Role of the Clp System in Stress Tolerance, Biofilm Formation, and Intracellular Invasion in *Porphyromonas gingivalis*[▽]

Cindy A. Capestany,¹ Gena D. Tribble,^{1†} Kazuhiko Maeda,¹
Donald R. Demuth,² and Richard J. Lamont^{1*}

Department of Oral Biology and Center for Molecular Microbiology, College of Dentistry, University of Florida, Gainesville, Florida 32610,¹ and Department of Periodontics, Endodontics and Dental Hygiene, University of Louisville School of Dentistry, Louisville, Kentucky 40292²

Received 8 October 2007/Accepted 23 November 2007

Clp proteases and chaperones are ubiquitous among prokaryotes and eukaryotes, and in many pathogenic bacteria the Clp stress response system is also involved in regulation of virulence properties. In this study, the roles of ClpB, ClpC, and ClpXP in stress resistance, homotypic and heterotypic biofilm formation, and intracellular invasion in the oral opportunistic pathogen *Porphyromonas gingivalis* were investigated. Absence of ClpC and ClpXP, but not ClpB, resulted in diminished tolerance to high temperatures. Response to oxidative stress was not affected by the loss of any of the Clp proteins. The *clpC* and *clpXP* mutants demonstrated elevated monospecies biofilm formation, and the absence of ClpXP also enhanced heterotypic *P. gingivalis*-*Streptococcus gordonii* biofilm formation. All *clp* mutants adhered to gingival epithelial cells to the same level as the wild type; however, ClpC and ClpXP were found to be necessary for entry into host epithelial cells. ClpB did not play a role in entry but was required for intracellular replication and survival. ClpXP negatively regulated the surface exposure of the minor fimbrial (Mfa) protein subunit of *P. gingivalis*, which stimulates biofilm formation but interferes with epithelial cell entry. Collectively, these results show that the Clp protease complex and chaperones control several processes that are important for the colonization and survival of *P. gingivalis* in the oral cavity.

Porphyromonas gingivalis, a gram-negative anaerobe, is a causative agent of severe and chronic manifestations of periodontal disease, one of the most common microbial infections of humans (24, 61). The primary ecological niche of *P. gingivalis* is the gingival sulcus, and within this compartment, *P. gingivalis* colonizes different microenvironments. On tooth surfaces, *P. gingivalis* is a constituent of the complex multispecies biofilm known as dental plaque. Colonization of dental plaque is favored by the attachment of *P. gingivalis* cells to substrata of antecedent colonizers. In particular, *P. gingivalis* can adhere to cells of *Streptococcus gordonii*, an early colonizer of plaque, and subsequently accumulate into heterotypic biofilm microcolonies (11, 34, 37). *P. gingivalis* also colonizes the tissues and cells of the gingival epithelium (1, 36). The organism is intensely invasive, and high numbers of *P. gingivalis* cells rapidly accumulate in the perinuclear area of gingival epithelial cells (GECs) (2). Both host and internalized bacterial cells remain viable, and indeed, *P. gingivalis* can suppresses host cell apoptosis through modulation of intrinsic apoptotic pathways (44). In addition to the oral habitat, *P. gingivalis* can also spread systemically and locate in heart and gestational tissues (32, 41), bone (67), and cerebrospinal fluid (25).

Transitioning among the various oral and nonoral and intra-

and extracellular locations will subject *P. gingivalis* to environmental stresses, including temperature and redox potential. *P. gingivalis* is well equipped to respond to these stressors through the production of a series of stress-related proteins. These include heat shock proteins (HSPs), such as GroES/GroEL (Hsp60), DnaK/DnaJ/GrpE (Hsp70/Hsp40/Hsp70 cofactor), and HtpG (Hsp90) (21, 59), along with superoxide dismutase, alkyl hydroperoxide reductase, rubrerythrin, and the transcriptional activator OxyR, which are involved in aerotolerance (15, 28, 46, 49, 50). *P. gingivalis* also possesses ClpB, ClpC, ClpP, and ClpX, which are members of the Clp (caseinolytic protease, Hsp100) family. The Clp protease proteolytic subunit, ClpP, is a cytoplasmic, barrel-shaped serine protease composed of two heptameric rings (5, 17). Narrow axial pores prohibit access of globular proteins to the internal catalytic chamber, and in order to gain proteolytic activity, ClpP must associate with a Clp ATPase partner that possesses nucleotide binding domains characteristic of the AAA+ superfamily of ATPases (5). The Clp ATPase regulatory proteins ClpA, ClpC, and ClpX form hexameric rings and possess a ClpP recognition tripeptide that allows association with the ClpP peptidase. The resulting Clp proteolytic complex specifically targets damaged or misfolded proteins for translocation and degradation (31, 33, 69). The ATPases determine the protease substrate specificity, in some cases by recognition of an 11-amino-acid degradation tag known as SsrA (30, 69). The ClpB and ClpL ATPases do not appear to associate with ClpP but rather function solely as chaperones with protein reactivation and remodeling activities (5, 20, 45, 76).

The Clp system is involved in general stress responses as well as in the maintenance of cell morphology and virulence. For

* Corresponding author. Mailing address: Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, FL 32610-0424. Phone: (352) 392-5067. Fax: (352) 392-2361. E-mail: rlamont@dental.ufl.edu.

† Present address: Department of Periodontics, Dental Branch, University of Texas Health Sciences Center at Houston, Houston, TX.

[▽] Published ahead of print on 7 December 2007.

example, in *Staphylococcus aureus*, ClpP and ClpC are required for heat stress, while ClpXP is important for survival under osmotic stress, oxidative stress, and cold (16). Clp mutants of *Salmonella*, *Streptococcus pneumoniae*, and *S. aureus* all demonstrate attenuated virulence (5, 22). ClpC is required for optimal adhesion and invasion of *Listeria monocytogenes* and promotes early escape of the organism from the phagosomal compartment of macrophages (55). ClpP is also involved in the adaptive response of *Listeria* in macrophages, and both ClpP and ClpC affect the control of PrfA-regulated genes (*inlA*, *inlB*, *actA*, and *hly*) (18, 47). Differential biofilm formation also has been observed in Clp mutants, and either enhancement or diminution of biofilm formation can be observed, depending on the organisms and the nature of the Clp protein deficiency (16, 40). Hence, while the overall phenotypic properties controlled by the Clp system are similar among organisms, different species can adapt the Clp components to perform distinct roles.

The roles of the Clp proteins in *P. gingivalis* have not been investigated in a systematic manner. Our initial proteomic studies found that ClpP, ClpC, and ClpX were upregulated in response to an epithelial cell environment and that an insertional mutation in *clpXP* reduced the ability of *P. gingivalis* to invade GECs (75). Similarly, insertional inactivation of *clpB* was found to reduce invasion of *P. gingivalis* in epithelial and endothelial cells (74). In this study, we utilized deletion mutants of *clpB*, *clpC*, and *clpXP*, along with their genetic complements, to assess the functions of the corresponding proteins in *P. gingivalis* stress responses; single- and mixed-species biofilm formation; and adhesion, entry, and survival in epithelial cells. In addition, the impacts of the *clp* mutations on the expression of well-characterized virulence factors of *P. gingivalis* were evaluated.

MATERIALS AND METHODS

Bacteria and culture conditions. *P. gingivalis* strain ATCC 33277 and derivatives were grown on sheep blood agar or in trypticase soy broth supplemented with yeast extract (1 $\mu\text{g ml}^{-1}$), menadione (1 $\mu\text{g ml}^{-1}$), and hemin (5 $\mu\text{g ml}^{-1}$) at 37°C under anaerobic conditions of 85% N₂, 10% H₂, and 5% CO₂. When necessary, erythromycin at 10 $\mu\text{g ml}^{-1}$, tetracycline at 1 $\mu\text{g ml}^{-1}$, or gentamicin at 100 $\mu\text{g ml}^{-1}$ was added to the medium. *S. gordonii* DL1 was grown in chemically defined medium (3), with sucrose (0.8%) as the carbon source. *Escherichia coli* TOP10, J53, and DH5 α were grown in Luria-Bertani (LB) broth containing, when necessary, ampicillin at 100 $\mu\text{g ml}^{-1}$. Solid medium was prepared by addition of 1.5% agar.

General molecular techniques. Recombinant-DNA techniques were performed as described previously (57). Restriction enzymes and DNA-modifying enzymes were purchased from Promega. *P. gingivalis* cells were lysed with Trizol (Invitrogen). Chromosomal DNA was isolated using a Wizard genomic DNA purification kit (Promega). RNA was extracted with phenol-chloroform and precipitated with isopropanol. RNA preparations were washed with 70% ethanol, dissolved in RNase-free H₂O, and treated with RNase-free DNase I (Ambion). The RNA was then purified on RNeasy columns (Qiagen). Plasmid DNA was isolated from *E. coli* by using a QiaMiniprep kit (Qiagen). Standard PCR conditions were 95°C for 6 min and 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min, followed by a final extension at 72°C for 7 min. For Southern blots, DNA probes were labeled and hybridization was detected using the Gene Images AlkPhos direct labeling and detection system (Amersham). *P. gingivalis* competent cells were prepared by suspending early-log-phase cells in electroporation buffer (10% glycerol, 1.0 mM MgCl₂). For electroporation, cells were incubated with 2 μg of a PCR product in Tris-EDTA buffer and pulsed with a Bio-Rad gene pulser at 2.5 kV.

Construction of mutant and complemented strains. Mutants of the *clpB* and *clpC* genes were created by allelic replacement. To create the $\Delta clpB$ mutant, a 4.4-kb region encompassing the entire *clpB* gene was PCR amplified with primers

ClpB-upper (5'-GGCTGAGGTCTAGAGACTGCCTGCGTTGTGGTTG-3') and ClpB-lower (5'-TGCGTATTTGGTGGTTATTG-3'). The resulting PCR product was digested with restriction enzymes XbaI and HindIII, and the fragment was cloned into the same restriction sites of pUC19. The resulting plasmid was then digested with PstI to remove a 2-kb internal fragment from *clpB*. This region was replaced with a 1.1-kb insert containing the *ermF* allele from pVA3000 (39). For $\Delta clpC$, PCR primers ClpC-upper (5'-GGGAAGGCGAATTCGGCGGAGGCGATACTACCAC-3') and ClpC-lower (5'-GGGCTTGCATGGCACCCATG-3') were used to amplify a 2.2-kb region containing the 3' region of the *clpC* gene. The resulting PCR product was digested with EcoRI and HindIII and cloned into the complementary sites of pUC18. The resulting plasmid was digested with SphI to remove a 303-bp internal fragment of *clpC*. This fragment was replaced by ligation to the *ermF* gene, contained on a 1.1-kb SphI fragment. For both the $\Delta clpB$ and $\Delta clpC$ mutants, the final plasmid constructs were linearized by digestion with ScaI and transformed into *P. gingivalis* strain 33277 by electroporation, and allelic replacement mutants were selected with erythromycin. Insertion of the replacement allele was confirmed by PCR and Southern hybridization, and loss of mRNA was established by reverse transcription-PCR (RT-PCR).

The $\Delta clpXP$ mutant was constructed by gene replacement using a PCR fusion technique (60). A DNA sequence containing 542 bp upstream of the *clpP* initiation codon was amplified using the primers CLPP-upF (5'-TCAGTGGCAGCGGAGATG-3') and CLPP-upR (5'-AACGGGCAATTTCTTTTGTGTCATTGTCCTTACTGTGATCATATTTGTTT-3'). A 799-bp region downstream of the *clpX* stop codon was amplified using CLPX-downF (5'-GTCCCTGAAAAATTCATCTTCGTAGTAGAAGTACCCCCAAGAGAATC-3') and CLPX-downR (5'-CCGCTGGGATAGGATGTA-3'). To replace the *clpXP* genes, an *ermF* cassette was amplified from pVA3000 by using primers ERMF-F (5'-ATGACAAAAAGAAATTGCCGTT-3') and ERMF-R (5'-CTACGAAGGATGAAATTTTCAGGGAC-3'), which contain 5' homology to CLPP-upR and CLPX-downF, and a fusion PCR product was produced (35). To confirm product size and purity, the fusion PCR product was size fractionated by agarose gel electrophoresis. The final construct was purified with the Wizard PCR cleanup system (Promega) and introduced into *P. gingivalis* by electroporation. A double-crossover recombination event was selected by plating with erythromycin selection. Insertion of the replacement allele was confirmed by PCR and Southern hybridization, and loss of mRNA was established by RT-PCR.

For complementation of the *clp* mutations, a region containing the appropriate *clp* open reading frame, plus no fewer than an additional 100 bp upstream and 30 bp downstream, was PCR amplified using ExTaq (Takara). The primers were engineered to contain the following restriction sites: SalI-HindIII for *clpB*, NheI-HindIII for *clpC*, and NheI-BamHI for *clpXP*. The shuttle vector plasmid pT-COW (19) was digested with the appropriate restriction enzymes to allow cloning of the PCR product into the *tetC* region. The resulting plasmids were transformed into *E. coli* TOP10 and selected on ampicillin plates. Colonies were screened by colony direct PCR and restriction digestion. The plasmids with the correct restriction profile were designated pT-ClpB, pT-ClpC, and pT-ClpXP, and these plasmids were introduced into the corresponding Δclp strain by conjugation. The conjugation reaction mixture also contained helper *E. coli* J53 containing R751, an IncP plasmid used to mobilize vectors from *E. coli* to a *Bacteroides* recipient. The conjugation mixture had a donor-to-recipient ratio of 0.2. The mating was performed in a candle jar on prerduced blood agar plates for 16 h, and transconjugants were selected with gentamicin and tetracycline. The presence of pT-COW-derived plasmid, and of the *ermF* gene on the chromosome in the transconjugants, was confirmed by PCR. The resulting strains were designated C $\Delta clpB$, C $\Delta clpC$, and C $\Delta clpXP$.

***P. gingivalis* biofilm formation.** Homotypic biofilm formation by *P. gingivalis* was quantified by a microtiter plate assay (51), as adapted for *P. gingivalis* (6). Parental and mutant strains in early log phase (2×10^8 cells) were incubated at 37°C anaerobically for 24 h. The resulting biofilms were washed, stained with 1% crystal violet, and destained with 95% ethanol. Absorbance at 595 nm was determined with a Benchmark microplate reader. Biofilm assays were repeated independently three times with each strain in triplicate.

Monospecies *P. gingivalis* biofilms and heterotypic *P. gingivalis*-*S. gordonii* biofilms on glass plates were generated and analyzed by confocal microscopy as described previously (6, 34). For homotypic biofilms, *P. gingivalis* strains in early log phase were labeled with 5 (and 6)-carboxyfluorescein succinimidyl ester (4 $\mu\text{g ml}^{-1}$; Molecular Probes). Labeled bacterial cells (5×10^7) were added to CultureWell chambered cover glass 16-well slides (Grace Bio Labs) and incubated anaerobically for 24 h at 37°C with rocking. For heterotypic *P. gingivalis*-*S. gordonii* biofilms, *S. gordonii* cells were labeled with hexidium iodide (15 $\mu\text{g ml}^{-1}$; Molecular Probes) and then cultured anaerobically at 37°C for 16 h with rocking in individual chambers of the CultureWell cover glass system. Fluorescein-la-

beled *P. gingivalis* cells (2×10^6 in prerduced phosphate-buffered saline [PBS]) were reacted with the *S. gordonii* biofilm for 24 h anaerobically at 37°C with rocking. The resultant single- or mixed-species biofilms were examined with a Bio-Rad MRC600 confocal scanning laser microscope (Kr/Ar) system with an MS plan 40 $\times$ 1.4-numerical-aperture objective. Images were digitally reconstructed (*x-z* section and *z* projection of *x-y* sections) with Image J V1.33u (NIH). Quantitation of *P. gingivalis*-specific fluorescence, along with the heights of the biofilms, was determined using DAIME software (13). Quantitation of *S. gordonii*-specific fluorescence ensured that equivalent levels of the streptococcal substratum were present in each experiment. Biofilm assays were repeated independently three times with each strain in triplicate.

GEC culture and *P. gingivalis* invasion. Primary cultures of GECs were generated as described previously (36, 48). Briefly, healthy gingival tissue was collected from patients undergoing surgery for removal of impacted third molars, following Institutional Review Board guidelines. Basal epithelial cells were separated and cultured in keratinocyte growth medium (Cambrix) at 37°C in 5% CO₂. GECs were used at passages 3 to 5 and at 80% confluence. Invasion assays were repeated three times in triplicate with GECs from different donors.

Overall invasion and survival of *P. gingivalis* in GECs were determined by an antibiotic protection assay (36). GECs were reacted with *P. gingivalis* strains at a multiplicity of infection (MOI) of 100 at 37°C in 5% CO₂ for 2 h. Infected GECs were incubated with metronidazole (200 μ g/ml) and gentamicin (300 μ g/ml) for 1 h to kill extracellular bacteria and lysed with sterile, distilled water for 15 min. The intracellular *P. gingivalis* bacteria released by cell lysis were enumerated by viable counting on blood agar plates.

Internalization of *P. gingivalis* strains was visualized and quantified by confocal microscopy. GECs were cultivated on glass coverslips (Lab-Tek) and reacted *P. gingivalis* strains at an MOI of 100 at 37°C in 5% CO₂ for 30 min. Cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS, and blocked overnight at 4°C in 10% normal goat serum in PBS. The cells were permeabilized for 20 min at room temperature (RT) with 10% normal goat serum and 0.2% saponin in PBS and then incubated with rabbit polyclonal antibody to *P. gingivalis* 33277 (72) at 1:5,000 for 2 h at RT. Fluorescein isothiocyanate (FITC)-conjugated Affini-Pure F(ab')₂ fragment goat anti-rabbit immunoglobulin G (heavy plus light chains; Jackson ImmunoResearch Laboratories) was added at 1:500 for 2 h at RT. Actin microfilaments were stained with tetramethyl rhodamine isocyanate-phalloidin (Sigma) at 1:200 for 15 min at RT. Images were acquired with a Bio-Rad MRC600 confocal scanning laser microscope (Kr/Ar) system with an MS plan 40 $\times$ 1.4-numerical-aperture objective. A series of fluorescent optical *x-y* sections were collected to create digitally reconstructed images (*x-z* section and *z* projection of *x-y* sections) with Image J 1.35c and Adobe Photoshop 6.0 software. Total *P. gingivalis* fluorescent accumulations in the stack of *z* projections representing whole cells were quantified with Image J 1.35c using the area calculator plugin.

Attachment of *P. gingivalis* strains to GEC was determined by an enzyme-linked immunosorbent assay (ELISA) as described previously (53). GECs were cultured on 96-well plates, fixed with 5% buffered formalin, and reacted with *P. gingivalis* strains at an MOI of 100 for 30 min at 37°C. Cells were washed with PBS, and surface bacteria were immunolabeled with *P. gingivalis* whole-cell antibodies (1:1,000) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (ICN Biochemicals). The adherence of each strain was determined by a colorimetric reaction using the 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system (Sigma).

Detection of Mfa, FimA, and SerB. For Western blotting, *P. gingivalis* strains in mid-log phase were lysed with radioimmunoprecipitation assay buffer (Santa Cruz) and proteins (10 μ g) separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane by electroblotting. Membranes were blocked and reacted with rabbit polyclonal antibodies against recombinant Mfa (rMfa), rFimA, and rSerB (1:5,000) followed by horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. Bound antibody was detected with an enhanced chemiluminescence system (Amersham).

The levels of cell surface Mfa and FimA proteins were determined by an ELISA with formalin-fixed whole cells. Bacteria were reacted with rMfa or rFimA antibodies (1:10,000) followed by peroxidase-conjugated secondary antibody. Antigen-antibody binding was determined by a colorimetric reaction with TMB. Antiserum to *P. gingivalis* 33277 whole cells was used as a control to ensure that consistent numbers of cells were fixed in each well.

Quantitative RT-PCR. Real-time RT-PCR was used to quantify mRNA levels for *mfa* and *fimA* by using the following primers, designed with Beacon Designer 2.0 software (Palo Alto, CA): *mfa*, 5'-TGCGGCGAAGTCGTATATG-3' and 5'-ATCTTCAGCACTCTCCACAAG-3'; and *fimA*, 5'-TTGTTGGGACTTGC TGCTCTTG-3' and 5'-TTCGGCTGATTGATGGCTTCC-3'. Total RNA was

reverse transcribed to cDNA by using Superscript III. Specific DNA standards for *mfa* and *fimA* were synthesized from chromosomal DNA by using standard PCR. Real-time RT-PCR was performed with a Bio-Rad iCycler using SYBR green Supermix (Bio-Rad). Results were analyzed with iCycler iQ Optical System software version 3.0a. The melt curve profiles were examined to verify a single peak for each sample, indicating primer specificity, and transcript copy number was calculated (73). RNA extracts were prepared in duplicate from independent experiments, and cDNA samples were loaded in triplicate.

RESULTS

The *clp* genes in *P. gingivalis*: construction of mutants and complemented mutant strains. The genome of the sequenced strain of *P. gingivalis*, W83, possesses *clpB* (PG1118), *clpC* (PG0010), *clpP* (PG0418), and *clpX* (PG0417), with the latter two adjacent to each other on the chromosome. *P. gingivalis* lacks *clpA*, which is usually present in gram-negative but absent in gram-positive bacteria (17), although *P. gingivalis* does have a relatively low GC content (48%). Proteins corresponding to each of the *P. gingivalis* *clp* gene products have been detected in strain 33277 (75), which was adopted in this study as it is more virulent than W83 in rodent models of periodontitis (29). RT-PCR demonstrated that *clpP* and *clpX* are cotranscribed in 33277 (not shown). Allelic replacement mutations were created in the *clpB* and *clpC* genes. The cotranscribed genes *clpP* and *clpX* were deleted together to produce a double knockout. RT-PCR showed that transcription of the upstream and downstream genes was unaffected by the mutations (not shown). Furthermore, all mutations were complemented with the wild-type allele(s) in *trans* using the *E. coli*-*P. gingivalis* shuttle vector pT-COW (19). As a control for the complemented strains, pT-COW without an insert was introduced into 33277.

Inactivation of *clpC* and *clpXP* impairs growth at high temperatures. The survival and growth of wild-type, mutant, and complemented mutant strains under conditions of elevated temperature or oxidative stress were compared to assess the contribution of the Clps to environmental stress resistance. For heat stress, *P. gingivalis* cells were cultured to mid-exponential phase and dilutions spotted in duplicate on blood agar plates which were incubated anaerobically for 5 days at either 37°C or 42°C. At the control temperature (37°C), the wild type and *clp* mutants maintained similar viabilities (Fig. 1A). At the higher temperature (42°C), the *clpC* and *clpXP* mutants showed 3-log decreases in viable counts compared to wild-type levels. In contrast, the *clpB* mutant formed colonies with sizes and numbers similar to those of the parental strain. These results differ from those of a study with *P. gingivalis* strain W83 that showed increased susceptibility to death at 50°C for a *clpB* mutant, although the difference between parent and mutant levels was less than 1 log (74). We did not observe a change in resistance to thermal stress in the 33277 *clpB* mutant at this higher temperature, and thus there may be some strain-specific effects in *P. gingivalis* related to the functionality of ClpB. Sensitivity to higher temperatures in the *clpC* and *clpXP* mutants was lost when the strains were genetically complemented (Fig. 1B). Exposure to 30 mM hydrogen peroxide or atmospheric air for 5 h did not result in any significant differences in viability between parental and mutant strains (not shown). The Clp system in strain 33277, therefore, does not appear to be involved in protection against oxidative stress; however, ClpC

FIG. 1. ClpC and ClpXP are required for optimal growth at high temperatures. *P. gingivalis* cultures at mid-exponential phase were serially diluted and spotted onto blood agar plates. The plates were incubated anaerobically at the indicated temperatures and colonies (CFU) counted. (A) Parental strain 33277 and *clp* mutants *ΔclpB*, *ΔclpC*, and *ΔclpXP*. (B) 33277 with pT-COW (CWT) and complemented strains CΔclpC and CΔclpXP. Experiments were repeated three times in duplicate with independent cultures. * denotes *P* values of <0.0001 (*t* test) for comparison to 33277.

and ClpXP are required for optimal resistance to thermal stress.

Homotypic biofilm formation by *P. gingivalis* is enhanced in the absence of ClpC and ClpXP. The ability of *P. gingivalis* to form biofilms on oral surfaces contributes to the persistence of the organism and increases the potential for periodontal tissue destruction. Stress responses, including those mediated by the Clp system, are known to be important for biofilm formation by other pathogenic organisms, such as *S. aureus*, *Streptococcus mutans*, and *Pseudomonas fluorescens* (16, 40, 51). Therefore, we examined the relative biofilm forming capabilities of the *clp* mutant strains of *P. gingivalis*. Monospecies biofilm formation was first tested in the microtiter plate assay with crystal violet staining (51). As shown in Fig. 2A, the *clpC* and *clpXP* mutants showed greater biofilm accumulation than the parental strain,

and biofilm formation by the complemented ClpC and ClpXP strains was restored to wild-type levels (Fig. 2B). To observe and quantify biofilm architectural features, biofilms of fluorescein-labeled *P. gingivalis* cells were generated on glass surfaces and then examined by confocal microscopy with digitally reconstructed image analysis. Biofilms formed by the ClpC- and ClpXP-deficient cells were visibly denser (Fig. 3A) than those of the parent, and total accumulation was significantly increased (Fig. 3B). Furthermore, the average heights of the *clpC* and *clpXP* mutant biofilms were significantly elevated compared to that of the parent (Fig. 3C). The biofilm accumulations of the complemented *clpC* and *clpXP* mutant strains were indistinguishable from those of the parental control (Fig. 3D and E). Hence, ClpC and ClpXP are required to constrain *P. gingivalis* homotypic biofilm development, and in the ab-

FIG. 2. ClpC and ClpXP control homotypic *P. gingivalis* biofilm growth. Microtiter plate biofilms after 24 h were stained with crystal violet and washed, and then the crystal violet was released with 95% ethanol. Biofilm accumulation was measured by absorbance at 595 nm. (A) Parental strain 33277 and *clp* mutants *ΔclpB*, *ΔclpC*, and *ΔclpXP*. (B) 33277 with pT-COW (CWT) and complemented strains CΔclpC and CΔclpXP. Results for the complemented strains are expressed as percentages of CWT biofilm growth. * denotes *P* values of <0.05 (*t* test) for comparison to 33277.

FIG. 3. ClpC and ClpXP regulate *P. gingivalis* monospecies biofilm architecture. Biofilm accumulations of fluorescein-labeled *P. gingivalis* strains on glass plates were viewed by confocal microscopy, and fluorescent optical *x-y* sections were reconstructed with Image J and analyzed with DAIME software. (A) Confocal laser scanning microscopy images of *x-y* and *x-z* projections of parental strain 33277 and *clp* mutants $\Delta clpB$, $\Delta clpC$, and $\Delta clpXP$. Magnification, $\times 40$. Total biofilm accumulation was measured by grain area analysis of 268.6- by 268.6- μm *x-y* sections for 33277, $\Delta clpB$, $\Delta clpC$, and $\Delta clpXP$ strains (B) or 33277 with pT-COW (CWT), $C\Delta clpC$, and $C\Delta clpXP$ (D). Average biofilm height of *P. gingivalis* accumulation was measured across three random *x-z* sections for 33277, $\Delta clpB$, $\Delta clpC$, and $\Delta clpXP$ strains (C) or CWT, $C\Delta clpC$, and $C\Delta clpXP$ (E). Results for the complemented strains are expressed as percentages of CWT levels. * denotes *P* values of < 0.05 (*t* test) for comparison to 33277.

sence of ClpC and ClpXP, more luxuriant biofilms are formed by *P. gingivalis*.

ClpXP deficiency enhances accumulation of heterotypic *P. gingivalis*-*S. gordonii* biofilms. The biofilms that develop in vivo on oral surfaces are complex multispecies communities (54). Successful bacterial colonizers of oral biofilms, therefore, are frequently capable of interacting synergistically with other biofilm inhabitants. Of particular relevance to this study, the presence of a substratum of the antecedent colonizer *S. gordonii* stimulates attachment and biofilm formation by *P. gingivalis* (34, 37). Hence, the role of the Clp proteins in the formation of mixed *P. gingivalis*-*S. gordonii* biofilms was determined. A biofilm of hexidium iodide-labeled *S. gordonii* cells was first generated on glass coverslips. Fluorescein-labeled *P. gingivalis* cells were reacted anaerobically with the *S. gordonii* biofilm, and accumulations of heterotypic biofilms were observed by confocal microscopy as described above. As the *P. gingivalis* cells were suspended in buffer, any increase in number is unlikely to be due to cell division. Therefore, this assay models one of the early stages of biofilm development: the recruitment, coadhesion, and accumulation of *P. gingivalis* cells from the planktonic phase into a sessile biofilm. This process is contingent on interbacterial signaling events and occurs prior to a further increase in number through growth and division (34). *x-y* and *x-z* images of the heterotypic *P. gingivalis*-*S. gordonii* biofilms are shown in Fig. 4A. *S. gordonii* cells developed biofilms that extensively covered the glass surface, and cells of parental *P. gingivalis*, along with those of the *clpC* mutant, formed discrete microcolony accumulations clearly separated from each other, consistent with previous studies (11). In contrast, while the *clpB* mutant exhibited the same degree of total accumulation, there were fewer microcolonies with a larger volume (Fig. 4B and C). The *clpXP* mutant formed more abundant accumulations within the mixed *P. gingivalis*-*S. gordonii* biofilm, and total fluorescence analysis verified the enhanced accumulation of the *clpXP* mutant compared to the parental level (Fig. 4B). Interestingly, the average biofilm heights across the *x-z* sections showed no increase in vertical accretion for the *clpXP* mutant (Fig. 4C). The complemented ClpB and ClpXP strains displayed heterotypic biofilm features similar to those of the parental control (Fig. 4D and E). Collectively, these results indicate that ClpB is required to maintain optimal surface area/volume ratios of heterotypic *P. gingivalis* microcolonies, whereas ClpXP is required for optimal spacing of the *P. gingivalis* microcolonies.

The Clp proteins are required for intracellular invasion and survival of *P. gingivalis*. In the subgingival crevice, *P. gingivalis* cells will encounter the host epithelial cells that line the gingival tissue and form both a physical barrier to microbial intrusion and an interactive interface that signals the presence of bacteria to the immune system. *P. gingivalis* cells can survive and replicate within GECs (36), and the organism can be detected within these cells in vivo (10). The role of the Clp proteins in the invasion of, and survival within, primary cultures of GECs was assessed by a standard antibiotic protection assay. All of the *clp* mutants exhibit significant decreases in intracellular survival compared to that of the parental strain (Fig. 5A). Additionally, complementation successfully restored the invasion phenotype to the parental levels (Fig. 5B). There-

FIG. 4. ClpB and ClpXP regulate heterotypic *P. gingivalis*-*S. gordonii* biofilms. *S. gordonii* stained with hexidium iodide (red) was cultured on glass plates. *P. gingivalis* strains were stained with fluorescein (green) and reacted with the *S. gordonii* biofilms for 24 h. Colocalized bacteria appear yellow. Mixed-species biofilm accumulations were viewed by confocal microscopy, and fluorescent optical *x-y* sections were reconstructed with Image J and analyzed with DAIME software. (A) Confocal laser scanning microscopy images of *x-y* and *x-z* projections of parental strain 33277 and *clp* mutants $\Delta clpB$, $\Delta clpC$, and $\Delta clpXP$. Magnification, $\times 40$. Total *P. gingivalis* accumulation was measured by grain area analysis of $268.6\text{-}\mu m$ *x-y* sections for 33277, $\Delta clpB$, $\Delta clpC$, and $\Delta clpXP$ strains (B) or 33277 with pT-COW (CWT) and $\Delta clpXP$ (D). Average biofilm height of *P. gingivalis* accumulation was measured across three random *x-z* sections for 33277, $\Delta clpB$, $\Delta clpC$, and $\Delta clpXP$ strains (C) or CWT and $\Delta clpB$ (E). Results for the complemented strains are expressed as percentages of CWT levels. * denotes *P* values of <0.05 (*t* test) for comparison to 33277.

fore, the Clp stress response system is essential for optimal invasion of *P. gingivalis* into GECs.

The overall invasive process can be divided into three broadly defined steps, comprising adherence, internalization, and intracellular survival. To dissect the contribution of the Clp proteins to each of these steps, mutants were tested for adherence and for internalization capability. Adherence was measured in an ELISA format with *P. gingivalis* antibodies and GECs that were formalin fixed to prevent internalization (53). All of the *clp* mutants adhered to GECs to the same level as the parental strain (Fig. 6), indicating that the defect in invasion is not the result of reduced adherence. Interestingly, in one previous study, loss of ClpB resulted in a reduction in adherence and internalization of epithelial cells for strain W83 (74). However, strain W83 is naturally deficient in GEC invasion, with levels over 1 log less than those of strain 33277 or of low-passage clinical isolates (36, 64). Thus, ClpB may play a different role in W83-epithelial cell interactions than in 33277.

Internalization was measured by confocal microscopy with *P. gingivalis* antibodies and FITC-labeled secondary antibodies. A series of fluorescent optical *x-y* sections were collected to create digitally reconstructed images throughout the volume of the cell (*x-z* section and *z* projection of *x-y* sections), and total *P. gingivalis* fluorescent accumulation in the stack of *z* projections representing whole cells was quantified. *P. gingivalis* wild-type cells rapidly entered GECs and accumulated in the perinuclear area (Fig. 7A), consistent with previous reports (2). The *clpB* mutant demonstrated levels of both internalization and intracellular location similar to those of the parent. In contrast, the *clpC* and *clpXP* mutants showed significant decreases in entry efficiency. Quantitation of intracellular *P. gingivalis* levels confirmed the results of the visual inspection (Fig. 7B). Internalization of complemented ClpC and ClpXP strains returned to parental control levels (Fig. 7C). These results suggest that ClpC and ClpXP are involved in the process of entry of *P. gingivalis* into GECs, whereas ClpB is required for intracellular survival.

ClpXP controls surface expression of Mfa protein. To gain greater insight into the potential role of the Clp stress response system in the mechanisms of control of the biofilm and invasive properties of *P. gingivalis*, we investigated the expressions of well-characterized virulence determinants that relate to these phenotypes, namely, the long (FimA) and short (Mfa) fimbriae and the SerB serine phosphatase (37, 52, 64, 72). Total bacterial proteins were extracted and probed with antibodies to FimA, Mfa, and SerB by Western blotting. The blot analysis showed that expression of Mfa was increased in the *clpXP* mutant (Fig. 8A). No change in expression of FimA or SerB was detected in any of the *clp* mutants. Furthermore, expression of Mfa was restored to wild-type levels in the complemented strain (Fig. 8B). The relative ratios of Mfa1/FimA in the parental and mutant strains were determined by scanning densitometry (Fig. 8C), which confirmed that Mfa is induced in the ClpXP mutant. To assess whether Mfa expression on the bacterial cell surface was also increased in the *clpXP* mutant, an ELISA with whole cells and antibodies to Mfa was performed (Fig. 8D). Surface exposure of Mfa was increased in the *clpXP* mutant compared to that in the parental strain but not in the *clpB* or *clpC* mutant strains. An increase in Mfa expression could be the result of an elevated rate of gene

FIG. 5. *clp* mutants are impaired in GEC invasion. GECs (1×10^5) were infected with *P. gingivalis* strains (1×10^7 bacterial cells) for 2 h. Extracellular bacteria were killed with gentamicin and metronidazole and intracellular bacteria released by lysis of the GECs. Numbers (CFU) of intracellular bacteria were determined by viable counting of the cell lysates. (A) Invasion of 33277, $\Delta clpB$, $\Delta clpC$, and $\Delta clpXP$ strains. (B) Invasion of C $\Delta clpB$, C $\Delta clpC$, and C $\Delta clpXP$, expressed as percentages of the invasion level of 33277 with pT-COW (CWT). * denotes *P* values of <0.0005 (*t* test) for comparison to 33277.

transcription or could result from effects on posttranscriptional processing. To begin to distinguish between these possibilities, we compared *mfa* mRNA levels in the parent and mutant strains by using quantitative real-time RT-PCR. The results showed no difference in *mfa* gene transcription (Fig. 8E), suggesting that the role of ClpXP in restricting Mfa protein exposure occurs at the protein processing level.

Differential expression of Mfa modulates *P. gingivalis* invasion. The Mfa fimbrial subunit protein is essential for heterotypic biofilm formation by *P. gingivalis* (37, 52). However, the function of Mfa in invasion of GEC by *P. gingivalis* has not been investigated. As the *clpXP* mutant expresses higher levels of Mfa and exhibits reduced invasion, we hypothesized that Mfa may be detrimental to invasion, and thus, an Mfa-deficient mutant would invade GEC more efficiently. Hence, the relative levels of invasion of *mfa*-null mutant and complemented mutant strains (52) were tested. As shown in Fig. 9, in the absence of Mfa, invasion of *P. gingivalis* was significantly enhanced, and

this effect was reversed in the complemented strain. These results suggest that the defect in invasion exhibited by the *clpXP* mutant is a consequence of the higher levels of surface Mfa expression.

DISCUSSION

Clp proteolytic complexes, consisting of a proteolytic core and associated ATPases, are a highly conserved bacterial stress response system. In addition, Clp proteins are required for the optimal expression of virulence in a number of pathogens (5, 17, 22). Despite overall homology, the numbers and types of Clp ATPases vary among organisms, and different species appear to utilize the individual components of the Clp system according to their unique environmental requirements. The oral anaerobic opportunistic pathogen *P. gingivalis* possesses the ClpP proteolytic subunit along with the ATPases ClpB, ClpC, and ClpX. To investigate the role of the Clp system in the stress responses and expression of virulence-associated phenotypes of the organism, mutants were constructed and complemented and the resulting strains tested for growth under a variety of stress conditions, the ability to form biofilms, and the capacity for invasion of epithelial cells.

While all the major families of HSPs are present in *P. gingivalis*, the functionality of these proteins in resistance to thermal stress has yet to be defined. In this study, we demonstrated that ClpC and ClpXP were required for optimal growth of *P. gingivalis* at a high temperature. ClpC and ClpP have also been shown to be required for growth of *S. aureus* at a high temperature (16), and ClpXP is important for survival of *S. aureus* under osmotic, oxidative, and cold stress. Loss of ClpXP did not affect resistance to oxidative stress in *P. gingivalis*; however, as a strict anaerobe, *P. gingivalis* possesses multiple mechanisms for responding to oxidative stress, including those involving superoxide dismutase, alkyl hydroperoxide reductase, rubrerythrin, HtrA, Dps, and the manganese transporter FeoB2 (28, 46, 49, 50, 56, 65), some of which are controlled by the transcriptional regulator OxyR (15). Hence, with multiple redundancy, a mutational analysis may not reveal individual contributions to oxidative-stress resistance. Loss of ClpB did not affect heat tolerance in *P. gingivalis* 33277, although ClpB

FIG. 6. Adherence to GECs is not affected by *clp* mutations. Formalin-fixed GECs were reacted with *P. gingivalis* strains at an MOI of 100 for 30 min. Binding was quantified with specific antibodies and colorimetric substrate reactions in an ELISA format. A fimbria-deficient mutant, $\Delta fimA$ (72), was used as a control for reduced adherence. * denotes *P* values of <0.005 (*t* test) for comparison to 33277.

FIG. 7. Differential effects of ClpB, ClpC, and ClpXP on internalization into GECs. Epithelial cells were cultured on glass plates and infected with *P. gingivalis* strains for 30 min. Bacterial cells were detected with *P. gingivalis* antibodies and FITC-secondary antibodies (green). The cell volume of the GECs was visualized with tetramethyl rhodamine isocyanate-phalloidin staining for actin (red). Infected cells were viewed by confocal microscopy, and fluorescent optical x - y sections were reconstructed and analyzed with Image J. (A) Confocal laser scanning microscopy images of a 0.2- μ m optical x - y section through GECs infected with 33277, Δ clpB, Δ clpC, and Δ clpXP strains. Magnification, $\times 40$. Quantitative image analysis of intracellular *P. gingivalis* fluorescence in the stack of z projections representing whole cells for wild-type (33277), Δ clpB, Δ clpC, and Δ clpXP strains (B) or 33277 with pT-COW (CWT), C Δ clpB, C Δ clpC, and C Δ clpXP strains (C). Intracellular *P. gingivalis* levels are expressed as percentages of CWT levels for complemented strains. * denotes P values of <0.01 (t test) for comparison to the wild type.

does play role in adaptation to thermal stress in *E. coli*, *S. aureus*, and *L. monocytogenes* (7, 16, 62). However, in addition to HSPs, universal stress protein A (UspA) (8) and the periplasmic serine protease HtrA (56) have been shown to contribute to heat tolerance in *P. gingivalis*. UspA and HtrA could thus compensate for loss of ClpB or allow ClpB to perform more exclusive roles in *P. gingivalis*, as is discussed further below.

The transition of bacteria from planktonic to biofilm mode often involves multiple adaptive responses, including stress responses. In the absence of ClpC and ClpXP, monospecies biofilm formation by *P. gingivalis* was enhanced, in terms of

both total accumulation and biofilm height. While the current study did not address the in vivo consequences of the in vitro increase in biofilm formation, the activities of ClpC and ClpXP can be predicted to regulate the biofilm-forming potential of *P. gingivalis* in vivo. Indeed, control of biofilm development in bacteria involves complex multilevel regulatory pathways that can either stimulate an increase in biomass or limit or stabilize biofilm accumulation according to environmental conditions. For example, in *S. aureus*, mutation of *clpP* increases biofilm formation (16). In contrast, the transcription factor RpoS limits *P. aeruginosa* biofilm depth (23, 68), and RpoS production is regulated at multiple levels, including transcription, translation, and proteolysis, in response to stress conditions (66). Activities controlled by LuxS, the AI-2 synthase, also repress biofilm formation by *Staphylococcus epidermidis* and *Helicobacter pylori* (9, 71). In *P. gingivalis*, control of biofilm accumulation has also been shown to require the leucine-rich surface protein InlJ (6), the cytoplasmic low-molecular-weight tyrosine phosphatase PtpA, and FtsH, an integral membrane zinc metalloprotease (60). Similar to ClpC and ClpX, FtsH is an AAA+ ATPase that is universally conserved in bacteria (26). Thus, the activities of a range of ATPase-dependent proteases in *P. gingivalis* are important for optimizing biofilm potential. The extent to which this potential is realized will depend on other bacterial, environmental, and contextual factors.

In vivo, oral biofilms are complex multispecies communities that develop through a variety of coadhesive, nutritional, metabolic, and signaling interactions among the constituent organisms (27, 54). *P. gingivalis* accumulates into heterotypic biofilm colonies with *S. gordonii* as a result of interbacterial attachment mediated by the long and short fimbriae of *P. gingivalis*, which interact with streptococcal GAPDH and Ssp surface proteins, respectively (43, 52). Binding of the short fimbrial protein Mfa to SspB provides the stimulus for biofilm accumulation, and this interaction is driven by an approximately 27-amino-acid binding domain of SspB termed BAR (4, 14). The binding and biofilm-inducing characteristics of BAR are dictated by the electrostatic and hydrophobic properties of a motif comprising the amino acid sequence NITVK (12). Similar to what was found for the monospecies biofilms, loss of ClpXP resulted in more abundant mixed *P. gingivalis* biofilms. Notably, the *clpXP* operon is not regulated at the transcriptional level in *P. gingivalis*-*S. gordonii* biofilms (60); hence, normal levels of these proteins are generally sufficient to control biofilm development. In contrast, loss of ClpB, while not significantly affecting total *P. gingivalis* accumulation, resulted in fewer, larger microcolonies. ClpB, which is thought not to associate with ClpP, may therefore have a more specific role in regulation of biofilm accumulation in the presence of *S. gordonii*. Considered together, these results indicate that unrestricted biofilm accumulation may be detrimental to *P. gingivalis*. The biofilms that accumulate in the oral cavity will experience fluctuations in nutrient availability and oxygen tension. Bacterial inhabitants, therefore, will be maximally competitive if they can optimize oxygen exposure and nutrient availability throughout their biofilm structures. Such constraints would provide for optimal biofilm proportions that vary among organisms and for the consequent development of pathways, such as those mediated by the Clp system in *P.*

FIG. 8. Expression of Mfa is increased in the absence of ClpXP. (A) Whole cells of *P. gingivalis* strains were lysed and blotted with antibodies to FimA, Mfa, or SerB. (B) Blot of complemented CΔclpXP strain with FimA and Mfa antibodies. (C) Scanning densitometric analysis of blots showing ratio of Mfa/FimA. (D) ELISA of formalin-fixed whole cells of wild-type (WT; 33277), ΔclpB, ΔclpC, and ΔclpXP strains probed with Mfa or FimA antibodies. *P. gingivalis* mutants deficient in FimA (ΔFimA) or Mfa (ΔMfa) were used as a control for antibody specificity. * denotes *P* values of <0.05 (*t* test) for comparison to the WT. (E) Quantitative real-time RT-PCR of *mfa* and *fimA* mRNA levels in WT and ClpXP-deficient strains.

gingivalis, to ensure that biofilms do not exceed the optimal size.

P. gingivalis is a facultative intracellular pathogen that can survive and replicate inside GECs. An intracellular location may protect the organism from immune activity and from antibiotics and may thus provide a reservoir of *P. gingivalis* that could lead to disease recrudescence. The invasion process is a multistep procedure that requires fimbria-mediated attachment of *P. gingivalis* to integrin receptors, subsequent signal transduction events that lead to cytoskeletal remodeling, and entry of the organism into the cytoplasm (36, 38, 72). Around 40% of the expressed proteome of *P. gingivalis* is differentially regulated as the organism adapts to an intracellular location (75). Among the regulated proteins are ClpC and ClpXP,

which show elevated expression in the presence of GEC components (75). Here, we found that the absence of ClpB, ClpC, or ClpXP significantly impaired the invasive capacity of *P. gingivalis*. While adhesion of all the mutant strains was normal, *clpC* and *clpXP* mutants were defective in entry into GECs. Thus, Clp proteolytic activity may be required for processing of proteins that are involved in the internalization process. A similar situation occurs with *Salmonella*, which requires the ClpXP protease to degrade FlhD and FlhC, master regulators of the synthesis of flagellum that are involved in entry into epithelial cells (58, 63). In contrast, ClpB seems to play no role in the entry of *P. gingivalis* into epithelial cells but is important for intracellular survival. Similarly, *S. aureus* cells lacking the ClpB chaperone are unable to replicate intracellularly in bo-

FIG. 9. Invasion of GECs is enhanced in the absence of Mfa. GECs (1×10^5) were infected with *P. gingivalis* strains (1×10^7 bacterial cells) for 2 h. Δmfa is an *mfa* mutant, and CΔmfa is the mutant complemented with the wild-type (WT) *mfa* gene (52). Extracellular bacteria were killed with gentamicin and metronidazole, and intracellular bacteria released by lysis of the GECs. Numbers (CFU) of intracellular bacteria were determined by viable counting of the cell lysates. Results are expressed as percentages of input bacteria that were recovered intracellularly. * denotes *P* values of <0.01 (*t* test) for comparison to the WT (33277).

vine T cells (16). ClpB is not directly involved in protein degradation (70); rather, in cooperation with DnaK/DnaJ, ClpB solubilizes protein aggregates (20, 45). Thus, the importance of ClpB for intracellular survival and for maintaining heterotypic biofilm architecture may be related to its ability to disaggregate and reactivate aggregated protein effectors.

The short fimbriae of *P. gingivalis* are 0.1 to 0.5 μm in length and are composed predominantly of the Mfa protein subunit. Mutants of *P. gingivalis* that lack Mfa show impaired formation of both monospecies biofilms (42) and heterotypic biofilms with *S. gordonii* (52). Consistent with this, in the absence of ClpXP, expression of Mfa was increased. Thus, the higher levels of homotypic and heterotypic biofilm formation in the *clpXP* mutant may result from elevated surface exposure of Mfa. Conversely, as loss of Mfa enhanced invasion, the increased expression of Mfa in the *clpXP* mutant could be the cause of the reduced invasion by this strain. We speculate that more abundant Mfa protein on the cell surface may disrupt physical parameters of FimA fimbrial surface presentation and impede the ability of the FimA fimbriae to activate their cognate integrin receptors. Upregulation of Mfa expression was not accompanied by an increase in *mfa* transcriptional activity. Hence, one possible mechanism by which ClpXP restricts Mfa expression may be through degradation of an inhibitor of Mfa synthesis or transport.

ACKNOWLEDGMENTS

This work was supported by grants DE12505, DE11111, and DE14605 from the NIDCR.

REFERENCES

- Balkovetz, D. F., and J. Katz. 2003. Bacterial invasion by a paracellular route: divide and conquer. *Microbes Infect.* 5:613–619.
- Belton, C. M., K. T. Izutsu, P. C. Goodwin, Y. Park, and R. J. Lamont. 1999. Fluorescence image analysis of the association between *Porphyromonas gingivalis* and gingival epithelial cells. *Cell. Microbiol.* 1:215–223.
- Bouvet, A., I. van de Rijn, and M. McCarty. 1981. Nutritionally variant streptococci from patients with endocarditis: growth parameters in a semi-synthetic medium and demonstration of a chromophore. *J. Bacteriol.* 146:1075–1082.
- Brooks, W., D. R. Demuth, S. Gil, and R. J. Lamont. 1997. Identification of a *Streptococcus gordonii* SspB domain that mediates adhesion to *Porphyromonas gingivalis*. *Infect. Immun.* 65:3753–3758.
- Butler, S. M., R. A. Festa, M. J. Pearce, and K. H. Darwin. 2006. Self-compartmentalized bacterial proteases and pathogenesis. *Mol. Microbiol.* 60:553–562.
- Capestany, C. A., M. Kuboniwa, I. Y. Jung, Y. Park, G. D. Tribble, and R. J. Lamont. 2006. Role of the *Porphyromonas gingivalis* InlJ protein in homotypic and heterotypic biofilm development. *Infect. Immun.* 74:3002–3005.
- Chastanet, A., I. Derre, S. Nair, and T. Msadek. 2004. *clpB*, a novel member of the *Listeria monocytogenes* CtsR regulon, is involved in virulence but not in general stress tolerance. *J. Bacteriol.* 186:1165–1174.
- Chen, W., K. Honma, A. Sharma, and H. K. Kuramitsu. 2006. A universal stress protein of *Porphyromonas gingivalis* is involved in stress responses and biofilm formation. *FEMS Microbiol. Lett.* 264:15–21.
- Cole, S. P., J. Harwood, R. Lee, R. She, and D. G. Guiney. 2004. Characterization of monospecies biofilm formation by *Helicobacter pylori*. *J. Bacteriol.* 186:3124–3132.
- Colombo, A. V., C. M. da Silva, A. Haffajee, and A. P. Colombo. 2007. Identification of intracellular oral species within human crevicular epithelial cells from subjects with chronic periodontitis by fluorescence in situ hybridization. *J. Periodontol. Res.* 42:236–243.
- Cook, G. S., J. W. Costerton, and R. J. Lamont. 1998. Biofilm formation by *Porphyromonas gingivalis* and *Streptococcus gordonii*. *J. Periodontol. Res.* 33:323–327.
- Daep, C. A., D. M. James, R. J. Lamont, and D. R. Demuth. 2006. Structural characterization of peptide-mediated inhibition of *Porphyromonas gingivalis* biofilm formation. *Infect. Immun.* 74:5756–5762.
- Daims, H., S. Lucker, and M. Wagner. 2006. daime, a novel image analysis program for microbial ecology and biofilm research. *Environ. Microbiol.* 8:200–213.
- Demuth, D. R., D. C. Irvine, J. W. Costerton, G. S. Cook, and R. J. Lamont. 2001. Discrete protein determinant directs the species-specific adherence of *Porphyromonas gingivalis* to oral streptococci. *Infect. Immun.* 69:5736–5741.
- Diaz, P. I., N. Slakeski, E. C. Reynolds, R. Morona, A. H. Rogers, and P. E. Kolenbrander. 2006. Role of *oxyR* in the oral anaerobe *Porphyromonas gingivalis*. *J. Bacteriol.* 188:2454–2462.
- Frees, D., A. Chastanet, S. Qazi, K. Sorensen, P. Hill, T. Msadek, and H. Ingmer. 2004. Clp ATPases are required for stress tolerance, intracellular replication and biofilm formation in *Staphylococcus aureus*. *Mol. Microbiol.* 54:1445–1462.
- Frees, D., K. Savijoki, P. Varmanen, and H. Ingmer. 2007. Clp ATPases and ClpP proteolytic complexes regulate vital biological processes in low GC, Gram-positive bacteria. *Mol. Microbiol.* 63:1285–1295.
- Gallot, O., E. Pellegrini, S. Bregenholt, S. Nair, and P. Berche. 2000. The ClpP serine protease is essential for the intracellular parasitism and virulence of *Listeria monocytogenes*. *Mol. Microbiol.* 35:1286–1294.
- Gardner, R. G., J. B. Russell, D. B. Wilson, G. R. Wang, and N. B. Shoemaker. 1996. Use of a modified *Bacteroides-Prevotella* shuttle vector to transfer a reconstructed β -1,4-D-glucanase gene into *Bacteroides uniformis* and *Prevotella ruminicola* B₁₄. *Appl. Environ. Microbiol.* 62:196–202.
- Goloubinoff, P., A. Mogk, A. P. Zvi, T. Tomoyasu, and B. Bukau. 1999. Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. *Proc. Natl. Acad. Sci. USA* 96:13732–13737.
- Goulhen, F., D. Grenier, and D. Mayrand. 2003. Oral microbial heat-shock proteins and their potential contributions to infections. *Crit. Rev. Oral Biol. Med.* 14:399–412.
- Henderson, B., E. Allan, and A. R. Coates. 2006. Stress wars: the direct role of host and bacterial molecular chaperones in bacterial infection. *Infect. Immun.* 74:3693–3706.
- Heydorn, A., B. Ersboll, J. Kato, M. Hentzer, M. R. Parsek, T. Tolker-Nielsen, M. Givskov, and S. Molin. 2002. Statistical analysis of *Pseudomonas aeruginosa* biofilm development: impact of mutations in genes involved in twitching motility, cell-to-cell signaling, and stationary-phase sigma factor expression. *Appl. Environ. Microbiol.* 68:2008–2017.
- Holt, S. C., and J. L. Ebersole. 2005. *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the “red complex”, a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol* 2000 38:72–122.
- Iida, Y., K. Honda, T. Suzuki, S. Matsukawa, T. Kawai, T. Shimahara, and H. Chiba. 2004. Brain abscess in which *Porphyromonas gingivalis* was detected in cerebrospinal fluid. *Br. J. Oral Maxillofac. Surg.* 42:180.
- Ito, K., and Y. Akiyama. 2005. Cellular functions, mechanism of action, and regulation of FtsH protease. *Annu. Rev. Microbiol.* 59:211–231.
- Jenkinson, H. F., and R. J. Lamont. 2005. Oral microbial communities in sickness and in health. *Trends Microbiol.* 13:589–595.
- Johnson, N. A., Y. Liu, and H. M. Fletcher. 2004. Alkyl hydroperoxide peroxidase subunit C (*ahpC*) protects against organic peroxides but does not affect the virulence of *Porphyromonas gingivalis* W83. *Oral Microbiol. Immunol.* 19:233–239.
- Katz, J., D. C. Ward, and S. M. Michalek. 1996. Effect of host responses on the pathogenicity of strains of *Porphyromonas gingivalis*. *Oral Microbiol. Immunol.* 11:309–318.
- Keiler, K. C., P. R. Waller, and R. T. Sauer. 1996. Role of a peptide tagging

- system in degradation of proteins synthesized from damaged messenger RNA. *Science* **271**:990–993.
31. Kessel, M., M. R. Maurizi, B. Kim, E. Kocsis, B. L. Trus, S. K. Singh, and A. C. Steven. 1995. Homology in structural organization between *E. coli* ClpAP protease and the eukaryotic 26 S proteasome. *J. Mol. Biol.* **250**:587–594.
 32. Kozarov, E., D. Sweier, C. Shelburne, A. Progulsk-Fox, and D. Lopatin. 2006. Detection of bacterial DNA in atheromatous plaques by quantitative PCR. *Microbes Infect.* **8**:687–693.
 33. Krüger, E., E. Witt, S. Ohlmeier, R. Hanschke, and M. Hecker. 2000. The Clp proteases of *Bacillus subtilis* are directly involved in degradation of misfolded proteins. *J. Bacteriol.* **182**:3259–3265.
 34. Kuboniwa, M., G. D. Tribble, C. E. James, A. O. Kilic, L. Tao, M. C. Herzberg, S. Shizukuishi, and R. J. Lamont. 2006. *Streptococcus gordonii* utilizes several distinct gene functions to recruit *Porphyromonas gingivalis* into a mixed community. *Mol. Microbiol.* **60**:121–139.
 35. Kuwayama, H., S. Obara, T. Morio, M. Katoh, H. Urushihara, and Y. Tanaka. 2002. PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors. *Nucleic Acids Res.* **30**:E2.
 36. Lamont, R. J., A. Chan, C. M. Belton, K. T. Izutsu, D. Vasek, and A. Weinberg. 1995. *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect. Immun.* **63**:3878–3885.
 37. Lamont, R. J., A. El-Sabaeny, Y. Park, G. S. Cook, J. W. Costerton, and D. R. Demuth. 2002. Role of the *Streptococcus gordonii* SspB protein in the development of *Porphyromonas gingivalis* biofilms on streptococcal substrates. *Microbiology* **148**:1627–1636.
 38. Lamont, R. J., and H. F. Jenkinson. 1998. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol. Mol. Biol. Rev.* **62**:1244–1263.
 39. Lee, S. W., J. D. Hillman, and A. Progulsk-Fox. 1996. The hemagglutinin genes *hagB* and *hagC* of *Porphyromonas gingivalis* are transcribed in vivo as shown by use of a new expression vector. *Infect. Immun.* **64**:4802–4810.
 40. Lemos, J. A., and R. A. Burne. 2002. Regulation and physiological significance of ClpC and ClpP in *Streptococcus mutans*. *J. Bacteriol.* **184**:6357–6366.
 41. Leon, R., N. Silva, A. Ovalle, A. Chaparro, A. Ahumada, M. Gajardo, M. Martinez, and J. Gamonal. 2007. Detection of *Porphyromonas gingivalis* in the amniotic fluid in pregnant women with a diagnosis of threatened premature labor. *J. Periodontol.* **78**:1249–1255.
 42. Lin, X., J. Wu, and H. Xie. 2006. *Porphyromonas gingivalis* minor fimbriae are required for cell-cell interactions. *Infect. Immun.* **74**:6011–6015.
 43. Maeda, K., H. Nagata, Y. Yamamoto, M. Tanaka, J. Tanaka, N. Minamino, and S. Shizukuishi. 2004. Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus oralis* functions as a cohesin for *Porphyromonas gingivalis* major fimbriae. *Infect. Immun.* **72**:1341–1348.
 44. Mao, S., Y. Park, Y. Hasegawa, G. D. Tribble, C. E. James, M. Handfield, M. F. Stavropoulos, O. Yilmaz, and R. J. Lamont. 2007. Intrinsic apoptotic pathways of gingival epithelial cells modulated by *Porphyromonas gingivalis*. *Cell. Microbiol.* **9**:1997–2007.
 45. Motohashi, K., Y. Watanabe, M. Yohda, and M. Yoshida. 1999. Heat-inactivated proteins are rescued by the DnaKJ-GrpE set and ClpB chaperones. *Proc. Natl. Acad. Sci. USA* **96**:7184–7189.
 46. Mydel, P., Y. Takahashi, H. Yumoto, M. Sztukowska, M. Kubica, F. C. Gibson III, D. M. Kurtz, Jr., J. Travis, L. V. Collins, K. A. Nguyen, C. A. Genco, and J. Potempa. 2006. Roles of the host oxidative immune response and bacterial antioxidant rubrerythrin during *Porphyromonas gingivalis* infection. *PLoS Pathog.* **2**:e76.
 47. Nair, S., E. Milohanic, and P. Berche. 2000. ClpC ATPase is required for cell adhesion and invasion of *Listeria monocytogenes*. *Infect. Immun.* **68**:7061–7068.
 48. Oda, D., and E. Watson. 1990. Human oral epithelial cell culture I. Improved conditions for reproducible culture in serum-free medium. *In Vitro Cell. Dev. Biol.* **26**:589–595.
 49. Ohara, N., Y. Kikuchi, M. Shoji, M. Naito, and K. Nakayama. 2006. Super-oxide dismutase-encoding gene of the obligate anaerobe *Porphyromonas gingivalis* is regulated by the redox-sensing transcription activator OxyR. *Microbiology* **152**:955–966.
 50. Okano, S., Y. Shibata, T. Shiroza, and Y. Abiko. 2006. Proteomics-based analysis of a counter-oxidative stress system in *Porphyromonas gingivalis*. *Proteomics* **6**:251–258.
 51. O'Toole, G. A., and R. Kolter. 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. *Mol. Microbiol.* **28**:449–461.
 52. Park, Y., M. R. Simionato, K. Sekiya, Y. Murakami, D. James, W. Chen, M. Hackett, F. Yoshimura, D. R. Demuth, and R. J. Lamont. 2005. Short fimbriae of *Porphyromonas gingivalis* and their role in coadhesion with *Streptococcus gordonii*. *Infect. Immun.* **73**:3983–3989.
 53. Park, Y., O. Yilmaz, I. Y. Jung, and R. J. Lamont. 2004. Identification of *Porphyromonas gingivalis* genes specifically expressed in human gingival epithelial cells by using differential display reverse transcription-PCR. *Infect. Immun.* **72**:3752–3758.
 54. Rosan, B., and R. J. Lamont. 2000. Dental plaque formation. *Microbes Infect.* **2**:1599–1607.
 55. Rouquette, C., C. de Chastellier, S. Nair, and P. Berche. 1998. The ClpC ATPase of *Listeria monocytogenes* is a general stress protein required for virulence and promoting early bacterial escape from the phagosome of macrophages. *Mol. Microbiol.* **27**:1235–1245.
 56. Roy, F., E. Vanterpool, and H. M. Fletcher. 2006. HtrA in *Porphyromonas gingivalis* can regulate growth and gingipain activity under stressful environmental conditions. *Microbiology* **152**:3391–3398.
 57. Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY.
 58. Schmitt, C. K., J. S. Ikeda, S. C. Darnell, P. R. Watson, J. Bispham, T. S. Wallis, D. L. Weinstein, E. S. Metcalf, and A. D. O'Brien. 2001. Absence of all components of the flagellar export and synthesis machinery differentially alters virulence of *Salmonella enterica* serovar Typhimurium in models of typhoid fever, survival in macrophages, tissue culture invasiveness, and calf enterocolitis. *Infect. Immun.* **69**:5619–5625.
 59. Shelburne, C. E., M. D. Coopamah, D. G. Sweier, F. Y. An, and D. E. Lopatin. 2007. HtpG, the *Porphyromonas gingivalis* HSP-90 homologue, induces the chemokine CXCL8 in human monocytic and microvascular vein endothelial cells. *Cell. Microbiol.* **9**:1611–1619.
 60. Simionato, M. R., C. M. Tucker, M. Kuboniwa, G. Lamont, D. R. Demuth, G. D. Tribble, and R. J. Lamont. 2006. *Porphyromonas gingivalis* genes involved in community development with *Streptococcus gordonii*. *Infect. Immun.* **74**:6419–6428.
 61. Socarransky, S. S., and A. D. Haffajee. 1992. The bacterial etiology of destructive periodontal disease: current concepts. *J. Periodontol.* **63**:322–331.
 62. Thomas, J. G., and F. Baneyx. 1998. Roles of the *Escherichia coli* small heat shock proteins IbpA and IbpB in thermal stress management: comparison with ClpA, ClpB, and HtpG in vivo. *J. Bacteriol.* **180**:5165–5172.
 63. Tomoyasu, T., T. Ohkishi, Y. Ukyo, A. Tokumitsu, A. Takaya, M. Suzuki, K. Sekiya, H. Matsui, K. Kutsukake, and T. Yamamoto. 2002. The ClpXP ATP-dependent protease regulates flagellum synthesis in *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* **184**:645–653.
 64. Tribble, G. D., S. Mao, C. E. James, and R. J. Lamont. 2006. A *Porphyromonas gingivalis* haloacid dehalogenase family phosphatase interacts with human phosphoproteins and is important for invasion. *Proc. Natl. Acad. Sci. USA* **103**:11027–11032.
 65. Ueshima, J., M. Shoji, D. B. Ratnayake, K. Abe, S. Yoshida, K. Yamamoto, and K. Nakayama. 2003. Purification, gene cloning, gene expression, and mutants of Dps from the obligate anaerobe *Porphyromonas gingivalis*. *Infect. Immun.* **71**:1170–1178.
 66. Venturi, V. 2003. Control of rpoS transcription in *Escherichia coli* and *Pseudomonas*: why so different? *Mol. Microbiol.* **49**:1–9.
 67. Welkerling, H., W. Geissdorfer, T. Aigner, and R. Forst. 2006. Osteomyelitis of the ulna caused by *Porphyromonas gingivalis*. *J. Clin. Microbiol.* **44**:3835–3837.
 68. Whiteley, M., M. G. Banger, R. E. Bumgarner, M. R. Parsek, G. M. Teitzel, S. Lory, and E. P. Greenberg. 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* **413**:860–864.
 69. Wiegert, T., and W. Schumann. 2001. SsrA-mediated tagging in *Bacillus subtilis*. *J. Bacteriol.* **183**:3885–3889.
 70. Woo, K. M., K. I. Kim, A. L. Goldberg, D. B. Ha, and C. H. Chung. 1992. The heat-shock protein ClpB in *Escherichia coli* is a protein-activated ATPase. *J. Biol. Chem.* **267**:20429–20434.
 71. Xu, L., H. Li, C. Vuong, V. Vadyvaloo, J. Wang, Y. Yao, M. Otto, and Q. Gao. 2006. Role of the LuxS quorum-sensing system in biofilm formation and virulence of *Staphylococcus epidermidis*. *Infect. Immun.* **74**:488–496.
 72. Yilmaz, O., K. Watanabe, and R. J. Lamont. 2002. Involvement of integrins in fimbriae-mediated binding and invasion by *Porphyromonas gingivalis*. *Cell. Microbiol.* **4**:305–314.
 73. Yin, J. L., N. A. Shackel, A. Zekry, P. H. McGuinness, C. Richards, K. V. Putten, G. W. McCaughan, J. M. Eris, and G. A. Bishop. 2001. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. *Immunol. Cell Biol.* **79**:213–221.
 74. Yuan, L., P. H. Rodrigues, M. Belanger, W. Dunn, Jr., and A. Progulsk-Fox. 2007. The *Porphyromonas gingivalis* *clpB* gene is involved in cellular invasion in vitro and virulence in vivo. *FEMS Immunol. Med. Microbiol.* **51**:388–398.
 75. Zhang, Y., T. Wang, W. Chen, O. Yilmaz, Y. Park, I. Y. Jung, M. Hackett, and R. J. Lamont. 2005. Differential protein expression by *Porphyromonas gingivalis* in response to secreted epithelial cell components. *Proteomics* **5**:198–211.
 76. Zolkiewski, M. 2006. A camel passes through the eye of a needle: protein unfolding activity of Clp ATPases. *Mol. Microbiol.* **61**:1094–1100.