

The *bcp* gene in the *bcp-recA-vimA-vimE-vimF* operon is important in oxidative stress resistance in *Porphyromonas gingivalis* W83

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SUMMARY

The ability of *Porphyromonas gingivalis* to overcome oxidative stress in the inflammatory environment of the periodontal pocket is critical for its survival. We have previously demonstrated that the *recA* locus, which carries the bacterioferritin co-migratory protein (*bcp*) gene and has a unique genetic architecture, plays a role in virulence regulation and oxidative stress resistance in *P. gingivalis*. To further characterize the *bcp* gene, which was confirmed to be part of the *bcp-recA-vimA-vimE-vimF* operon, we created a *P. gingivalis* *bcp*-defective isogenic mutant (FLL302) by allelic exchange. Compared with the wild-type, FLL302 had a similar growth rate, black pigmentation, β -hemolysis and UV sensitivity. Although there was no change in the distribution of gingipain activity, there was a 30% reduction in both Arg-X and Lys-X activities in the mutant strain compared with the wild-type. When exposed to 0.25 mM hydrogen peroxide, *P. gingivalis* FLL302 was more sensitive than the wild-type. In addition, the cloned *P. gingivalis* *bcp* gene increased resistance to 0.25 mM hydrogen peroxide in a *bcp*-defective *Escherichia coli* mutant. The mutant also demonstrated decreased aerotolerance when compared with the wild-type. *Porphyromonas gingivalis* FLL302 and the

wild-type strain had similar virulence profiles in a mouse model of virulence. These observations suggest that the *bcp* gene may play a role in oxidative stress resistance but has a decreased functional significance in the pathogenic potential of *P. gingivalis*.

INTRODUCTION

Porphyromonas gingivalis is a black-pigmented, gram-negative bacillus commonly identified as a major etiological agent in adult periodontitis. This periodontal pathogen possesses several 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 (Genco & Slots, 1984; Mayrand & Holt, 1988; Nakayama, 1994). Survival of this organism in the periodontal pocket would necessitate mechanisms to surmount oxidative stress caused by exposure to air or reactive oxygen intermediates such as hydrogen peroxide (H₂O₂), superoxide (O₂⁻) and the hydroxyl radical (OH⁻) generated by neutrophils and macrophages (Chapple, 1996; Salyers, 1994). Oxidative stress is defined as a perturbation in the balance

between pro-oxidants and antioxidants in favor of the pro-oxidants. Two major mechanisms used by bacteria to overcome oxidative stress include the use of (i) endonuclease gene products encoded by *nei*, *nth*, *fpg* and *mutY* (Michaels & Miller, 1992; Grollman & Moriya, 1993; Nash *et al.*, 1996; Radicella & Boiteux, 1997; Boiteux & Radicella, 1999) and (ii) antioxidant enzymes such as superoxide dismutase, alkyl hydroperoxidase subunits C and F (AhpC/F) and catalase (Amano *et al.*, 1986, 1992; Nakayama, 1994; Baillon *et al.*, 1999; Rocha & Smith, 1999; Springer *et al.*, 2001). Toxic oxygen metabolites can be neutralized by superoxide dismutase, catalase and peroxidase, all of which are generally expressed by aerobic and many anaerobic bacteria (Beaman & Beaman, 1984; Rocha *et al.*, 1996). Although *P. gingivalis* is oxygen tolerant (Amano *et al.*, 1988) and expresses superoxide dismutase activity (Nakayama, 1990; Choi *et al.*, 1991; Nakayama, 1994), it is missing a catalase gene (Amano *et al.*, 1986; Nakayama, 1990, 1994; Choi *et al.*, 1991; Nelson *et al.*, 2003). However, protection against oxidative damage may also use another unique mechanism in *P. gingivalis*. Cell surface heme acquisition has been postulated to be a defense mechanism against reactive oxygen species in *P. gingivalis* (Smalley *et al.*, 1998). The storage of the heme on the cell surface, which gives the organism its characteristic black pigmentation, can form μ -oxo dimers in the presence of reactive oxygen species and can give rise to the catalytic degradation of hydrogen peroxide (Smalley *et al.*, 1998, 2000).

In addition to the aforementioned antioxidant enzymes, recent reports have demonstrated a role for the bacterioferritin co-migratory protein (Bcp) in oxidative stress resistance (Jeong *et al.*, 2000; Kong *et al.*, 2000; Wang *et al.*, 2005; Limauro *et al.*, 2008). The Bcp is part of the thiol-specific antioxidant/alkyl hydroperoxidase family and functions in a similar way to AhpC in detoxifying hydrogen peroxide (Jeong *et al.*, 2000). The *bcp* gene in *P. gingivalis* has been identified upstream of the *recA*, *vimA*, *vimE* and *vimF* genes and could be part of the same operon (Nelson *et al.*, 2003). Previous reports have attributed the function of DNA repair and protease activation and maturation to the genes in this operon (Abaibou *et al.*, 2001; Fletcher *et al.*, 1997; Vanterpool *et al.*, 2004, 2005a,b). To determine a role for the *P. gingivalis* *bcp* gene in oxidative stress resistance, we cloned and expressed this gene in *Escherichia coli*,

and created a *P. gingivalis* *bcp*-defective mutant by allelic exchange. We now report that the *bcp* homologue in *P. gingivalis* may play an important role in protecting this organism against toxic hydrogen peroxide but does not demonstrate a discernible difference in virulence in the mouse model tested.

METHODS

Bacterial strains and culture conditions

Porphyromonas gingivalis W83 was grown in brain–heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) supplemented with hemin ($5 \mu\text{g ml}^{-1}$), and cysteine (0.1%). The *E. coli* strains were grown in Luria–Bertani (LB) broth. Unless otherwise stated, all cultures were incubated at 37°C. The *P. gingivalis* was maintained in an anaerobic chamber (Coy Manufacturing, Ann Arbor, MI) in 10% H₂, 10% CO₂ and 80% N₂. Growth rates for *P. gingivalis* strains were determined spectrophotometrically (optical density at 600 nm). Hemolysis and pigmentation were determined by incubation of *P. gingivalis* on Brucella Blood agar (Anaerobe Systems, Morgan Hill, CA) for 7–10 days.

DNA isolation and analysis

The *P. gingivalis* chromosomal DNA was prepared by the method of Marmur (1961). For plasmid DNA analysis, DNA extraction was performed following the alkaline lysis procedure of Birnboim & Doly (1979). 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 (Boehringer Mannheim Corporation, Indianapolis, IN). DNA fragments were separated by electrophoresis [1% 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 (Bio 101 Inc., La Jolla, CA). *Porphyromonas gingivalis* chromosomal DNA and the plasmid pVA2198 were digested with *Bam*HI and *Pst*I (Roche, Indianapolis, IN), respectively, as specified by the manufacturer. Southern blot alkaline transfer was performed according to the method of Chomczynski (1992). The polymerase chain reaction (PCR)-amplified 0.5-kilobase (kb) *bcp* (P1 and P2, Table 1) and 2.1-kb *ermF-ermAM* (P3 and P4) genes

Table 1 Oligonucleotide primers used in this study

Primers	Oligonucleotide sequence	Characteristics	References
P1	5' ATGACCTTTTTGTTACCTTTG 3'	<i>bcp</i> 0.5 LEFT	This study
P2	5' TTATCCGTTTAGTATCTGATC 3'	<i>bcp</i> 0.5 RIGHT	This study
P3	5' TATTAGGCCTATAGCTTCGCTATT 3'	<i>ermStu</i> LEFT	This study
P4	5' AATTAGGCCTTAGTAACGTGTAACCTT 3'	<i>ermStu</i> RIGHT	This study
P5	5' CGTCCCGTCTTGGTCTATC3'	<i>bcp</i> PrimEx2	This study
P6	5'GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA 3'	5' RACE Adapter	FirstChoice [®] RLM-RACE Kit Manual
P7	5'GCTGATGGCGAATGAACACTG 3'	5' RACE Outer primer	FirstChoice [®] RLM-RACE Kit Manual
P8	5'CGCGGATCCGAACACTGCGTTTGTGCTGGCTTTGATG 3'	5' RACE Inner primer	FirstChoice [®] RLM-RACE Kit Manual
P9	5' ATGATACAAATAGGAGACCGT 3'	<i>bcp</i> ORF L	This study
P10	5' TATGTTTATCTCCGGAATAAGGACTCTAAGAACCCTAT 3'	<i>bcp</i> M39L	This study
P11	5' ATACAAATAGGAGGCCTTATTCCTGAGATTCTGGGATA 3'	<i>bcp</i> M39R	This study
P12	5' CTCTAAGGCAAATTTAGGAAG 3'	<i>bcp</i> LEFT	This study
P13	5' GATGAAGATACAGTTGTATTG 3'	<i>bcp</i> RIGHT	This study
P14	5' TACCTGTTTTTGTGACCGG 3'	<i>vimA</i> reverse	Abaibou <i>et al.</i> , (2001)
P15	5' ATGCCCATCCCTCTATACCTG 3'	<i>vimA</i> forward	Abaibou <i>et al.</i> , (2001)
P16	5' ATGGCAGAAGAAAAGATACCC 3'	<i>recA</i> forward	Fletcher <i>et al.</i> , (1997)
P17	5' TGAATGTTTGTGCGAATGG 3'	<i>recA</i> reverse	Fletcher <i>et al.</i> , (1997)

were labeled with digoxigenin and used as probes in hybridization experiments. DNA labeling, hybridization and detection were performed using the digoxigenin High prime Labeling and Detection Starter kit II (Roche) according to the manufacturer's instructions.

DNA sequencing

Nucleotide sequences were determined by the dideoxy-chain termination method (Devereux *et al.*, 1984) at the DNA core facility of Loma Linda University (Loma Linda, CA) or at Retrogen (Retrogen, Inc., San Diego, CA). Oligonucleotide primers used in sequencing reactions were obtained from Invitrogen (Invitrogen Corp., Carlsbad, CA) and IDT (Integrated DNA Technologies, Coralville, IA). Nucleotide sequences were analysed using the SEQUENCER software package (Gene Codes Corporation, Ann Arbor, MI).

PCR analysis of RNase-treated chromosomal DNA from *P. gingivalis*

The 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 template DNA (0.5 ng), 1 µM each primer, 0.2 mM dNTPs in 1× Expand High Fidelity System buffer, was denatured for 2 min at 94°C, then 1.73 U Expand High Fidelity System enzyme was added (Roche). The PCR 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 analysis of DNase-treated RNA extracted from *P. gingivalis*

Total RNA was extracted from *P. gingivalis* W83 and *P. gingivalis* FLL302 grown to mid-log phase [optical density at 600 nm (OD₆₀₀) of 0.7] using the RNAwiz RNA kit (Ambion, Austin, TX) as previously described (Johnson *et al.*, 2004). Extracted RNA was subjected to DNase treatment using the DNA-free™ Kit (Ambion) according to the manufacturer's protocol. Reverse transcription and PCR amplification were performed with a Perkin-Elmer Cetus

DNA thermal Cycler (Perkin Elmer Corporation) also as previously described (Abaibou *et al.*, 2001). The final products were analysed by 1% agarose gel electrophoresis.

Transcription start site (TSS) mapping of the *bcp* gene

To map the 5' region of the *bcp* transcript, the First-Choice[®] RLM-RACE kit (Ambion) was used according to the defined protocols. Total RNA harvested from *P. gingivalis* grown to mid-log ($OD_{600} \sim 0.6$) was sequentially treated with calf intestinal phosphatase and tobacco acid phosphatase. A 5' adapter sequence (P6) was ligated to the treated RNA by T4 RNA ligase before the sequence was reverse transcribed. An outer PCR using the 5'RACE outer primer (P7) and the *bcp* reverse primer (P2) was carried out. A nested PCR using the product from the outer PCR as template was performed using the 5'RACE inner primer (P8) and the same *bcp* reverse primer (P2). Minus template and minus tobacco acid phosphatase reactions were run as controls. The resulting inner PCR products were sequenced to reveal the TSS, which would be the nucleotide immediately upstream of the ligated adapter sequence.

Cloning and expression of the *P. gingivalis bcp* gene

Two possible open reading frames (ORF) for the *bcp* gene were amplified using primer pairs P1 and P2 or P9 and P2 by PCR. The amplified fragments for each ORF were TOPO[®] TA cloned into the pTrcHis2 expression vector (Invitrogen) and transformed into *E. coli* TOP10 cells. The transformed *E. coli* cells were incubated with shaking at 37°C for 1 h and 50- μ l aliquots were plated on LB agar plates containing ampicillin (50 μ g ml⁻¹) and incubated overnight at 37°C. Plasmid DNA was extracted from ampicillin-resistant colonies and digested with *Eco*RI (New England Biolabs, Beverly, MA) to confirm the orientation of the *bcp* gene. Two plasmids with the *bcp* gene cloned in opposite orientations relative to each other for each *bcp* ORF were chosen and designated pFLL301.3 or pFLL301.4 (forward orientation) and pFLL301.2 or pFLL301.5 (reverse orientation). The expression of the *bcp* gene was then induced for up

to 5 h using 1 mM isopropyl- β -D-galactopyranoside (IPTG). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was then performed with a 4–12% Bis–Tris separating gel (Invitrogen). The samples were electrophoresed at a constant voltage of 200 V for 45 min then stained with SimplyBlue SafeStain (Invitrogen) for 1 h, destained in distilled water for 30 min and visualized.

Complementation of *E. coli bcp*-defective mutant

The *E. coli* KD2301 (Jeong *et al.*, 2000), a *bcp*-defective mutant, was transformed by electroporation with the plasmid pFLL301.3 or pFLL301.4 (carrying the *bcp* gene in the forward orientation) and pFLL301.2 or pFLL301.5 (carrying the *bcp* gene in the reverse orientation). Additionally, the KD2301 strain was transformed with pFLL26, a plasmid containing a cloned 2.1-kb fragment encoding the *bcp-recA-vimA* genes (Fletcher *et al.*, 1997). Transformed *E. coli* cells were plated on LB agar plates with ampicillin (50 μ g ml⁻¹) and grown overnight at 37°C. One ampicillin-resistant transformant, each carrying the appropriate plasmid, was compared with *E. coli* KD2301 (Jeong *et al.*, 2000). All strains were grown overnight in LB broth; 100 μ l of each of the overnight cultures was subcultured into 100 ml LB broth and grown to mid log-phase (OD_{600} 0.4). Then, 100 μ l aliquots of each of the cultures were then plated onto LB agar with and without 1 mM IPTG. Ten microliters of a 3% hydrogen peroxide solution 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 three times, each in triplicate.

Mutagenesis of the cloned *bcp* gene

To facilitate the subsequent mutagenesis of the *bcp* gene with the *ermF-ermAM* antibiotic cassette, a unique *Stu*I restriction site was created in the *bcp* gene by the PCR-based overlap extension method described previously (Horton *et al.*, 1990, 1993; Horton, 1995). Primers 10 and 11 were engineered with restriction sequences for *Stu*I at the Nucleic Acid Core facility at Loma Linda University. In two separate PCR using primer pairs P11 and P12 and

P10 and P13 (Table 1), fragments of the *bcp* gene with flanking regions (1.0 and 0.8 kb, respectively) were amplified from *P. gingivalis* W83 chromosomal DNA. The amplified DNA fragments were electrophoresed on a 1% agarose gel at 110 V for 45 min, purified by the Gene Clean II kit (Bio 101 Inc.) and then used as a template for the third PCR using primers P12 and P13 (Table 1). The final 1.8-kb PCR amplified fragment with the *bcp* gene and flanking regions was identified by 1% agarose gel electrophoresis and purified. The 1.8-kb fragment containing the *bcp* gene and flanking regions constructed by the PCR-based overlap extension method (Horton *et al.*, 1990, 1993; Horton, 1995) was cloned into the PCR2.1 TOPO[®] TA cloning vector (Invitrogen). The recombinant plasmid carrying the cloned *bcp* gene and flanking DNA (pFLL301.1) was digested with *Stu*I. The pVA2198 containing the 2.1-kb *ermF-ermAM* cassette was digested with *Sac*I and *Bam*HI and treated with Klenow (Roche). The blunt-ended *ermF-ermAM* antibiotic cassette was ligated into the *Stu*I site of pFLL301.1. The recombinant plasmid pFLL301.1 was used as a donor to electroporate *P. gingivalis* W83. Electroporation of *P. gingivalis* was performed as previously reported (Fletcher *et al.*, 1995).

Hydrogen peroxide sensitivity testing and determination of tolerance to air

Porphyromonas gingivalis W83 and *P. gingivalis* FLL302 the *bcp*-defective isogenic mutant strain were tested for sensitivity to hydrogen peroxide as previously described (Johnson *et al.*, 2004). Both strains were also tested for resistance to killing by atmospheric oxygen using a method similar to one previously described (Diaz *et al.*, 2006). Both *P. gingivalis* W83 and FLL302 were grown to mid-log ($OD_{600} \sim 0.8$). A 10^{-6} dilution of each culture was made with pre-warmed BHI medium and 20 μ l of the dilution was plated on each BHI agar plate. Test plates were immediately incubated at 37°C in air for 30 min or 1 h before being returned to anaerobic incubation for 4–6 days. Control plates were only incubated under anaerobic conditions. Percentage survival was determined as: (colony-forming units on plates exposed to air/colony-forming units on control plates) \times 100. All experiments were performed three times, each in triplicate.

Bacterial cell fraction preparation and protease activity assay

Preparations of whole-cell culture media were performed as previously reported (Abaibou *et al.*, 2000). Briefly, bacterial cultures were centrifuged at 6000 *g* for 30 min at 4°C. The supernatant (cell-free) was removed from the cell pellet and further clarified by ultracentrifugation (100,000 *g*, 60 min, 4°C) to yield vesicle-free (supernatant) and vesicle (pellet) fractions. The cell pellet from the first centrifugation was washed three times and resuspended in 0.1 M Tris–HCl buffer. The presence of Arg-X and Lys-X activity in each fraction (cell-free, cell suspension, vesicle-free and vesicles) was determined using a microplate reader (Bio-Rad, Hercules, CA) according to the methods of Potempa *et al.* (1998).

Ultraviolet sensitivity measurements

The UV sensitivity experiments were performed as previously described (Fletcher *et al.*, 1997). All experiments were performed in triplicate.

Virulence testing

Both *P. gingivalis* strain W83 and the isogenic mutant strain FLL302 were tested for invasiveness in a murine model as previously described (Fletcher *et al.*, 1995). Briefly, actively growing overnight cultures of *P. gingivalis* W83 and FLL302, each grown in BHI were centrifuged and washed in sterile phosphate-buffered saline. Each strain was then adjusted to 1×10^{10} bacterial ml^{-1} dose and 3×10^9 bacteria ml^{-1} dose in phosphate-buffered saline. Female BALB/c mice were challenged with 100 μ l of each bacterial culture from each strain and at each concentration at two sites on the dorsal surface (200 μ l total). The mice were 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 (Loma Linda University Animal Research Committee, 2010).

Statistical analysis

Statistical analysis was performed using Student's *t*-test.

RESULTS

The *bcp* gene is part of a multigene transcriptional unit

The *recA-vimA-vimE-vimF* was previously shown to be part of the same multigene transcriptional unit (Abaibou *et al.*, 2001; Vanterpool *et al.*, 2004, 2005a). To further confirm if the *bcp* gene was part of the same transcriptional unit, reverse transcription (RT-) PCR analysis of the DNase-treated RNA extracted from *P. gingivalis* W83 was amplified using *bcp* forward (P1) and *vimA* reverse (P14) oligonucleotide primers. A 2.4-kb fragment [*bcp* (0.5 kb), *recA* (1.0 kb) and *vimA* (0.9 kb)] was amplified only in the presence of reverse transcriptase.

TSS mapping of the *bcp* gene

RLM-RACE was performed to determine the start site for this transcriptional unit. As shown in Fig. 1, an adenine residue was identified as the TSS. It is noteworthy that this residue is located 25 bases downstream from the start of the current predicted ORF of the *bcp* gene (<http://www.oralgen.lanl.gov>). An ATG start codon is observed 23 nucleotide bases downstream of the TSS (Fig. 1B). This ORF would encode for a 151-amino-acid protein in contrast to the previous 167-amino-acid protein (<http://www.oralgen.lanl.gov>). A comparison of the Bcp proteins from *P. gingivalis* W83 and *P. gingivalis* ATCC 33277 shows 98% homology. Together they are similar to the Bcp proteins of other oral anaerobes and of *E. coli* (Fig. 1C).

Complementation of *E. coli bcp*-defective mutant with *P. gingivalis bcp* homologue

Bcp is known to play a functional role in oxidative stress resistance in *E. coli* (Jeong *et al.*, 2000). To determine any functional similarity between the *E. coli* and *P. gingivalis* Bcp proteins chromosomal DNA from *P. gingivalis* W83 was subjected to PCR analysis using primers (P9 and P2; Table 1), which would amplify the 0.5-kb *bcp* gene. This purified DNA fragment was inserted into pTrcHis2 TOPO[®] TA vector (Invitrogen) under the control of the *trc* promoter (Mulligan *et al.*, 1985; Brosius *et al.*, 1985). The inserts in the resulting recombinant plasmids designated pFLL301.4 (correct orientation) and pFLL301.5 (opposite orientation) were confirmed by nucleotide sequencing analysis. In the presence of IPTG *E. coli* KD2301, a *bcp*-defective mutant transformed with pFLL301.4 (*bcp* homologue), showed increased resistance when exposed to hydrogen peroxide (Table 2). A recombinant plasmid carrying the *bcp* ORF from the previously predicted ORF had no effect on hydrogen peroxide sensitivity of the *bcp*-defective *E. coli* strain, either in the presence or absence of IPTG (Table 2). None of the chimeric constructs with the *bcp* gene in the reverse orientation had any effect on the level of resistance of the *bcp*-defective *E. coli* strain to hydrogen peroxide even in the presence of IPTG (Table 2). To further confirm if the *P. gingivalis* Bcp was expressed in *E. coli*, cell lysates of *E. coli* transformants carrying pFLL301.3, pFLL301.2, pFLL301.4 or pFLL301.5 were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The predicted protein was observed to be expressed

Figure 1 Identification of the transcriptional start site (TSS) of the *bcp* gene of *Porphyromonas gingivalis* by RLM-RACE. (A) Total RNA was harvested from a mid log-phase culture ($OD_{600} \sim 0.6$) of *P. gingivalis* W83 and sequentially treated with calf intestinal phosphatase and tobacco acid phosphatase (TAP). After ligation of a 5' RACE adapter sequence (P6, Table 1) the RNA was reverse transcribed. An outer polymerase chain reaction (PCR) using primers P7 and P2 was performed (data not shown) and the product from this outer primer reaction was used as the template for the nested PCR using primers P8 and P2. No-template –TAP reactions (lane 1) and –TAP (lane 2) reactions were run as controls. The inner PCR revealed no PCR product in the absence of template DNA (lane 3) but yielded a fragment slightly larger than 0.5 kb (lane 4) with template DNA. The size of observed PCR products was based on the 1 kb DNA marker (M) used. (B) Sequencing analysis of the RLM-RACE inner PCR product revealed the transcription start site (TSS) to be an 'A' (indicated by arrows) located immediately at the junction of the ligated 5' adapter sequence (in italics). A partial sequence of the *bcp* gene is shown for reference. This TSS is at the –23 position upstream of an ATG. (C) Sequence alignment of *P. gingivalis* Bcp proteins and Bcp homologues in common oral anaerobes and *Escherichia coli* (CLUSTAL W version 2.0). *Pg*, *Porphyromonas gingivalis*; *Tf*, *Tannerella forsythensis*; *Pi*, *Prevotella intermedia*; *Bf*, *Bacteroides fragilis*; *Aa*, *Aggregatibacter actinomycetemcomitans*; *Ec*, *Escherichia coli*. Asterisks indicate positions that have a fully conserved residue; whereas conservative replacements are indicated by a full stop or a colon.

Table 2 Complementation of *Escherichia coli* *bcp*-defective mutant

Background strain	Plasmid ¹	Zone of inhibition (cm) ² mean (SD)	
		-IPTG	+IPTG
<i>E. coli</i> BL21(DE3)	–	1.58 (0.10)	1.35 (0.28)
<i>E. coli</i> KD2301	–	1.25 (0.08)	1.25 (0.10)
<i>E. coli</i> KD2301	pFLL26 (Fletcher <i>et al.</i> , 1997)	1.23 (0.05)	1.20 (0.09)
<i>E. coli</i> KD2301	pFLL301.2 (<i>bcp</i> gene in reverse orientation)	1.08 (0.12)	1.10 (0.11)
<i>E. coli</i> KD2301	pFLL301.3 (<i>bcp</i> gene in forward orientation)	1.08 (0.08)	1.12 (0.08)
<i>E. coli</i> KD2301	pFLL301.4 (<i>bcp</i> gene in forward orientation)	1.03 (0.05)	0.87 (0.08)*
<i>E. coli</i> KD2301	pFLL301.5 (<i>bcp</i> gene in reverse orientation)	1.08 (0.08)	1.07 (0.08)

¹Plasmids containing various constructs of the *bcp* gene from *Porphyromonas gingivalis* W83 were used to transform the *E. coli* *bcp*-defective mutant, KD2301.

²Sensitivity to hydrogen peroxide was determined by disk inhibition assays. The data shown are an average of three independent experiments. Complementation was determined as a statistically significant difference ($P < 0.05$) in resistance to hydrogen peroxide between the KD2301 mutant and the complemented strain and is indicated by an asterisk (*).

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

An isogenic *P. gingivalis* W83 mutant defective in the *bcp* gene was constructed by allelic exchange mutagenesis. The recombinant plasmid pFLL301, which carries the *ermF-ermAM* cassette in the unique *StuI* site of the *bcp* 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 12 clindamycin-resistant colonies after 6 days of incubation. Randomly chosen colonies were further plated on *Brucella* blood agar 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 two randomly chosen colonies and the wild-type W83 strain was digested with *Bam*HI and subjected to Southern blot analysis. If the *bcp* gene was interrupted by the *ermF-ermAM* cassette, a 4.2-kb fragment should be observed when probed with the digoxigenin-labeled *bcp* DNA. The expected 4.2- and 2.1-kb fragments were observed in the two clindamycin-resistant strains and the wild-type, respectively (data not shown). In addition, when hybridization was performed with the digoxigenin-labeled *erm* DNA no

hybridization was seen in wild-type DNA. However, the expected 4.2- and 9.2-kb fragments were observed in the two clindamycin-resistant colonies and the pVA2198 control plasmid, respectively (data not shown). Further confirmation of the clindamycin-resistant colonies was performed by PCR using primers P1 and P2 (Table 1) for the 0.5-kb *bcp* gene fragment. If the *bcp* gene was interrupted by the *ermF-ermAM* cassette, a 2.6-kb fragment was expected to be amplified. The expected 2.6- and 0.5-kb fragments were observed in the two clindamycin-resistant strains and the wild-type W83, respectively (data not shown). Taken together, these results suggest the insertional inactivation of the chromosomal *bcp* gene with the 2.1-kb *ermF-ermAM* antibiotic cassette. One strain designated *P. gingivalis* FLL302 was randomly chosen from the two *bcp*-defective mutants for further studies.

RT-PCR analysis of *P. gingivalis* FLL302 and W83

To further confirm the inactivation of the *bcp* gene in *P. gingivalis* FLL302 and to evaluate any polar effects on the *recA* and *vimA* genes, total RNA was isolated from the wild-type W83 and the *bcp*-defective mutant FLL302 grown to mid log-phase. Specific oligonucleotide primers for the *bcp*, *recA* and *vimA* genes (Table 1) were used in RT-PCR analysis. As primers specific for the *bcp*, *recA* and *vimA* genes would yield 0.5-, 1.0- and 0.9-kb fragments, respectively, a 0.5-kb fragment for the *bcp* gene should be amplified in the wild-type strain but should be missing in *P. gingivalis* FLL302. Furthermore, as *recA* and

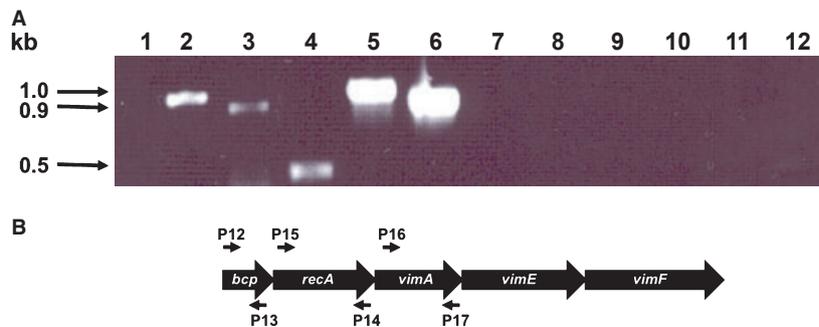


Figure 2 (A) Reverse transcription–polymerase chain reaction (RT-PCR) analysis of RNA extracted from *Porphyromonas gingivalis* W83 and FLL302. Total RNA was extracted from FLL302 (lanes 1–3 and 7–9) and W83 (lanes 4–6 and 10–12) grown to mid log-phase (OD_{600} , 0.6) and subjected to RT-PCR. *Porphyromonas gingivalis* FLL302: lane 1, primers (P1 and P2, Table 1) plus reverse transcriptase; lane 2, primers (P16 and P17, Table 1) plus reverse transcriptase; lane 3, primers (P14 and P15, Table 1) plus reverse transcriptase; *P. gingivalis* W83: lane 4, primers (P1 and P2, Table 1) plus reverse transcriptase; lane 5, primers (P16 and P17, Table 1) plus reverse transcriptase; lane 6, primers (P14 and P15, Table 1) plus reverse transcriptase; lanes 7–12, corresponding primer sets for the *bcp*, *recA* and *vimA* genes minus reverse transcriptase (negative controls). All lanes contain 5 μ l of the amplified mixture. (B) Location of the primers used in RT-PCR analysis.

vimA are a part of the *bcp-recA-vimA* transcriptional unit, 1.0- and 0.9-kb fragments should be amplified in the wild-type strain, which should be absent in the mutant strain. As shown in Fig. 2A (lanes 4–6), fragments of 0.5, 1.0 and 0.9 kb in size were amplified in the wild-type strain using *bcp*, *recA* and *vimA* specific primers (Fig. 2B), respectively. In contrast, only 1.0- and 0.9-kb fragments using the same primer pairs were amplified in the *bcp*-defective mutant FLL302 (Fig. 2A, lanes 1–3). There were no amplified fragments observed for either the wild-type strain or the mutant when reverse transcriptase was absent in the reaction mix (Fig. 2A, lanes 7–12). To test if a read-through from the *erm* cassette could result in the expression of the downstream genes (*recA* and *vimA*), primers P11 (5' oligonucleotide primer for the *erm* cassette) and P2 (3' primer for the *bcp* gene) were used in RT-PCR analysis of total RNA isolated from *P. gingivalis* FLL302. There was no amplified product observed in RNA from *P. gingivalis* FLL302 (data not shown). Taken together, these data confirm the inactivation of the *bcp* gene in *P. gingivalis* FLL302 and may suggest that the *recA* and *vimA* genes can be independently expressed in *P. gingivalis* W83.

UV sensitivity of *P. gingivalis* FLL302

The *recA* gene plays a role in DNA repair in *P. gingivalis* (Fletcher *et al.*, 1997). The RT-PCR showed that the *recA* gene was expressed in *P. gingivalis*

FLL302 so we expected that the sensitivity to UV should be similar to that of the wild-type. The *P. gingivalis* FLL302, FLL32 (*recA*-defective isogenic mutant) and the wild-type W83 were exposed to UV radiation at 2000 and 4000 μ J. After 2000 μ J UV irradiation there was 15 and 11% survival of the wild-type W83 and the isogenic *bcp*-defective mutant FLL302, respectively (data not shown). In contrast, there was no survival of the *recA*-defective mutant FLL32 at 2000 μ J UV irradiation (data not shown). No survival of any of the *P. gingivalis* strains was observed after exposure to 4000 μ J of UV irradiation. Taken together these data indicate that under the same physiological conditions the UV sensitivity of the wild-type *P. gingivalis* W83 and the *bcp*-defective mutant FLL302 is similar.

Sensitivity of *P. gingivalis* FLL302 to hydrogen peroxide and tolerance to air

Because the *P. gingivalis* Bcp can increase the resistance of *E. coli* to peroxides, *P. gingivalis* W83 and the isogenic mutant *P. gingivalis* FLL302 were assessed for sensitivity to hydrogen peroxide. Hydrogen peroxide was prepared at a concentration of 0.25 mM in BHI. In contrast to the parent strain, *P. gingivalis* FLL302 demonstrated greater sensitivity to hydrogen peroxide at a concentration of 0.25 mM (3A). Sensitivity of both bacterial stains was similar when a higher concentration was used (0.5 mM) (data not shown). Taken together, these data

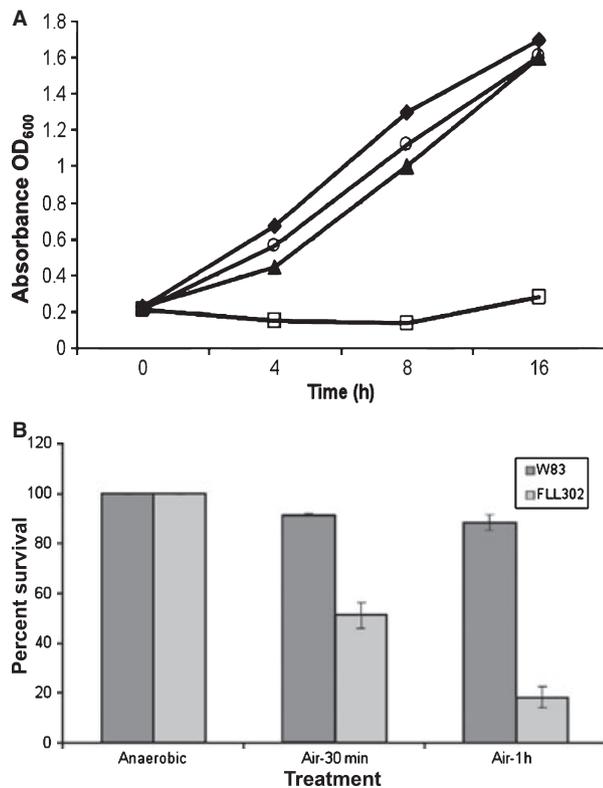


Figure 3 Sensitivity of the *Porphyromonas gingivalis bcp* mutant to hydrogen peroxide. The *P. gingivalis* was grown to early log-phase (OD_{600} 0.2) in brain–heart infusion broth and 0.25 mM hydrogen peroxide (▲ W83, ◻ FLL302) was then added to the cell cultures and incubated for a further 16 h. Cell cultures without hydrogen peroxide (◆ W83, ◊ FLL302) were used as controls. The results shown are representative of three independent experiments performed in triplicate.

suggest that *P. gingivalis* FLL302 has an increased sensitivity to hydrogen peroxide compared with the wild-type W83. In addition, when exposed to air, the *bcp* mutant, FLL302, demonstrated decreased aerotolerance when compared with the wild-type (Fig. 3B).

Protease distribution and activity of *P. gingivalis* FLL302

We have previously shown that the *vimA* gene is involved in protease activation and distribution in *P. gingivalis* W83 (Abaibou *et al.*, 2001). Because the *vimA* transcript was expressed in the *bcp*-defective mutant FLL302 we expected that the proteolytic activity and distribution would be similar to that in the wild-type. Strains of *P. gingivalis* W83 and *P. gingivalis*

FLL302 were assayed for proteolytic activity using *N*- α -benzoyl-DL-arginine *p*-nitroanilide and Ac-Lys-*p*-nitroanilide HCl. In late exponential growth phase cultures, both the arginine-X and lysine-X proteolytic activities of *P. gingivalis* FLL302 were 70% of the activity compared with the wild-type W83 (Fig. 4).

Taken together, these data suggest that under the same physiological conditions, there is a 30% reduction in total proteolytic activity of *P. gingivalis* FLL302 when compared with the wild-type W83 whereas the distribution remained similar in both strains.

Virulence testing of *P. gingivalis* FLL302

Protection against peroxide damage during the course of an infection plays a major role in pathogenesis (Nathan & Shiloh, 2000). As *P. gingivalis* FLL302 was more sensitive to hydrogen peroxide and had decreased proteolytic activity when compared with W83, its virulence potential in the mouse model was assessed. Our previous reports showed that the 50% lethal dose of *P. gingivalis* W83 in the BALB/c mouse model is approximately 3×10^9 bacteria per animal (Fletcher *et al.*, 1995). Within 6 days, four of ten animals challenged with *P. gingivalis* (wild-type) at a dose of 3×10^9 bacteria per animal had developed spreading, ulcerative abdominal lesions and died (data not shown). The surviving animals appeared cachectic and hunched with ruffled hair. However, all six recovered before the end of the 14-day observation period. Similarly, within 4 days, four of ten animals challenged with *P. gingivalis* FLL302 (*bcp*-defective mutant) at a dose of 3×10^9 bacteria per animal had developed spreading, ulcerative abdominal lesions and died (data not shown). 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 after challenge. All animals challenged with a dose of 1×10^{10} bacteria per animal of the wild-type (10 of 10) or the *P. gingivalis* FLL302 (10 of 10) died by 48 h (data not shown). Although the mice did not display lesions at the dorsal surface site of injection, they had developed spreading, ulcerative abdominal lesions. These data suggest that there is no detectable difference in the virulence potential of the *bcp*-defective mutant *P. gingivalis* FLL302 compared with the parent strain.

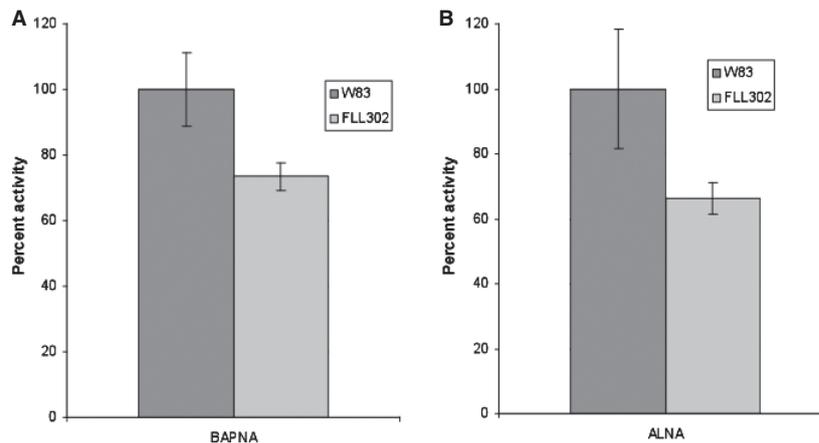


Figure 4 Arg-X and Lys-X cysteine protease activity in *Porphyromonas gingivalis* strains W83 and FLL302. The *P. gingivalis* was grown to late log-phase OD_{600} (1.2) in 50 ml brain–heart infusion broth supplemented with hemin and vitamin K. Activity against *N*- α -benzoyl-DL-arginine *p*-nitroanilide (BAPNA) and Ac-Lys-*p*-nitroanilide HCl (ALNA) was tested in whole cell culture according to the method of Potempa *et al.* (1998). The Arg-X (A) and Lys-X (B) activities of W83 were assumed to be 100%; this activity measured in *P. gingivalis* FLL302 represents 70% of the activity of the wild-type strain W83 for both cysteine proteases. The results shown are representative of three independent experiments performed in triplicate.

DISCUSSION

Periodontal disease is a chronic infection marked by massive inflammation (Seymour *et al.*, 1993). This inflammatory process is marked by the recruitment of neutrophils and macrophages to the site of infection to effect the clearance of the offending organism(s) through the oxidative burst (Van Dyke *et al.*, 1985). As a result, the inflammatory microenvironment of the periodontal pocket is also a highly oxidatively stressful environment characterized by several reactive oxygen intermediates (Beaman & Beaman, 1984; Genco & Slots, 1984). Bacterial exposure to these reactive oxygen intermediates can result in damage to bacterial membranes and nucleic acids (Imlay & Linn, 1988). Bacteria have therefore developed several mechanisms to subvert the damage caused under oxidative stress through the use of antioxidant enzymes such as superoxide dismutase, AhpC and Bcp (Nakayama, 1994; Rocha & Smith, 1999; Jeong *et al.*, 2000). Bcp has been reported to detoxify hydrogen peroxide in *E. coli* and so plays a role in oxidative stress resistance (Jeong *et al.*, 2000). In this study we examined the role of the *bcp* gene in the oxidative stress resistance and pathogenesis. It was our hypothesis that the *bcp* gene in *P. gingivalis* W83 would play a significant role in oxidative stress and virulence.

In a previous communication we hypothesized that the *bcp* gene may be part of a unique transcriptional unit together with the *recA*, *vimA*, *vimE* and *vimF* genes in *P. gingivalis* (Abaibou *et al.*, 2001). In this study we have confirmed *bcp* as part of the multi-gene transcriptional unit. The *recA* and *vim* genes in *P. gingivalis* are involved in DNA repair and protease activation/distribution, respectively (Fletcher *et al.*, 1997; Abaibou *et al.*, 2001). This *bcp-recA-vimA-vimE-vimF* genetic architecture would be advantageous to *P. gingivalis* because DNA repair activity, protease activation and oxidative stress resistance could be co-ordinately regulated. The significance of this observation would be consistent with the ability of *P. gingivalis* to use the heme on its surface as an oxidative sink to neutralize reactive oxygen intermediates (Smalley *et al.*, 1998, 2000). Because *P. gingivalis* proteases are major virulence factors involved in heme acquisition and binding to the bacterial membrane (Okamoto *et al.*, 1998; Genco & Dixon, 2001; Sroka *et al.*, 2001), this could be an important strategy for survival in the periodontal pocket.

In our analysis of the *bcp* transcript from cells in the exponential growth phase we identified the TSS to be an adenine residue. Interestingly, this was located within the current predicted ORF for the *bcp* gene. Identification of another in-frame start codon 23 bases downstream of that predicted for the ORF

of Bcp suggests that the Bcp protein may be smaller than predicted. When the current predicted ORF is aligned with other Bcp proteins, including that of another *P. gingivalis* strain, ATCC 33277, the *P. gingivalis* Bcp appears to have additional amino acid residues in its N-terminal region that are not conserved with the other proteins. Based on the transcript observed in our experiments, a smaller Bcp protein would be consistent with the observed size of Bcp that is seen in other bacterial species. This presents two interesting scenarios: either *in silico* prediction of the Bcp product is incorrect or there is possibly an alternative *bcp* gene product depending on differential regulation based on growth phase, stress or other environmental factors. These hypotheses would need to be further evaluated and validated.

The inactivation of *bcp* in *E. coli* does not significantly change its sensitivity to hydrogen peroxide (Kong *et al.*, 2000). However, complementation using the smaller Bcp homologue from *P. gingivalis* significantly increases its resistance to hydrogen peroxide. It is possible that the Bcp protein in *P. gingivalis* may use hydrogen peroxide as a substrate. Bioinformatic analysis of the *P. gingivalis* Bcp (<http://tigr-blast.tigr.org/cmrb-blast>) revealed that it was 60% similar to the *E. coli* Bcp. Although both proteins share sequence similarity, the differences in their sequences could account for differences in their specificity for or efficiency in degrading hydrogen peroxide. Collectively, these data suggest that the Bcp homologue in *P. gingivalis* can detoxify hydrogen peroxide.

Inactivation of the *P. gingivalis bcp* gene allowed us to determine a role for the *bcp* gene in oxidative stress. Comparison of the sensitivities of the wild-type W83 and the *bcp*-defective mutant FLL302 to hydrogen peroxide and exposure to air demonstrated that the Bcp plays a significant role in oxidative stress resistance. These findings are consistent with other reports that suggest similar roles for Bcp (Jeong *et al.*, 2000; Attack *et al.*, 2008).

RT-PCR analysis of the DNase-treated RNA from *P. gingivalis* FLL302 (*bcp*-defective mutant) revealed that both the *recA* and *vimA* genes can be independently expressed. Because the *ermF-ermAM* antibiotic cassette was constructed with transcriptional stop signals (Fletcher *et al.*, 1995) it is unlikely that a read-through of this cassette would facilitate the

expression of the down stream *recA* and *vimA* genes. This would also be consistent with the failure to RT-PCR amplify any product from the *bcp*-defective mutant using a specific 5' oligonucleotide primer for the *erm* cassette and a 3' primer from the *bcp* or *recA* genes. From this study however, our observations of the independent expression of the *recA* and *vimA* genes could further help to explain the multiple hybridizing bands previously observed when RNA from the wild-type strain was probed with the *recA* gene (Abaibou *et al.*, 2001). Taken together, these data suggest that there may be some differential expression of the genes in the *bcp-recA-vimA* transcriptional unit. Preliminary analysis of the nucleotide sequence of this locus indicates putative promoter regions for each gene (data not shown). The significance of this expression pattern in *P. gingivalis* is under further investigation in the laboratory. Because the gingipains are downregulated at elevated temperature (Percival *et al.*, 1999), typical of the inflammatory microenvironment of the periodontal pocket, it is tempting to speculate that under conditions of oxidative stress and where a decrease in proteolytic activity is desirable, an ability to differentially express genes in the *bcp-recA-vimA-vimE-vimF* operon may be an important strategy for its adaptation to conditions of oxidative stress.

Consistent with previous reports on *P. gingivalis* FLL33, a *recA*-defective mutant (Fletcher *et al.*, 1997) and *P. gingivalis* FLL92, a *vimA*-defective mutant (Abaibou *et al.*, 2001), the independent expression of the *recA* and *vimA* gene in *P. gingivalis* FLL302 should not alter UV sensitivity and gingipain activity/distribution in this mutant. The *bcp*-defective mutant was black-pigmented, and had a similar growth rate, β -hemolysis and UV sensitivity as the parent strain. There was a 30% reduction in the Arg-X and Lys-X cysteine protease activities in *P. gingivalis* FLL302 though the distribution was similar to the wild-type *P. gingivalis* W83 strain (data not shown). The source of the reduction in proteolytic activity is currently unknown but it is likely that the reduced expression of the *vimA* gene, as demonstrated in the FLL302 mutant, may play a role.

Reduced gingipain activity is correlated with reduced virulence in *P. gingivalis* (Fletcher *et al.*, 1997; Abaibou *et al.*, 2001). Further, protection against peroxide damage during the course of an

infection is important for survival of the invading microorganism (Miller & Britigan, 1997). Results of our virulence studies in the mouse model suggest that there is no discernible difference in the virulence potential of *P. gingivalis* FLL302, the *bcp*-defective mutant, compared with the wild-type strain. It is likely that the level of gingipain activity of *P. gingivalis* FLL302 is not significant enough to alter its virulence potential. Furthermore, the *bcp* defect by itself might not be enough to alter the virulence potential of *P. gingivalis*. These data are consistent with similar experiments conducted with a *P. gingivalis* *ahpC* mutant (Johnson *et al.*, 2004) and a *Salmonella typhimurium* *ahpC*-defective mutant (Taylor *et al.*, 1998). 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 (Taylor *et al.*, 1998). However, we cannot rule out the possibility that the similarity in virulence potential observed between the wild-type and the *bcp*-defective mutant may be partly the result of the mouse abscess model of virulence used in these experiments. It is possible that in other mouse models of virulence, we may be able to mimic more closely the conditions of the periodontal cavity, possibly allowing for subtle differences in the attenuation of virulence to be observed. A second likely explanation for the inability of a single oxidative stress resistance gene to affect virulence in this mouse model, could be the presence of other redundant mechanisms of oxidative stress resistance upregulated during the infectious process. Recent reports on mechanisms of oxidative stress resistance in *P. gingivalis* indicate a role for several genes including *sod*, *dps*, *oxyR* (Diaz *et al.*, 2006), *FeoB2* (He *et al.*, 2006) and *rubrerythrin* in this process (Amano *et al.*, 1992; Sztukowska *et al.*, 2002; Ueshima *et al.*, 2003). In addition, a survey of the *P. gingivalis* genome [<http://www.oral-gen.lanl.gov/>] has identified other genes (*NADH oxidase*, *oxyR*, *thiol peroxidase* and *thioredoxin*) that may play a role in oxidative stress defense. Although many of the genes may play some role in oxidative stress, their role in virulence has not been investigated and remains unknown.

We have constructed an isogenic mutant of *P. gingivalis* that is defective in the *bcp* gene, which is part of the *bcp-recA-vimA* transcriptional unit. Further, we have demonstrated that the genes in this operon can be differentially expressed. While *P. gingivalis*

FLL302, the *bcp*-defective mutant, showed *in vitro* sensitivity to hydrogen peroxide, reduced aerotolerance and its virulence potential in a mouse model were unaltered when compared with the wild-type. This suggests that there may be other redundant mechanism(s) involved in oxidative stress defense in *P. gingivalis*. Further studies characterizing the relationship of the *bcp* gene to other oxidative stress genes may give insight into the global regulation of these genes in oxidative stress defense in *P. gingivalis*.

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