INFECTION AND IMMUNITY, Apr. 2000, p. 1980–1987 Vol. 68, No. 4
0019-9567/00/$04.00+0

Characterization of Heat-Inducible Expression and Cloning of HtpG (Hsp90 Homologue) of *Porphyromonas gingivalis*

DENNIS E. LOPATIN,[1]* ALLISON COMBS,[1] DOMENICA G. SWEIER,[1] J. CHRISTOPHER FENNO,[1] AND SANGEETA DHAMIJA[2]

Department of Biologic and Materials Sciences[1] and Department of Oral Medicine, Pathology, and Oncology,[2] School of Dentistry, University of Michigan, Ann Arbor, Michigan 48109-1078

Received 13 October 1999/Returned for modification 12 November 1999/Accepted 14 December 1999

***Porphyromonas gingivalis* is implicated in the etiology of periodontal disease. Associations between microbial virulence and stress protein expression have been identified in other infections. For example, Hsp90 homologues in several microbial species have been shown to contribute to virulence. We previously reported that *P. gingivalis* possessed an Hsp90 homologue (HtpG) which cross-reacts with human Hsp90. In addition, we found that elevated levels of serum antibody to Hsp90 stress protein in individuals colonized with this microorganism were associated with periodontal health. However, the role of HtpG in *P. gingivalis* has not been explored. Therefore, we cloned the *htpG* gene and investigated the characteristics of HtpG localization and expression in *P. gingivalis*. *htpG* exists as a single gene of 2,052 bp from which a single message encoding a mature protein of approximately 68 kDa is transcribed. Western blot analysis revealed that the 68-kDa polypeptide was stress inducible and that a major band at 44 kDa and a minor band at 40 kDa were present at constitutive levels. Cellular localization studies revealed that the 44- and 40-kDa species were associated with membrane and vesicle fractions, while the 68-kDa polypeptide was localized to the cytosolic fractions.**

Stress responses are characterized by the preferential synthesis of a distinct array of proteins. These proteins are a part of the evolutionarily conserved mechanisms used by cells to deal with the stresses of their environment (15, 36, 37). Although these proteins were originally shown to be synthesized following heat shock (18, 19), a variety of environmental stresses (oxidative stress or heavy metals and toxins) have been shown to induce the expression of these proteins, resulting in a change in the nomenclature from heat shock to stress proteins. The stress proteins have been categorized into families based on the approximate molecular weight of the prototype member (e.g., Hsp60, Hsp70, and Hsp90 families, etc.).

Associations between microbial virulence and stress protein expression have been identified. Johnson et al. (12, 13) reported that the stress response was directly related to *Salmonella enterica* serovar *Typhimurium* virulence. Ensgraber and Loos (6) reported that a 66-kDa stress protein of *S. enterica* serovar *Typhimurium* was responsible for binding of the bacterium to intestinal mucus. Protection against a variety of infectious agents is thought to be due to antibodies directed against specific stress proteins that often involve conserved epitopes. In systemic fungal infections involving *Candida albicans*, patient survival is associated with the presence of antibodies reactive with a 47-kDa fragment of the 90-kDa stress proteins of the fungus (3, 25, 26). Hsp90 has been reported to participate in the assembly and disassembly of protein complexes, in the translocation of specific proteins through intracellular membranes, and in assisting in protein folding (42, 45). Proteins of the Hsp90 family bind to steroid hormone receptors, viral and cellular kinases, actin, and tubulin. Due to the critical roles of these stress protein families, considerable sequence and structural conservation (as much as 50%) is evident in species as diverse as bacteria, plants, insects, and mammals (29).

Limited data on stress proteins associated with putative periodontal pathogens are available. There have been studies which reported (i) detection of common stress proteins in oral microorganisms (7–9), (ii) cloning of these stress proteins (10, 24, 43), (iii) demonstration of potent induction of bone resorption (16, 30), or (iv) purification of specific heat shock proteins from various Hsp families (4, 10, 14, 20, 23, 39, 40, 44). There have been several studies that reported an association between periodontal disease and titers of antibody to Hsp65 and Hsp90 in serum (17, 22, 24, 31). In addition, there is evidence that the iron-modifiable protein of *Porphyromonas gingivalis* is a stress protein (2). However, among periodontal pathogens, the Hsp90 homologues have been virtually neglected in the literature. The exception was one report of an "accidental" cloning of the gene encoding the Hsp90 homologue, HtpG, of *Actinobacillus actinomycetemcomitans* (43).

Clinical studies from our laboratory suggest that serum antibodies reactive with purified human Hsp90 are elevated in individuals with good periodontal health. This association appears to be protective only in individuals with *P. gingivalis* in their subgingival dental plaque (22). Subsequently, we reported that *P. gingivalis* expressed a 68-kDa protein reactive with antibodies directed against human Hsp90 (21). The antibody reactivity and the presence of a 68-kDa polypeptide are consistent with the existence of a member of the HtpG family, the Hsp90 homologue found in prokaryotes. In addition, we showed by immunoelectron microscopy that the *P. gingivalis* anti-Hsp90-reactive epitope is associated with the cell membrane fraction and extracellular vesicles (21).

Given these observations and the evidence for the importance of protective anti-Hsp90 antibodies in fungal infections, we believed that further studies of the HtpG in *P. gingivalis* were important. We hypothesize that this protein is important in the virulence of *P. gingivalis*. By characterizing the expression of this HtpG we may begin to understand the role it plays in microbial virulence.

* Corresponding author. Mailing address: Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, 1011 North University Ave., Campus Box 1078, Ann Arbor, MI 48109-1078. Phone: (734) 647-3912. Fax: (734) 764-2425. E-mail: lopatin@umich.edu.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *P. gingivalis* ATCC 33277, SUNYaB A7A1-28, ATCC 53978 (W50), W83, and 381 were grown under anaerobic conditions at 37°C in an atmosphere of 95% N_2 and 5% CO_2 either on Anaerobic Medium 1 plates (Remel) or in Mycoplasma Broth Base (BBL) supplemented with 5 μg of hemin per ml and 5 μg of menadione per ml in an anaerobic GasPak150 (BBL). *P. gingivalis* cultures were grown from early phase (A_{600} = 0.06) to stationary phase (A_{600} = 0.6) and then split (under anaerobic conditions) to enable culturing for an additional hour at 37 or 45°C. The cultures were centrifuged for 10 min at 12,000 × *g*. The cell pellets or culture supernates were treated with either 10% trichloroacetic acid (TCA) and incubated overnight at room temperature for protein precipitation or with Trizol (Gibco BRL) for RNA extraction.

Escherichia coli strains DH5α, JM109 (Promega), and JB45 (1) were used. *E. coli* JM109 was used for all cloning studies and was cultured aerobically in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg/ml) as appropriate. *E. coli* JB45 was used in Southern blotting and was grown aerobically in LB medium supplemented with 100 μg of kanamycin per ml. *E. coli* DH5α was also used in Southern blotting and was grown aerobically in LB medium. *A. actinomycetemcomitans* ATCC 43718 was cultured in a manner similar to the method used for *P. gingivalis*.

Cell fractionation. The cells were pelleted by centrifugation (12,000 × *g*, 20 min) and then resuspended in phosphate-buffered saline containing protease inhibitors (21). Protease inhibitors were used only in this experiment because the cells could not be fractionated in the presence of TCA. All subsequent steps were processed with TCA, as described above. The culture supernatant containing the vesicles was retained for subsequent processing. Each whole-cell suspension was processed four times in a French press. The lysates were centrifuged at 12,000 × *g* for 20 min to pellet the remaining intact cells. The clarified lysate was ultracentrifuged for 2 h at 200,000 × *g* to pellet the cell membranes. The reserved culture supernatant was also ultracentrifuged at 100,000 × *g* to harvest the extracellular vesicles.

Western immunoblotting. TCA-precipitated samples were centrifuged at 13,000 × *g* for 15 min at 4°C. The precipitates were solubilized in NuPage LDS sample buffer (Novex) with 50 mM dithiothreitol, boiled for 10 min at 100°C, assayed for protein concentration, and then stored at −80°C. Then, 300 ng of protein per lane was loaded onto 4 to 12% Bis-Tris 1.0-mm NuPAGE gels (Novex) and electrophoresed in morpholinepropanesulfonate-sodium dodecyl sulfate (MOPS-SDS) running buffer (Novex). Proteins in the gels were either stained with Coomassie blue or transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and then probed with anti-Hsp90 antibodies. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin G antibody. Blots were treated for chemiluminescence (ECL; Amersham) and then exposed to X-ray film (Kodak X-Omat AR). Antibodies used in these studies included (i) polyclonal rabbit anti-human Hsp90 (21), (ii) polyclonal rabbit anti-*E. coli* HtpG (a gift of James Bardwell), and (iii) monoclonal anti-*Achlya ambisexualis* Hsp90 (SPA-830; StressGen).

Preparation of DNA and cloning of the *htpG* locus. Genomic DNA for PCR amplification and Southern blotting was isolated from mid-log-phase cultures of *P. gingivalis*, *E. coli*, and *A. actinomycetemcomitans* by using the Wizard Genomic DNA Purification Kit (Promega) and stored at 4°C until used. PCR products were generated with *Taq* DNA polymerase (PCR SuperMix; Life Technologies, Inc.) using *P. gingivalis* ATCC 33277 DNA as a template. Oligonucleotide primers were synthesized by the University of Michigan Biomedical Research Core Facilities and were gel purified by using the QIAquick Gel Extraction Kit (Qiagen). Primer design was based on preliminary sequence data obtained from the websites of the *P. gingivalis* Genome Project (http://www.forsyth.org/pgpg/) and The Institute for Genomic Research (http://www.tigr.org). The following primers were used in this study: 3547I (5′-ATG AGT AAG AAA GGA ACA-3′), 3546I (5′-TAT TTG CCG AGG AGC CCC-3′), 7752I (5′-GGC GGG TAC CAT AAG TAA GAA AGG AAC AAT CGG GGT AAC G-3′), and 733J (5′-GGC GGA GCT CGC ACA ACT CAC TAT TCA C-3′). Restriction enzyme sites were engineered into the primers as necessary to facilitate cloning and, in primer 7752I, the putative *htpG* start codon was modified to ATA. PCR products were cloned in pBluescript II KS(+). DNA sequencing was performed at the University of Michigan Biomedical Research Core Facilities.

Southern blot analysis. Southern analysis was performed using standard procedures as described by Sambrook et al. (35). Genomic DNA was quantified spectrophotometrically at 260 nm and was digested with *Bam*HI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, or *Xho*I in accordance with the manufacturer's recommendations (Promega and Gibco BRL). Digested genomic DNA (0.5 μg per lane) was electrophoresed through a 0.8% Tris-acetate-EDTA agarose gel. The DNA was transferred by capillary action to a Hybond-N+ membrane (Amersham) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The DNA was UV cross-linked, and the membranes were prehybridized with 5% nonfat dry milk in 6× SSC at 65°C. The DNA probe was random primer labeled with ^{32}P using Rediprime (Amersham), and unincorporated label was removed by using G-25 Sephadex Quick Spin columns (Boehringer Mannheim). After overnight hybridization at 65°C with the heat-denatured DNA probe, the blots were washed twice in 2× SSC–0.1% SDS at 65°C for 30 min and then twice in 0.1× SSC–0.5% SDS at 65°C for 15 min. The membranes were then exposed to X-ray film (Kodak X-Omat AR).

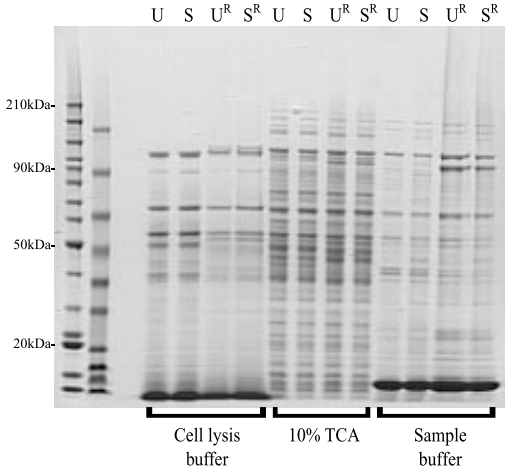

FIG. 1. Effect of sample processing on PAGE analysis. *P. gingivalis* ATCC 33277 was grown to mid-log phase (A_{600} = 0.23) as described in Materials and Methods. The bacterial suspensions were split under anaerobic conditions and cultured for an additional hour at 37°C (U) or 45°C (S). The cells were harvested by centrifugation for 10 min at 12,000 × *g* and then placed in LDS sample buffer, cell lysis buffer or 10% TCA, followed by a final resuspension in LDS sample buffer. Proteins in the gels were then stained with Coomassie blue. Lanes labeled with a superscript "R" were reduced with dithiothreitol. Those without the superscript were not reduced.

Northern blot analysis. Trizol-harvested RNA was resuspended in 100 μl of H_2O and stored at −80°C. RNA concentrations were determined spectrophotometrically at 260 nm. Then, 10 μg of total RNA/lane was electrophoresed through a 1% agarose gel (2.2 M formaldehyde, 1× MOPS) for 4 h at 100 V and transferred by capillary action to a Hybond XL membrane (Amersham) in 2× SSC. Equal loading of RNA in each lane was confirmed by ethidium bromide staining. The blots were UV cross-linked and then prehybridized with 100 μg of denatured salmon sperm DNA per ml, 50% formamide, 0.2% SDS, and 10× Denhardt's solution in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) for 1.5 h at 42°C. The DNA probe was labeled as described above. After overnight hybridization of the heat-denatured DNA probe with the membrane at 42°C, the blots were washed twice for 15 min with 5× SSC–0.1% SDS at room temperature, twice for 15 min with 1× SSC–0.1% SDS at 37°C, and once for 15 min with 0.1 × SSC–0.1% SDS at 37°C. Blots were then exposed to X-ray film (Kodak X-Omat AR).

Computer analysis. Developed films for Northern and Western blots were scanned and analyzed with NIH Image version 1.61. Sequence analyses were done using MacVector version 6.5 and SeqVu 1.1 (34).

Nucleotide sequence accession number. The GenBank accession number for the *P. gingivalis* ATCC 33277 *htpG* nucleotide sequence is AF176245.

RESULTS

Proteolysis effects. Our initial attempts to detect immunoreactivity with our panel of anti-Hsp90 antibodies and to demonstrate the inducibility of an Hsp90 stress response were not reproducible between experiments. Because of our prior concerns regarding *P. gingivalis* proteases, we assessed several extraction protocols (protease inhibitor cocktail and TCA extraction) aimed at circumventing proteolysis. Figure 1 compares the results of three extraction protocols on *P. gingivalis* (ATCC 33277; A_{600} = 0.23). The bacterial cells were cultured at 37 or 45°C for 1 h before extraction (previously determined to be optimal for induction). As shown, there were significant losses of protein bands in both the cell lysis buffer (protease cocktail) and in the SDS sample buffer alone compared to the use of TCA. There was a significant loss of both high- and low-molecular-weight species, with large amounts of degradation products visible at the bottom of the gel. After we observed these results, all subsequent Western blots were performed with TCA-precipitated preparations. The TCA pre-

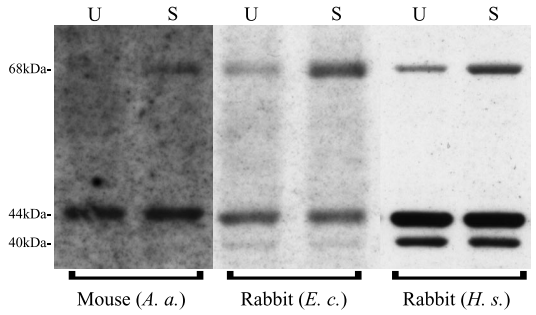

FIG. 2. Comparison of anti-Hsp90 and anti-HtpG reactivities with *P. gingivalis* in Western blot analysis. *P. gingivalis* ATCC 33277 was grown to mid-log phase (A_{600} = 0.32) at 37°C, split under anaerobic conditions, and cultured for an additional hour at 37°C (U) or 45°C (S). Membranes were probed with one of three antibodies (monoclonal anti-*A. ambisexualis* Hsp90 (*A. a.*), rabbit anti-*E. coli* HtpG (*E. c.*), and rabbit anti-human Hsp90 (*H. s.*). Molecular masses of the three major bands (68, 44, and 40 kDa) are indicated.

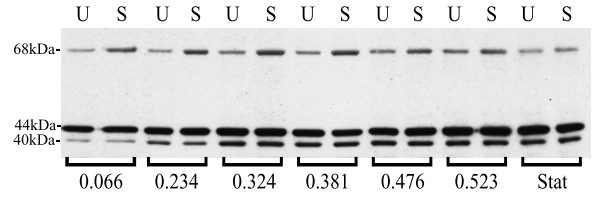

FIG. 3. Effect of growth phase of *P. gingivalis* on expression of HtpG. *P. gingivalis* ATCC 33277 was grown from early log (A_{600} = 0.07) to stationary phase at 37°C, split under anaerobic conditions, and cultured for an additional hour at 37°C (U) or 45°C (S). The membranes were probed with rabbit anti-human Hsp90. Molecular masses of the three major bands (68, 44, and 40 kDa) are indicated (upper panel).

cipitates were resuspended in SDS sample buffer for electrophoresis, and 300 ng of total protein was loaded per well. Western blot analysis (not shown) performed in the absence of TCA revealed that the *P. gingivalis* HtpG was quite susceptible to proteolysis. In contrast, other stress proteins, such as the DnaK and the GroEL homologues, which were used in unrelated studies as controls for stress protein induction in *P. gingivalis*, appeared to be quite resistant to proteolysis and could be detected even when polyacrylamide gel electrophoresis (PAGE) analysis was performed in the absence of TCA or protease inhibitors.

Comparison of anti-Hsp90 antibodies used in Western blot analysis. Our initial studies were performed with an anti-Hsp90 murine monoclonal antibody. This antibody was raised against a conserved epitope of *A. ambisexualis* shown to react with multiple species (33). Two additional antibodies subsequently became available for use in the analysis of *P. gingivalis* Western blots: rabbit anti-*E. coli* HtpG, a generous gift of James Bardwell, and rabbit anti-human Hsp90 prepared in our laboratory (21). Reactivity with *P. gingivalis* ATCC 33277 whole cells at mid-log phase (A_{600} = 0.32) stressed at 45°C or held at a control temperature of 37°C is shown in Fig. 2. All antibodies detected 68- and 44-kDa bands and demonstrated heat inducibility of the 68-kDa band. The monoclonal antibody did not detect the 40-kDa band. The anti-*E. coli* HtpG antibody detected trace levels of the 40-kDa band, while the anti-human Hsp90 antibody reacted strongly with all three bands. In all cases, only the 68-kDa band appeared to be heat inducible.

Effect of growth phase on HtpG expression. The relationship between HtpG expression and growth phase was examined. Prior to these experiments, growth studies were performed (data not shown) to establish the relationship between A_{600} and bacterial growth phase under our conditions. *P. gingivalis* ATCC 33277 was exposed to either 37 or 45°C for 1 h. The relationship between growth phase and the intensity of each of the three major bands is illustrated in Fig. 3. As shown, inducibility of the 68-kDa band appears to increase until the culture reaches an A_{600} of approximately 0.32. This corresponds to the mid-log phase of the culture. As the culture ages, inducibility appears to decrease and baseline levels in the "unstressed" control cultures appear to increase.

Kinetics of HtpG expression following heat shock. We next assessed the time course of HtpG expression following exposure to stress. It should be noted that what we are measuring is the accumulation of product in the cell and not an instantaneous measurement of synthesis of the protein. Such measurements would have to be determined in pulse-labeling experiments which we felt were not necessary for these studies. Expression of anti-Hsp90-reactive bands by *P. gingivalis* ATCC 33277 (A_{600} = 0.34) after a 1-h heat shock at 45°C is shown in Fig. 4. As depicted in this figure, for as long as 4 h after heat stress, the 68-kDa band remains elevated over its control. In contrast, while the intensity of the 40- and 44-kDa bands fluctuate over time, no significant differences are obvious when stressed and control categories are compared at each time point.

Subcellular localization of the anti-Hsp90-reactive substances. In order to determine if there was compartmentalization of the different anti-Hsp90-reactive polypeptides we fractionated stressed and unstressed *P. gingivalis* into membrane and cytosol fractions. *P. gingivalis* ATCC 33277 was grown to early log phase (A_{600} = 0.15) and then cultured for an additional hour at 37 or 45°C. The cells were harvested, treated with TCA, and processed in a French press as described earlier. The culture supernatant containing the vesicles was retained for subsequent processing.

The results of the fractionation are shown in the immunoblot in Fig. 5. The whole culture reveals the typical staining pattern (rabbit anti-human Hsp90) with the 68-kDa band (trace) in the stressed culture and the 44- and 40-kDa bands in both. Prior to ultracentrifugation, there is little reactive protein detectable in the culture supernatant (Sup1). Upon ultracentrifugation one sees the typical staining pattern in the pelleted vesicles (Fig. 5, Vesicles) of a major 44-kDa band and a minor 40-kDa band in both the stressed and unstressed categories and a trace 68-kDa band in the stressed category (not visible in the figure). Similar patterns are seen in the washed whole cells (Cells), the French press lysate (Lysate), and the low-speed

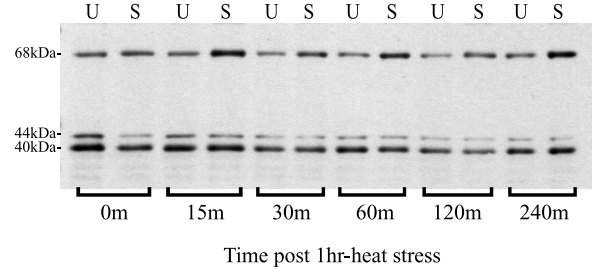

FIG. 4. Kinetics of expression of HtpG following heat shock of *P. gingivalis*. *P. gingivalis* ATCC 33277 was grown to mid-log (A_{600} = 0.34) phase at 37°C, split under anaerobic conditions, and cultured for an additional hour at 37°C (U) or 45°C (S). Pairs of cell cultures (stressed and unstressed) were harvested by centrifugation at 0, 15, 30, 60, 120, and 240 min poststress. Membranes were probed with rabbit anti-human Hsp90. Molecular masses of the three major bands (68, 44, and 40 kDa) are indicated.

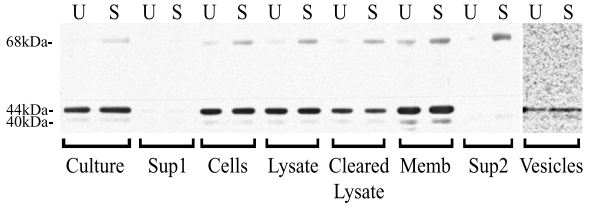

FIG. 5. Location of HtpG in subcellular fractions of *P. gingivalis*. *P. gingivalis* ATCC 33277 was grown to early log (A_{600} = 0.15) phase at 37°C, split under anaerobic conditions, and cultured for an additional hour at 37°C (U) or 45°C (S). The bacteria were fractionated as described in Materials and Methods. The fractions (left to right) included a whole-cell culture (Culture), a culture supernatant (Sup1), whole cells (Cells), French press product (Lysate), a supernatant fraction of French press product (Cleared Lysate), an ultracentrifuge-pelleted membrane fraction of cleared lysate (Memb), an ultracentrifuge supernatant of cleared lysate (Sup2), and an ultracentrifuge-pelleted vesicle fraction from culture supernate (Vesicles). Membranes were probed with rabbit anti-human Hsp90. Molecular masses of the three major bands (68, 44, and 40 kDa) are indicated.

centrifugation supernatant (Cleared Lysate). Upon ultracentrifugation of the cleared lysate into the membrane fraction and the cytosol fraction evidence of compartmentalization of the HtpG polypeptides is visible. The membrane fractions show intense staining by the 44-kDa band, while the stressed cytosol (Sup2) reveals an intensely staining 68-kDa band and a trace amount of the 40-kDa band.

HtpG induction in *P. gingivalis* strains. We compared the immunoreactivity of five strains of *P. gingivalis*: ATCC 33277, 381, A7A1-28, ATCC 53978 (W50), and W83. All cultures were grown to early log phase, heat stressed as described previously, and then evaluated by Western blot analysis with rabbit anti-human Hsp90 antibody. As shown in Fig. 6A, there appeared to be no significant interstrain differences in HtpG immunoreactivity, induction levels, or molecular weight.

Cloning and sequence analysis of *P. gingivalis htpG*. The recent release of preliminary *P. gingivalis* W83 genomic sequence data provided information necessary to clone the gene encoding HtpG of strain 33277. The *A. actinomycetemcomitans htpG* sequence (43) was used to identify DNA encoding HtpG in the unassembled contigs contained in the first preliminary release of *P. gingivalis* genomic DNA sequences by PGGP. We chose *A. actinomycetemcomitans* because it is also a gram-negative oral species and because *htpG* sequences from more closely related species were not available. By aligning the deduced *A. actinomycetemcomitans* HtpG amino acid sequence with six-frame translation of the available *P. gingivalis* sequences, two short regions of high homology were identified. Nondegenerate oligonucleotide primers were designed based on these sequences, using third-position codon biases of other *P. gingivalis* genes. PCR amplification from *P. gingivalis* 33277 with primers 3547I and 3546I generated a 0.6-kb DNA product. DNA sequencing confirmed its identity as the *P. gingivalis* sequence lying between the regions of homology identified with *A. actinomycetemcomitans* (data not shown). This PCR product was used as a probe in subsequent Southern and Northern analyses. Under high-stringency conditions, this probe hybridized with DNA of all *P. gingivalis* strains (ATCC 33277, SUNYaB A7A1-28, ATCC 53978, and 381) but did not hybridize with *E. coli* (JB45 and DH5α) or *A. actinomycetemcomitans* (ATCC 43718) (data not shown).

Subsequently, through analysis of more recent releases of *P. gingivalis* genomic sequence data, we identified a 2,052-bp open reading frame encompassing the 0.6-kb PCR product. This product corresponded to the 5′ end of the putative *htpG* gene. *P. gingivalis* ATCC 33277 DNA was subjected to PCR using oligonucleotide primers 7752I and 733J derived from sequences flanking the putative *htpG* locus of *P. gingivalis* W83. The PCR product was cloned in pBluescript II KS(+). The restriction map of the cloned *htpG* of strain 33277 corresponded to that predicted from the contig sequence of strain W83. DNA sequencing of both strands of the entire cloned 33277 *htpG* showed complete sequence identity between *P. gingivalis* W83 and ATCC 33277 *htpG* (data not shown).

BLAST searches yielded numerous heat shock genes with homology to the *P. gingivalis htpG*. Figure 7 shows that the deduced HtpG peptides of *E. coli* and *A. actinomycetemcomitans* are very similar to each other (>77% identity), while *P. gingivalis* HtpG shares <30% identity with either of these species. The deduced 684-residue *P. gingivalis* HtpG peptide has a predicted molecular mass of 78 kDa and includes a C-terminal domain not found in *E. coli* and *A. actinomycetemcomitans*. That the identical sequence was determined independently from two strains of *P. gingivalis* argues strongly that this was not a sequencing artifact. No HtpG sequences available through the National Center for Biotechnology Information BLAST server appeared to share significant homology with the C-terminal domain of *P. gingivalis* HtpG, suggesting that this region is unique.

Northern blot analysis. Northern blot analysis of *htpG* transcription provided information on HtpG expression beyond that shown by Western blotting. Figure 6B shows a Northern blot of total RNA from *P. gingivalis* ATCC 33277, 381, A7A1-28, ATCC 53978 (W50), and W83 probed with the ^{32}P-labeled 0.6-kb heat-denatured DNA fragment encoding the N-terminal region of *P. gingivalis* HtpG. Unstressed *P. gingivalis* does not have significant amounts of *htpG* transcript. RNA from heat-stressed cultures contained a major hybridizing band at approximately 2.2 kb, which could encode a peptide of up to 80 kDa, demonstrating a rapid upregulation of transcription following heat stress from virtually undetectable levels.

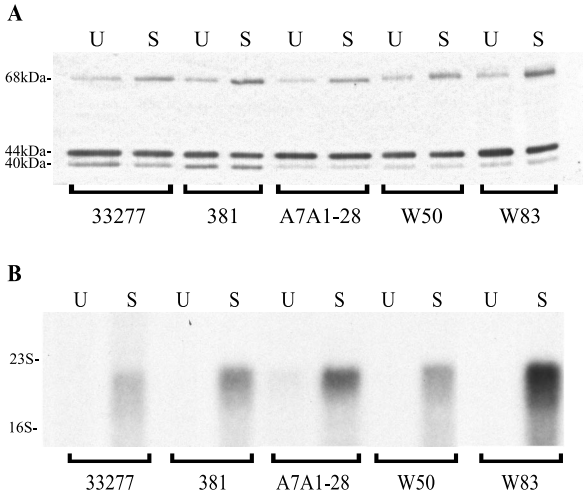

FIG. 6. Comparison of HtpG expression by five strains of *P. gingivalis* (ATCC 33277, 381, A7A1-28, and ATCC 53978 [W50] and W83). (A) Expression of HtpG protein following heat shock of *P. gingivalis*. Immunoreactivity with the rabbit anti-human Hsp90 antibody in Western blot analysis is shown in the upper panel. All cultures were grown to early log phase and heat stressed as described in the text. (B) Expression of *htpG* mRNA transcript following heat shock of *P. gingivalis*. Northern blot of RNA from heat-stressed (S, 45°C) and unstressed (U, 37°C) RNA from cultures of *P. gingivalis* strains is shown. Ten micrograms of total RNA was electrophoresed and probed with the ^{32}P-labeled 0.6-kb heat-denatured DNA fragment encoding the N-terminal region of *P. gingivalis* HtpG. The location of the 23S and 16S rRNAs are indicated.

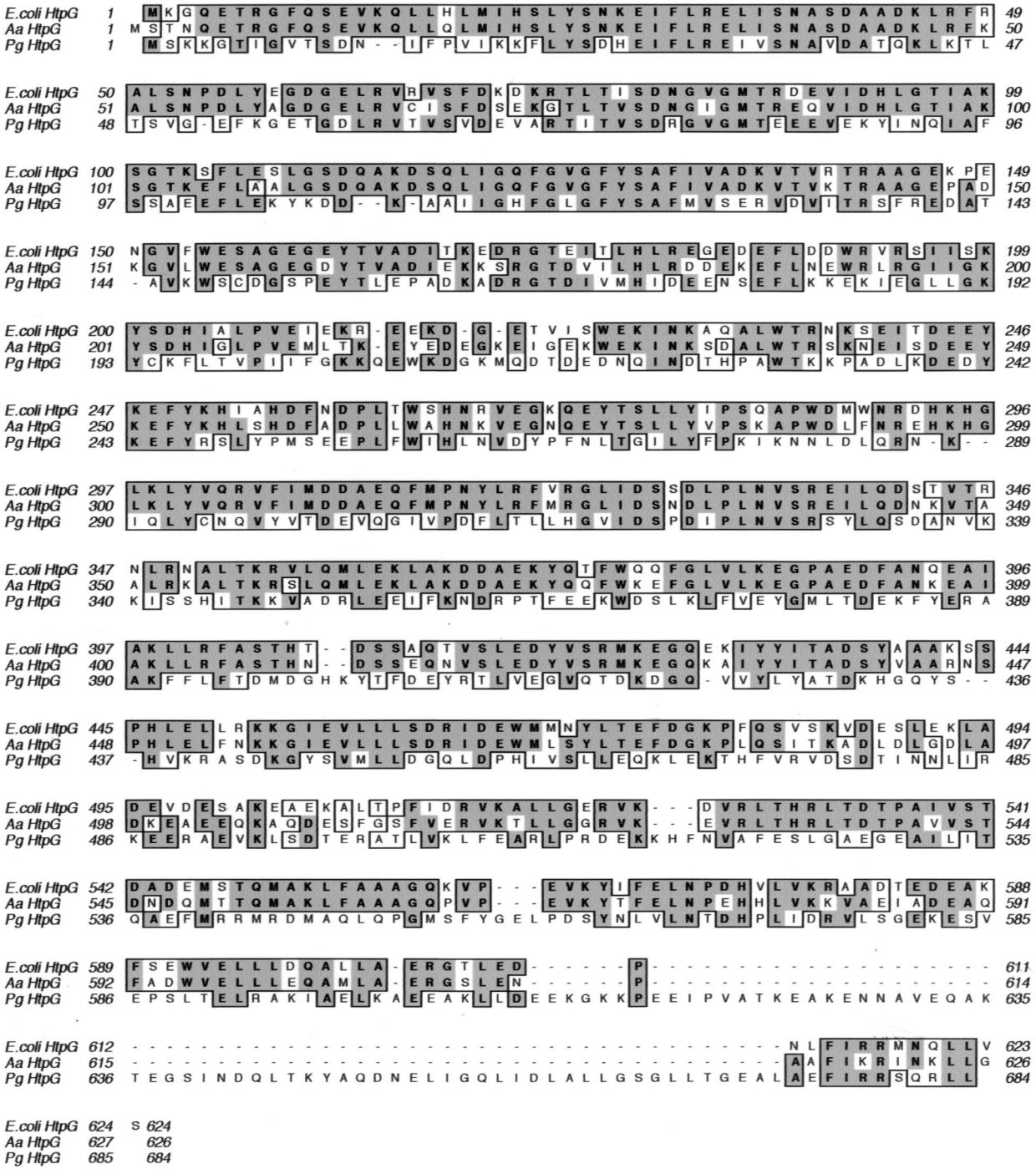

FIG. 7. Alignments of deduced amino acid sequences of *P. gingivalis*, *E. coli*, and *A. actinomycetemcomitans* HtpG. Optimal alignment of the deduced HtpG peptide sequences of *E. coli* (1), *A. actinomycetemcomitans* (43), and *P. gingivalis* ATCC 33277. Shaded residues are identical between at least two of the sequences. Boxed residues are conserved between at least two of the sequences (34).

DISCUSSION

Investigation of the nature of the HtpG protein of *P. gingivalis* has been driven by two compelling factors. First, we recently reported a protective relationship between the presence of anti-Hsp90 serum antibodies and oral health (22). This relationship was significant only when the individuals were colonized with *P. gingivalis*. This is of interest, since most investigators have reported that serum antibodies to periodontal pathogens were elevated as the severity of the disease worsened (reviewed in reference 5). Antibody levels that increase as the disease worsens probably reflect one of several processes. The antibodies may simply reflect the presence of the pathogen's stimulation of the immune system or they may contribute to the pathology of the lesion in a hypersensitivity mechanism. Second, there is evidence in studies of fungal in-

fections for serum antibodies reactive with members of the Hsp90 family that have been found to be protective (3, 25, 26). It is significant that we have found an immune response to a component of a periodontal pathogen that is associated with health. Subsequent analyses of this bacterial stress protein will address its participation in probable mechanisms that contribute to its virulence.

Our previous studies examined the localization of Hsp90 cross-reactive proteins (epitopes shared with human Hsp90) in *P. gingivalis* (21). Those studies revealed that *P. gingivalis* possessed 68-, 44-, and 40-kDa polypeptides that reacted strongly with the rabbit anti-human Hsp90 polyclonal antibodies. The reactive epitopes tended to localize to membranes and extracellular vesicles and were probably accessible to the external environment. The present study suggests that the 44- and 40-kDa forms (apparent fragments) of HtpG tended to be localized in the membrane fractions and that the 68-kDa forms (the intact protein) was found predominantly in the cytosol. This result was similar to the findings in which the *C. albicans* Hsp90 shared epitopes with human Hsp90 (25). In those studies, two polypeptides could be detected, one at 90 kDa (the size of native eukaryotic Hsp90) and one at 47 kDa (presumably a degradation product). It should be noted that, in eukaryotic systems, the Hsp90 homologue generally has a molecular mass in the range of 80 to 90 kDa, while the prokaryotic homologue, HtpG, has a molecular mass of approximately 68 kDa (11). Given the similarities with our findings, we felt that further investigation of the nature of the Hsp90 homologue (HtpG) of *P. gingivalis* was necessary in order to study its potential contribution to the virulence of the microorganism.

Because of earlier reports by Vayssier et al. (40) regarding the protease-derived artifacts in SDS-PAGE of *P. gingivalis* proteins, we were concerned about the effects of such artifacts in our analyses. It is clear that immediate extraction with TCA was much more effective at preserving protein integrity than either adding a protease inhibitor cocktail or boiling the samples in SDS sample buffer. Therefore, we employed immediate TCA extraction routinely in all subsequent experiments, except in the fractionation studies. As shown in Fig. 1, the resolution and clarity of the protein bands in the TCA-precipitated samples is remarkable compared to the other treatments. This finding has significant implications for the interpretation of numerous past studies of antibody reactivity (enzyme-linked immunosorbent assay or Western blots) performed in the absence of stringent procedures for inhibiting *P. gingivalis* proteases.

Since no antibodies specific for the *P. gingivalis* HtpG protein were available, we took advantage of the high level of epitope conservation in the stress protein families. We used a commercially available monoclonal antibody directed against a conserved epitope of the Hsp90 protein of the water mold *A. ambisexualis*, which reacted with the Hsp90 proteins of numerous species (33), including humans and *P. gingivalis*. In addition, we used a polyclonal rabbit antibody previously made in our laboratory against purified human Hsp90 protein (21) and a second rabbit polyclonal antibody prepared by James Bardwell against *E. coli* HtpG protein. All of these antibodies detected 68- and 44-kDa bands in *P. gingivalis* extracts. This is consistent with findings of 90- and 47-kDa bands by Matthews et al. in *C. albicans* (27). Since the monoclonal antibody reacted with both the 44- and 68-kDa bands, it is highly likely that the 44-kDa band is derived from the 68-kDa band. An additional 40-kDa band was detected by both polyclonal antibodies but not the monoclonal antibody, suggesting that the 40-kDa band is a degradation fragment that does not contain the epitope. The monoclonal antibody that we have used recognizes an epitope in the C-terminal portion of the human Hsp90 protein (32). Sequence comparisons between human Hsp90 and *P. gingivalis* HtpG suggest that this epitope may be located near the C terminus of the HtpG. The monoclonal antibody does not appear to detect this epitope in *E. coli* (not shown), which is supported by the lack of sequence homology with *P. gingivalis* in the C-terminal regions of their respective HtpG proteins. Thus, we believe that the 44-kDa polypeptide represents a C-terminal fragment of the 68-kDa polypeptide. The source of the 40-kDa polypeptide is not clear from these results, but we suspect that it is derived from the 44-kDa polypeptide.

Based on our Northern blot analyses, a single transcript encodes the HtpG protein, supporting our belief that the 40- and 44-kDa bands are derived from a single 68-kDa polypeptide. This finding was consistent for all five strains tested. This is confirmed by our Southern blot analyses, which reveal the presence of a single *htpG* gene (data not shown). Since the Northern blotting does not reveal the presence of transcripts that could encode these smaller polypeptides, it is likely that the 40- and 44-kDa polypeptides are the products of posttranslational events.

Our experiments which examined inducibility of the HtpG stress responses revealed that the 68-kDa polypeptide was generally detected at the lowest levels compared to the two other polypeptides (even following heat shock), and it was the only polypeptide that increased significantly and appears to be continuously expressed for at least 4 h following heat shock. While the 44-kDa band was generally more intense than the 40-kDa band, there was some variability in intensity when individual experiments were compared. However, comparisons of paired stressed and unstressed categories for each band showed no intra- or interexperiment differences regardless of the growth phase or other stress conditions. We hypothesize that the anti-Hsp90-reactive 40- and 44-kDa peptides detected with the monoclonal and polyclonal antibodies represent an accumulation of stable HtpG breakdown (or processed) products derived from either basal level production of the 68-kDa protein or from prior stress-induced responses. The probable scenarios are as follows: (i) after translation of the 68-kDa protein it is translocated to a membrane compartment which stabilizes or protects a 40- or 44-kDa fragment of the protein from proteolysis or (ii) after translation of the 68-kDa polypeptide specific posttranslational modifications generate the 44- and 40-kDa proteins. To support a potential role of HtpG as a virulence factor of *P. gingivalis* it was important to demonstrate that HtpG localized in the cell envelope and, thus, could be accessible for interaction with cytoplasmic components of the host cell. Currently, we favor the hypothesis that the fragmentation occurs subsequent to membrane association because, in the cell fractionation studies, the lower-molecular-mass fragments are found to be membrane associated, while the 68-kDa protein is found in both membrane-associated and soluble fractions.

We did not see major differences in expression of the HtpG protein, either at the protein or transcript levels when different strains of *P. gingivalis* were examined. Since other investigators have attributed various degrees of virulence to these strains, we hypothesize that other putative virulence factors work in concert with HtpG in the overall pathology associated with this microorganism. It is logical to predict a multistep virulence process which depends on mechanisms of adherence and/or attachment, invasion and, ultimately, damage (reviewed in reference 28). Deviation in any one of these steps would be expected to disrupt the overall virulence pathway of the pathogen.

Analysis of the cloned gene yielded a sequence of 2,052 bp which we believe to contain the entire *htpG* gene of *P. gingivalis*. The deduced *P. gingivalis* HtpG polypeptide is approximately 60 residues longer (684 amino acids) than other prokaryotic HtpG homologues and has <30% overall identity with *E. coli* and *A. actinomycetemcomitans* HtpGs. Note that the *htpG* sequence of strain 33277 was identical to that of strain W83 and that both strains contained this apparently unique carboxyl-terminal domain. Studies in progress will further characterize this region of HtpG. While the deduced sequence of *P. gingivalis* HtpG would predict a molecular size of approximately 78 kDa, compared to 68 kDa that was determined by SDS-PAGE, there is precedence for this disparity. In describing the purification and properties of *E. coli* HtpG, Spence and Georgopoulos (38) reported a discrepancy between the predicted molecular mass (from the DNA sequence) of 71 kDa compared to the experimentally deduced molecular mass of 65.5 kDa. While no conclusive evidence was presented, this discrepancy was attributed to the shape of the denatured protein or its highly acidic isoelectric point. Based on computer analysis, the *P. gingivalis* HtpG is predicted to have a pI of 4.8. The carboxyl region of *P. gingivalis* HtpG is not significantly homologous with other prokaryotic HtpG proteins, including that of *E. coli*. In contrast, *E. coli* and *A. actinomycetemcomitans* HtpGs are very nearly the same size and share more than 75% identity. Thus, we feel that the *P. gingivalis* HtpG may have unique biological features not shared with other prokaryotic HtpG proteins that have been cloned to date. These differences may contribute to the disease association that we reported previously.

ACKNOWLEDGMENTS

We acknowledge the assistance of Neal Van Poperin in the preparation and preliminary characterization of the anti-human Hsp90 antibody used in this study. In addition, we thank James Bardwell for the generous gift of anti-*E. coli* HtpG antibody.

This work was supported by an award from the National Institutes of Health and NIDCR DE11117.

REFERENCES

1. **Bardwell, J. C., and E. A. Craig.** 1988. Ancient heat shock gene is dispensable. J. Bacteriol. **170:**2977–2983.
2. **Bramanti, T. E., and S. C. Holt.** 1990. Iron-regulated outer membrane proteins in the periodontopathic bacterium, *Bacteroides gingivalis*. Biochem. Biophys. Res. Commun. **166:**1146–1154.
3. **Burnie, J. P., and R. C. Matthews.** 1991. Heat shock protein 88 and *Aspergillus* infection. J. Clin. Microbiol. **29:**2099–2106.
4. **Chen, C. K., A. DeNardin, D. W. Dyer, R. J. Genco, and M. E. Neiders.** 1991. Human immunoglobulin G antibody response to iron-repressible and other membrane proteins of *Porphyromonas* (*Bacteroides*) *gingivalis*. Infect. Immun. **59:**2427–2433.
5. **Ebersole, J. L., D. Cappelli, M. J. Steffen, D. E. Willmann, and D. S. O'Dell.** 1996. Host response assessment in recurring periodontitis. J. Clin. Periodontol. **23:**258–262.
6. **Ensgraber, M., and M. Loos.** 1992. A 66-kilodalton heat shock protein of *Salmonella typhimurium* is responsible for binding of the bacterium to intestinal mucus. Infect. Immun. **60:**3072–3078.
7. **Goulhen, F., A. Hafezi, V. J. Uitto, D. Hinode, R. Nakamura, D. Grenier, and D. Mayrand.** 1998. Subcellular localization and cytotoxic activity of the GroEL-like protein isolated from *Actinobacillus actinomycetemcomitans*. Infect. Immun. **66:**5307–5313.
8. **Hinode, D., D. Grenier, and D. Mayrand.** 1995. Purification and characterization of a DnaK-like and a GroEL-like protein from *Porphyromonas gingivalis*. Anaerobe **1:**283–290.
9. **Hinode, D., R. Nakamura, D. Grenier, and D. Mayrand.** 1998. Cross-reactivity of specific antibodies directed to heat shock proteins from periodontopathogenic bacteria and of human origin. Oral Microbiol. Immunol. **13:**55–58.
10. **Hotokezaka, H., H. Hayashida, N. Ohara, H. Nomaguchi, K. Kobayashi, and T. Yamada.** 1994. Cloning and sequencing of the groESL homologue from *Porphyromonas gingivalis*. Biochim. Biophys. Acta **1219:**175–178.
11. **Jakob, U., and J. Buchner.** 1994. Assisting spontaneity: the role of Hsp90 and small Hsps as molecular chaperones. Trends Biochem. Sci. **19:**205–211.
12. **Johnson, K., I. Charles, G. Dougan, D. Pickard, P. O'Gaora, G. Costa, T. Ali, I. Miller, and C. Hormaeche.** 1991. The role of a stress-response protein in *Salmonella typhimurium* virulence. Mol. Microbiol. **5:**401–407.
13. **Johnson, K. S., I. G. Charles, G. Dougan, I. A. Miller, D. Pickard, P. O'Goara, G. Costa, T. Ali, and C. E. Hormaeche.** 1990. The role of a stress-response protein in bacterial virulence. Res. Microbiol. **141:**823–825.
14. **Kadri, R., D. Devine, and W. Ashraf.** 1998. Purification and functional analysis of the DnaK homologue from *Prevotella intermedia* OMZ 326. FEMS Microbiol. Lett. **167:**63–68.
15. **Kaufmann, S. H.** 1990. Heat shock proteins and the immune response. Immunol. Today **11:**129–136.
16. **Kirby, A. C., S. Meghji, S. P. Nair, P. White, K. Reddi, T. Nishihara, K. Nakashima, A. C. Willis, R. Sim, M. Wilson, et al.** 1995. The potent bone-resorbing mediator of *Actinobacillus actinomycetemcomitans* is homologous to the molecular chaperone GroEL. J. Clin. Investig. **96:**1185–1194.
17. **Koga, T., T. Kusuzaki, H. Asakawa, H. Senpuku, T. Nishihara, and T. Noguchi.** 1993. The 64-kilodalton GroEL-like protein of *Actinobacillus actinomycetemcomitans*. J. Periodontal Res. **28:**475–477.
18. **Lindquist, S.** 1986. The heat-shock response. Annu. Rev. Biochem. **55:**1151–1191.
19. **Lindquist, S., and E. A. Craig.** 1988. The heat-shock proteins. Annu. Rev. Genet. **22:**631–677.
20. **Lokensgard, I., V. Bakken, and K. Schenck.** 1994. Heat shock response in *Actinobacillus actinomycetemcomitans*. FEMS Immunol. Med. Microbiol. **8:**321–328.
21. **Lopatin, D. E., E. Jaramillo, C. A. Edwards, A. Combs, and C. E. Shelburne.** 1999. Immunogold localization of an Hsp90 homologue in *Porphyromonas gingivalis*. FEMS Microbiol. Lett. **181:**9–16.
22. **Lopatin, D. E., C. E. Shelburne, N. Van Poperin, C. J. Kowalski, and R. A. Bagramian.** 1999. Humoral immunity to stress proteins and periodontal disease. J. Periodontol. **70:**1185–1193.
23. **Lu, B., and B. C. McBride.** 1994. Stress response of *Porphyromonas gingivalis*. Oral Microbiol. Immunol. **9:**166–173.
24. **Maeda, H., M. Miyamoto, H. Hongyo, A. Nagai, H. Kurihara, and Y. Murayama.** 1994. Heat shock protein 60 (GroEL) from *Porphyromonas gingivalis*: molecular cloning and sequence analysis of its gene and purification of the recombinant protein. FEMS Microbiol. Lett. **119:**129–135.
25. **Matthews, R., and J. Burnie.** 1992. Acquired immunity to systemic candidiasis in immunodeficient mice: role of antibody to heat-shock protein 90. J. Infect. Dis. **166:**1193–1195.
26. **Matthews, R. C., J. P. Burnie, D. Howat, T. Rowland, and F. Walton.** 1991. Autoantibody to heat-shock protein 90 can mediate protection against systemic candidosis. Immunology **74:**20–24.
27. **Matthews, R. C., J. P. Burnie, and S. Tabaqchali.** 1984. Immunoblot analysis of the serological response in systemic candidosis. Lancet **ii:**1415–1418.
28. **Meyer, D. H., K. P. Mintz, and P. M. Fives-Taylor.** 1997. Models of invasion of enteric and periodontal pathogens into epithelial cells: a comparative analysis. Crit. Rev. Oral Biol. Med. **8:**389–409.
29. **Morimoto, R. I., A. Tissleres, and C. Georgopoulos.** 1990. The stress response function of the proteins, and perspectives, p. 1–36. *In* R. I. Morimoto, A. Tissleres, and C. Georgopoulos (ed.), Stress proteins in biology and medicine. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Nair, S. P., S. Meghji, K. Reddi, S. Poole, A. D. Miller, and B. Henderson.** 1999. Molecular chaperones stimulate bone resorption. Calcified Tissue Int. **64:**214–218.
31. **Nakano, Y., Y. Inai, Y. Yamashita, S. Nagaoka, T. Kusuzaki-Nagira, T. Nishihara, N. Okahashi, and T. Koga.** 1995. Molecular and immunological characterization of a 64-kDa protein of *Actinobacillus actinomycetemcomitans*. Oral Microbiol. Immunol. **10:**151–159.
32. **Nemoto, T., N. Sato, H. Iwanari, H. Yamashita, and T. Takagi.** 1997. Domain structures and immunogenic regions of the 90-kDa heat-shock protein (HSP90). Probing with a library of anti-HSP90 monoclonal antibodies and limited proteolysis. J. Biol. Chem. **272:**26179–26187.
33. **Riehl, R. M., W. P. Sullivan, B. T. Vroman, V. J. Bauer, G. R. Pearson, and D. O. Toft.** 1985. Immunological evidence that the nonhormone binding component of avian steroid receptors exists in a wide range of tissues and species. Biochemistry **24:**6586–6591.
34. **Riek, R. P., M. D. Handschumacher, S. S. Sung, M. Tan, M. J. Glynias, M. D. Schluchter, J. Novotny, and R. M. Graham.** 1995. Evolutionary conservation of both the hydrophilic and hydrophobic nature of transmembrane residues. J. Theor. Biol. **172:**245–258.
35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Sauk, J. J.** 1990. Stress proteins in development and disease. Crit. Rev. Oral Biol. Med. **1:**235–245.
37. **Shinnick, T. M.** 1991. Heat shock proteins as antigens of bacterial and parasitic pathogens. Curr. Top. Microbiol. Immunol. **167:**145–160.
38. **Spence, J., and C. Georgopoulos.** 1989. Purification and properties of the *Escherichia coli* heat shock protein, HtpG. J. Biol. Chem. **264:**4398–4403.

39. **Stamm, L. V., F. C. Gherardini, E. A. Parrish, and C. R. Moomaw.** 1991. Heat shock response of spirochetes. Infect. Immun. **59:**1572–1575.
40. **Vayssier, C., D. Mayrand, and D. Grenier.** 1994. Detection of stress proteins in *Porphyromonas gingivalis* and other oral bacteria by Western immunoblotting analysis. FEMS Microbiol. Lett. **121:**303–307.
41. **Weidner, M. F., D. Grenier, and D. Mayrand.** 1996. Proteolytic artifacts in SDS-PAGE analysis of selected periodontal pathogens. Oral Microbiol. Immunol. **11:**103–108.
42. **Welch, W. J.** 1991. The role of heat-shock proteins as molecular chaperones. Curr. Opin. Cell Biol. **3:**1033–1038.
43. **Winston, J. L., S. I. Toth, B. A. Roe, and D. W. Dyer.** 1996. Cloning and characterization of the *Actinobacillus actinomycetemcomitans* gene encoding a heat-shock protein 90 homologue. Gene **179:**199–204.
44. **Yoshida, A., Y. Nakano, Y. Yamashita, H. Yu, M. Ohishi, and T. Koga.** 1997. Isolation and characterization of the dnaKJ operon from *Actinobacillus actinomycetemcomitans*. DNA Sequence **8:**93–98.
45. **Young, R. A.** 1990. Stress proteins and immunology. Annu. Rev. Immunol. **8:** 401–420.

Editor: E. I. Tuomanen