydrogen peroxide and t-butyl hydrogen peroxide. *P. gingivalis* was grown to early log phase (OD_{600} of 0.2) in BHI broth supplemented with hemin and vitamin K. H_2O_2 (0.5 mM; ● W83, ■ FLL141) was then added to the cell cultures and further incubated for 16 h. Cell cultures without H_2O_2 (◆ W83, ▲ FLL141) were used as controls. The results shown are representative of three independent experiments.

hydroperoxide at all concentrations tested (data not shown). Taken together, this suggests that *P. gingivalis* FLL141 has an increased sensitivity to organic peroxides compared to the wild-type W83.

Virulence testing of *P. gingivalis* FLL141

Protection against peroxide damage during the course of an infection plays a significant role in pathogenesis (26). Since *P. gingivalis* FLL141 was more sensitive to peroxides than W83, its virulence potential in the mouse model was assessed. We had previously shown that the LD_{50} of *P. gingivalis* W83 in the BALB/c mouse model is approximately 3×10^9 (14). Within 48 h, two of four animals challenged with *P. gingivalis* (wild-type) at a dose of 3×10^9 had developed spreading, ulcerative abdominal lesions and died. The surviving animals appeared cachectic and hunched with ruffled hair; however, all recovered before the end of the 14-day observation period. Similarly, at 48 h, five of 10 animals challenged with *P. gingivalis* FLL141 (*ahpC*-defective mutant) at a dose of 3×10^9 had developed spreading, ulcerative abdominal lesions and died. All of the surviving animals appeared cachectic and hunched with ruffled hair and also recovered during the 14-day observation period. In both groups, the lesions began healing by day 6 post-challenge. All animals challenged with a 1×10^{10} dose of the wild-type (five of five) or the *P. gingivalis* FLL141 (10 of 10) died by 48 h. Although the mice did not display lesions at the dorsal surface site of injection, all had developed spreading, ulcerative abdominal lesions. These data suggest that there is no detectable difference in the virulence potential of the *ahpC*-defective mutant *P. gingivalis* FLL141 compared with the parent strain.

Discussion

Bacterial exposure to reactive oxygen intermediates results in oxidative stress that causes damage to nucleic acids, proteins, and cell membranes. In several bacterial strains including *Salmonella typhimurium*, *E. coli*, and the anaerobic bacterium *B. fragilis*, this oxidative stress results in the induction of several proteins that help to reduce the detrimental effects of these reactive oxygen species (32, 37, 43). AhpC, one such protein, is essential for the elimination of alkyl hydroperoxides (38). The inflammatory nature of the *P. gingivalis*-associated diseases suggests that the ability to overcome oxidative stress may be critical in the pathogenesis of this anaerobic organism. Thus it was a logical hypothesis that the *ahpC* gene in this organism might play a significant role in its pathogenicity.

An analysis of the *P. gingivalis* genome indicated the presence of an *ahpC* homolog that appears to have a similar genetic architecture to that observed in other bacteria (27). When expressed in *E. coli*, this gene encoded for a protein of the expected size and complemented the *ahpC* defect in that strain. This indicated that the *P. gingivalis ahpC* homolog that shares 75% similarity with the *E. coli ahpC* gene [*http://tigrblast.tigr.org/cmr-blast*] has a similar function in that strain.

In this study, inactivation of the *ahpC* gene in *P. gingivalis* has allowed us to confirm the role of this gene in oxidative stress. A comparison of the sensitivities of the *ahpC*-defective mutant *P. gingivalis* FLL141 and the parent strain to hydrogen peroxide suggests that the *ahpC* gene in *P. gingivalis* plays a significant role in oxidative stress resistance. This is consistent with similar observations in *B. fragilis* and other pathogenic bacteria, where *ahpC* has an important role in the resistance to damage from peroxides (5, 34). Although not determined in this study, it was also shown that in *B. fragilis* this gene is up-regulated by either oxygen exposure or addition of exogenous hydrogen peroxide (34).

RT-PCR analysis of the *ahpC* and *ahpF* genes in *P. gingivalis* in our study showed that they are part of the same transcriptional unit. In addition, a 0.7 kb fragment was revealed in further RT-PCR analysis of the *ahpC*-defective mutant *P. gingivalis* FLL141 using *ahpF*-specific oligonucleotide primers. Taken together, these data suggest that *ahpC and ahpF* could be transcribed as moncistronic and polycistronic RNA. In most bacteria there are two subunits of the alkyl hydroperoxide reductases, a 22 kDa AhpC subunit and a 52 kDa AhpF subunit, responsible for substrate binding and flavoprotein sulfide oxidoreductase activities, respectively (38, 40). AhpC usually reduces the alkyl hydroperoxides to the corresponding alcohols and the AhpF reduces the oxidized AhpC by transferring electrons from NADH or NADPH (30, 40). In a previous report (34) differential monocistronic and polycistronic transcriptional units for *ahpCF* have been demonstrated in *B. fragilis*. In other reports *E. coli* and *S. typhimurium* have been shown to transcribe *ahpCF* mRNA polycistronically (38). The significance, however, of these expression patterns is unclear.

The *ahpC*-defective mutant was black-pigmented and had similar growth rate, and β-hemolytic and proteolytic profiles as the parent strain. While this suggests that the inactivation of the *ahpC* gene did not affect the proteases, one of its major virulence factors, its increased sensitivity to hydrogen peroxide could impact its pathogenicity. Protection against peroxide damage during the course of an infection is important for survival of the invading microorganism, especially during its interaction with macrophages and other polymorphonuclear cells (22). Virulence studies in the mouse model using the parent strain and the isogenic mutant *P. gingivalis* FLL141 suggest that there is no discernible difference in the virulence potential of the *ahpC*-defective mutant compared to the wild-type strain. These data are consistent with similar experiments conducted with a *S. typhimurium ahpC*-defective mutant. Results from these studies confirmed increased sensitivity of the *ahpC* mutant to peroxides *in vitro* but no change in its virulence in a mouse model (41).

The inability of the disrupted *ahpC* gene to affect the virulence potential of *P. gingivalis* could raise questions about its *in vivo* significance. Colonization of the periodontal pocket is often dependent on the coaggregation of several bacterial species (31). These interacting species that are present in the biofilm may reduce the oxygen concentration to levels that can be adequately detoxified by enzymes produced by the anaerobes (8, 20). A recent report by Diaz et al. have demonstrated the importance of *Fusobacterium nucleatum* and *P. gingivalis* coaggregation in protecting against oxidative stress (13). In this study, *F. nucleatum* supported the growth of *P. gingivalis* in an oxygenated and carbon dioxide-depleted environments. In addition to this strategy, it is also possible that other redundant mechanism(s) may be present in *P. gingivalis* that could be up-regulated during the infectious process, and may be important in protection against damage due to oxidative stress. A survey of the *P. gingivalis* genome [http://www.oralgen.lanl.gov/] reveals the presence of several genes (including *oxyR*, *dps*, *bcp* rubrerythrin, and NADH oxidase) that might potentially be involved in mediation of oxidative stress defense. The *bcp and* NADH oxidase genes have been identified as members of the TSA/alkyl-hydroperoxide peroxidase C (AhpC) family (17). The *bcp and* NADH oxidase genes are widespread among pathogenic bacteria including *P. gingivalis* and function in *E. coli* and *Amphibacillis xylanus*, respectively, as a general hydroperoxide peroxidases (17, 28). It has been recently shown that both Dps and rubrerythrin both function in the detoxification of peroxides and contribute significantly to the survivability of *P. gingivalis* (39, 42). Their significance in the virulence potential of *P. gingivalis* is unknown. In addition, *oxyR* has been identified as a regulator of many oxidative stress genes including *ahpCF*, *dps*, and *katG* (not present in *P. gingivalis*), in the peroxide response of a similar anaerobe, *B. fragilis* (32). Generally, in obligate anaerobes and some facultative anaerobes, *oxyR* appears to be up-regulated in situations where other oxidative stress genes are impaired (10). However, the function of the OxyR protein in *P. gingivalis* is still unknown.

In conclusion, we have shown that the *P. gingivalis ahpC* gene can be expressed in *E. coli* and is functionally similar to the AhpC of this organism in its protection against oxidative stress. While the *P. gingivalis ahpC* defective mutant showed increased sensitivity to organic peroxides when compared to the wild-type strain, this mutant had a similar virulence profile in a mouse model, suggesting there might be multiple mechanisms for oxidative stress resistance. Further characterization of the relationship between the *ahpC* gene and the other putative oxidative stress defense genes in *P. gingivalis* should give us insight into the possible mechanism(s) for oxidative stress resistance in anaerobes.

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