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Cellular localization of a Hsp90 homologue in *Porphyromonas gingivalis*

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Abstract

We previously reported an association between elevated serum antibody titers to the 90-kDa human heat shock protein (Hsp90), periodontal health and colonization by *Porphyromonas gingivalis*. In this study, we examined the cellular localization of the Hsp90 homologue of *P. gingivalis*. Cultures of *P. gingivalis* were heat-stressed (45°C) and examined for localization of the Hsp90 homologue. Heat stress induced a 4–5-fold increase in anti-Hsp90 antibody reactivity over that of the unstressed controls. Western blot analysis revealed two bands (44 and 68 kDa) that reacted with anti-Hsp90 antibodies. The 68-kDa band was heat-inducible, while the 44-kDa band was not. Immunogold staining revealed that the Hsp90 homologue localized principally to the membrane and extracellular vesicles. Subcellular fractionation confirmed that the Hsp90 homologue was primarily membrane-associated.

Keywords: Heat shock; Extracellular vesicle; 90-kDa human heat shock protein; *Porphyromonas gingivalis*

1. Introduction

Stress responses, characterized by the preferential synthesis of a distinct array of proteins, are evolutionarily conserved mechanisms used by cells to survive the changes in their environment [1]. Associations between virulence and stress protein expression have been identified. Johnson et al.[2] reported that the stress response was directly related to *Salmonella typhimurium* virulence. Ensgraber and Loos [3] reported that a 66-kDa heat shock protein of *S. typhimurium* was responsible for binding of the bacterium to intestinal mucus. In systemic fungal infections involving *Candida albicans*, it has been reported that patient survival is associated with the presence of antibodies reactive with the 47-kDa fragments of the 90-kDa stress proteins of the fungus [4].

There is a noticeable paucity of data on stress proteins associated with Gram-negative anaerobic pathogens. Reports by Lu and McBride [5] and Hinode et al. [6] demonstrated that *Porphyromonas gin-*

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givalis expresses families of stress proteins common to other bacteria. In addition, there is evidence that the iron-modifiable protein of *P. gingivalis* [7] probably represents a nutrition-induced stress protein. Studies from our laboratory suggest that serum antibodies reactive with this protein family are present in health and may protect from colonization and subsequent tissue damage mediated by *P. gingivalis* [8]. Because of this finding, we initiated a study to localize the 90-kDa human heat shock protein (Hsp90) homologue in *P. gingivalis* as the first step in determining the potential role of Hsp90 in the virulence of this microorganism.

2. Materials and methods

2.1. Cultivation, heat stress and processing of *P. gingivalis*

P. gingivalis strains ATCC 33277 and SUNYaB A7A1-28 were grown under anaerobic conditions at 37°C in an atmosphere of 95% N_2 and 5% CO_2 on enriched trypticase soy agar supplemented with menadione (5 μg ml^{-1}), hemin (5 μg ml^{-1}) and 2% sheep blood. Broth (Mycoplasma Broth, supplemented with hemin and menadione) cultures grown under anaerobic conditions at 37°C to the mid- to late-exponential phase were divided, with one portion maintained at 37°C, while the other was incubated at 45°C. After 1 h, the cultures were centrifuged for 20 min at 12 000 × *g*. The supernatant culture fluid was removed and the cell pellets were washed three times in sterile phosphate-buffered saline (PBS, 0.05 M PO_4, 0.15 M NaCl, pH 7.4).

2.2. Particle concentration immunofluorescence assay (PCFIA)

PCFIA analysis was performed using a monoclonal antibody directed against Hsp90 to demonstrate that the cells had actually undergone a stress response. The pelleted cells were combined with 5 ml of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Pefabloc SC (AEBSF; Boehringer Mannheim), 1% Triton X-100 pH 6.8) for 1–2 h at 4°C. The suspended cells were sonicated for two 15-s intervals on ice. Lysate was then diluted to 10 ml in 0.1 M sodium phosphate (pH 7.0) and combined with 10 mg of carbodiimide and 1 ml of carboxyl Epicon™ latex particles (0.8 μM, 5% w/v, IDEXX) and incubated for 1–2 h at 37°C with rotation. The lysate-coated particles were then washed three times in 0.1 M sodium phosphate and resuspended in 20 ml PBS+0.2% sterile milk proteins (Milk diluent/Blocking Solution, KPL Laboratories). Twenty μl of the coated particles was dispensed into each well of a 96-well filtration plate (Epicon™ Assay Plate, IDEXX) to which was then 20 μl added of an anti-Hsp90 monoclonal antibody (5 μg ml^{-1}; StressGen Biotechnologies, Victoria, B.C., Canada, SPA-830) and the plate was incubated 30 min at room temperature. The monoclonal antibody was directed against a Hsp90 epitope of the water mold *A. ambisexualis*. This antibody has been shown to react with a Hsp90 epitope common to numerous species, including human [9]. The particles were washed and 20 μl of FITC-labelled goat anti-mouse IgG (5 μg ml^{-1}, KPL Laboratories) was added. Following an additional 15-min incubation, the particles were washed three times and the bound fluorescence was measured at 535 nm.

2.3. Western blot analysis of stressed and control cultures

Processing of *P. gingivalis* employed 10% trichloroacetic acid (TCA) extraction to minimize artifact-producing degradation of cellular proteins by *P. gingivalis* proteases [10]. The cell pellets were resuspended in 10% TCA and incubated for 1 h at room temperature. The samples were then centrifuged at 13 000 × *g* for 15 min at 4°C. The pelleted material was resuspended in 1 × NuPAGE™ sample buffer (Novex, San Diego, CA, USA), containing 2% sodium dodecyl sulfate (SDS) and 50 mM dithiothreitol, and heated at 100°C for 10 min. The samples were electrophoresed on 4–12% SDS-polyacrylamide gel electrophoresis (PAGE) gels (NuPAGE™) in 1 × MOPS running buffer. After electroblotting onto nitrocellulose membranes and blocking with 5% non-fat dry milk in 1 × TBS buffer, the membranes were probed with anti-Hsp90 antibody and the reactive bands detected with horseradish peroxidase-conjugated goat anti-mouse or rabbit IgG antibody and chemiluminescence (ECL, Amersham).

2.4. Subcellular fractionation of P. gingivalis

Unstressed *P. gingivalis* cells were fractionated by a modification of the method of Kim et al. [11]. Two hundred and fifty-ml cultures at the mid-exponential phase were centrifuged at 12 000 × *g* for 20 min. The culture supernatant was centrifuged at 100 000 × *g* for 2 h to pellet the vesicles. The cell pellet was resuspended in PBS, pH 7.5, containing protease inhibitors (30 μM aprotinin, 2 mM benzamidine, 215 μM leupeptin, 10 μM pepstatin, 2 mM PMSF and 2 mM TLCK). After passage through a French press (= 1000 psi) four times, the suspension was centrifuged at 12 000 × *g* for 20 min to remove unbroken cells. The supernatant fluid was centrifuged at 200 000 × *g* for 2 h. The resultant vesicles, pellet and supernatant fluid were precipitated in 10% TCA and stored at −80°C prior to Western blot analysis.

2.5. Immunogold staining of stressed and unstressed cultures

The immunogold labelling methods described by Progulske-Fox et al. [12] were followed. Pelleted cultures of *P. gingivalis* were placed into microcentrifuge tubes containing buffered 2% paraformaldehyde/0.25% glutaraldehyde and fixed for 30 min at 4°C. After dehydration in ethanol, unicryl resin was added to the cell pellets and allowed to infiltrate for 2 days. The cell pellets were then exposed to an ultraviolet light source and allowed to cure at −10°C for 3 days. Sections (70–90 nm) were cut with a diamond knife and collected on carbon-coated formvar-reinforced 300-mesh nickel grids.

Grids containing the sections were floated on droplets of fresh 0.1 M NH_4Cl for 1 h, followed by a 15-min rinse buffer solution (0.01 M Tris-HCl, 0.15 M NaCl, pH 7.2). This was followed by a rinse in filtered bovine serum albumin (BSA) buffer (0.01 M Tris-HCl, 0.5 M NaCl, 0.025% NaN_3, 1.0% BSA fraction V, pH 7.2) for 30 min. The grids were blotted and placed on droplets containing the primary antibodies. The primary antibodies included the monoclonal anti-Hsp90 antibody and a murine monoclonal anti-human OKT8 antibody (Ortho Diagnostic Systems, Raritan, NJ, USA). Both antibodies were diluted (1:50) and incubated overnight at 4°C. The grids were washed in rinse buffer for 30 min, floated on a filtered solution of carbowax buffer (0.01 M Tris-HCl, 0.15 M NaCl, 0.025% NaN_3, 0.2% carbowax (PEG 20 M), pH 7.2) for 30 min, blotted and placed in the secondary antibody (goat anti-mouse IgGM conjugated to 10-nm gold particles (Goldmark Biologicals, Phillisburg, NJ, USA)) diluted 1:50 in carbowax buffer and incubated for 1 h at room temperature. Following two washes in rinse buffer and a final wash in distilled water, the grids were either left unstained or double-stained with uranyl acetate and lead citrate and examined in a transmission electron microscope.

3. Results

3.1. Hsp90 homologue induction

PCFIA analysis revealed a significant ($P = 0.05$) increase in anti-Hsp90 antibody reactivity of *P. gingivalis* cells following heat stress. Constitutive (37°C) expression of the anti-Hsp90-reactive antigens in the cellular extract of strain ATCC 33277 was

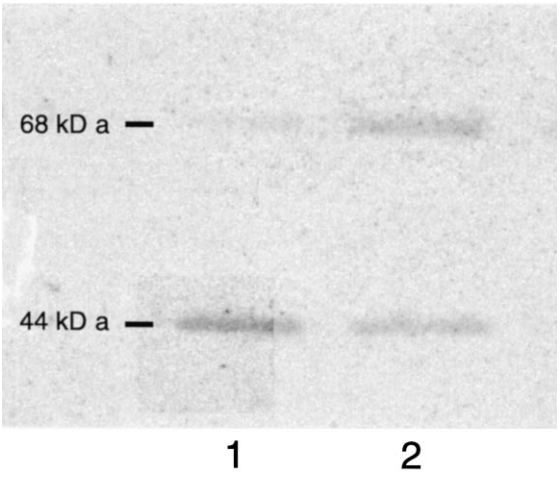

Fig. 1. Western blot analysis of *P. gingivalis* ATCC 33277 whole cell extracts electrophoresed on 4–12% SDS-PAGE gels in 1 × MOPS running buffer and electroblotted onto nitrocellulose membranes. The membranes were probed with a monoclonal anti-Hsp90 antibody (StressGen Biotechnologies, SPA-830) and the reactive bands detected with an alkaline phosphatase-conjugated goat anti-mouse antibody and chemiluminescence. Lane 1, unstressed (37°C) culture; lane 2, heat-stressed (45°C for 1 h) culture.

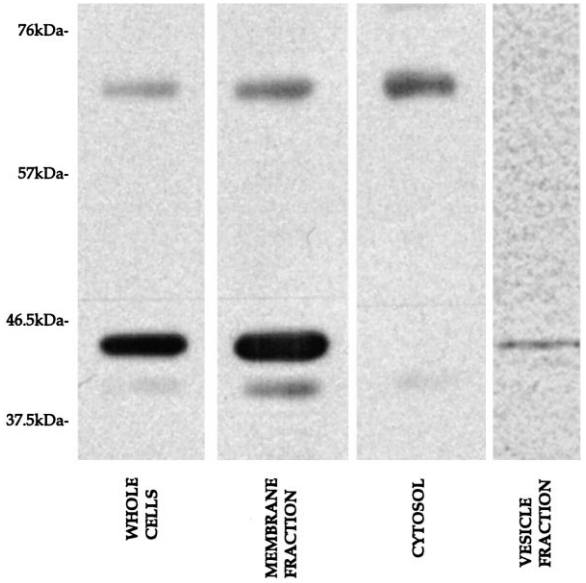

Fig. 2. Western blot analysis of subfractions of stressed *P. gingivalis* ATCC 33277. The membranes were probed with anti-Hsp90 antibody (StressGen Biotechnologies, SPA-830). First lane (left to right), unfractionated whole cells; lane 2, membrane fraction; lane 3, cytosol fraction; lane 4, extracellular vesicle fraction.

5974 ± 1230 RFU and stressed (45°C) expression was 27 834 ± 8574 RFU or approximately a 4–5-fold increase. The A7A1-28 strain yielded similar significant results.

3.2. Western blot analysis of cellular extracts

Reactivity of *P. gingivalis* cellular extracts with the monoclonal anti-Hsp90 antibody is shown in Fig. 1. Two prominent immunoreactive bands were detected. The first band, located at approximately 68 kDa, the expected molecular mass of prokaryotic Hsp90 homologues, demonstrated heat-inducibility. The blots of the unstressed cultures (37°C, lane 1) revealed that this protein was expressed constitutively. Heat stress (45°C, lane 2) induced a 3-fold increase in this protein expression over constitutive levels. A second band was located at approximately 44 kDa. This band stained more intensely than the unstressed 68-kDa band but did not appear to be heat-inducible. The identity of these two bands was confirmed using two additional polyclonal antibodies, the first prepared against *Escherichia coli* htpG product (*E. coli* Hsp90 homologue) and the second prepared against human Hsp90 protein (data not shown).

3.3. Subcellular fractionation

Western blot analysis of subcellular fractions of stressed *P. gingivalis* (Fig. 2) revealed that the anti-Hsp90-reactive antigen was localized predominantly in the membrane fractions of *P. gingivalis*. Consistent with whole cell extracts, membrane fractions possessed a strongly staining 44-kDa band, as well as a weaker 68-kDa band. In contrast, cytosol fractions stained only for the 68-kDa band. Vesicle staining was consistent with membrane staining and revealed only a 44-kDa band.

3.4. Immunogold localization of Hsp90 homologue

In order to confirm the findings of the cell fractionation studies, we performed immunogold staining of fixed sections of *P. gingivalis* cultures using the monoclonal antibody. An unrelated monoclonal antibody of the same immunoglobulin class and isotype (IgG1) was used to control for non-specific

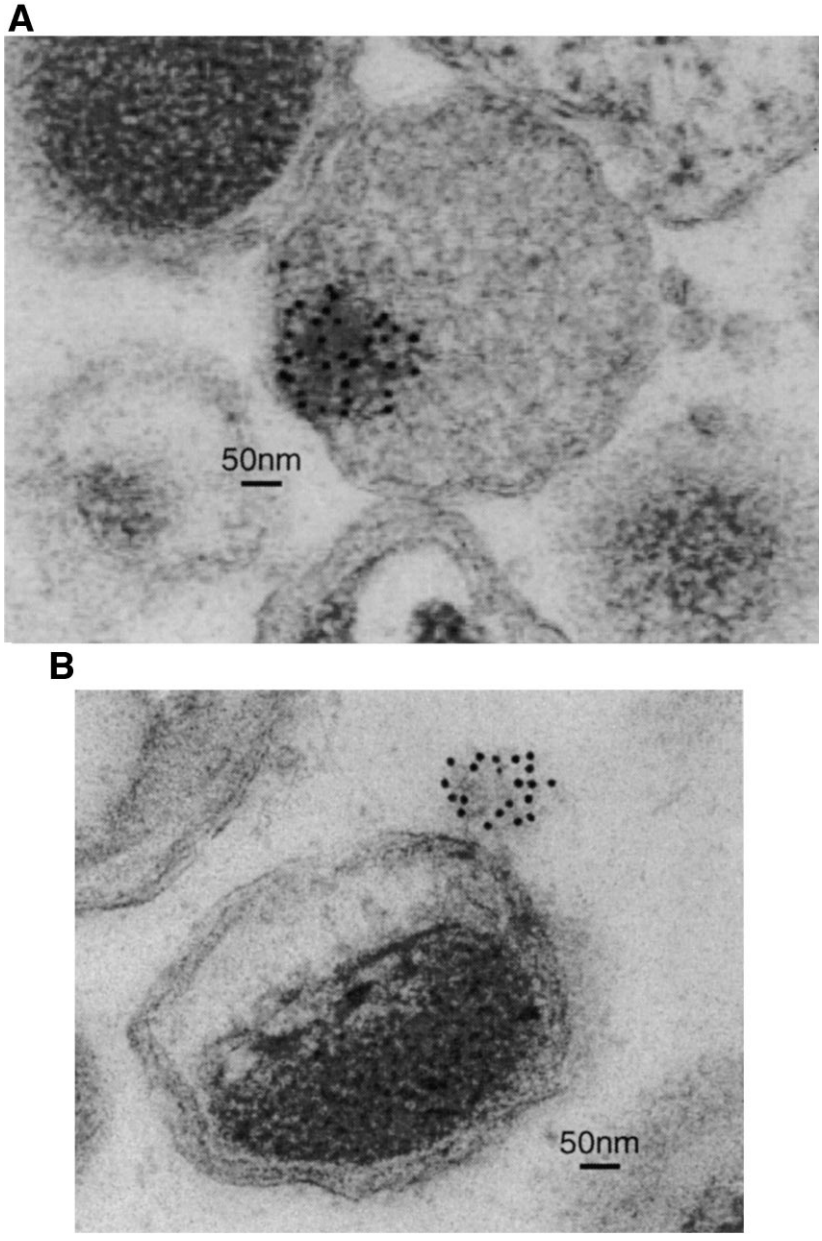

Fig. 3. Immunogold staining of *P. gingivalis* cells. Unicryl resin-embedded *P. gingivalis* (ATCC 33277 and A7A1-28) were sectioned (70–90 nm) and stained with monoclonal anti-Hsp90 antibody (primary) and with goat anti-mouse IgGM conjugated to 10-nm gold particles (secondary antibody). A shows staining of heat-stressed A7A1-28 'budding' membrane-associated vesicle. B shows staining of heat-stressed A7A1-28 extracellular vesicle. The bar represents 50 nm.

staining. The results of our localization study are shown in Fig. 3 and Table 1. Less than 10% of the *P. gingivalis* cells in any field were labelled with the anti-Hsp90 antibody. In those cells that were labelled, we observed a diffuse localization of the immunogold to the cytoplasmic areas, not generally associated with identifiable organelles, usually only 2–6 particles labelling the interior of the cell. As

Table 1
Distribution of immunogold particles in stressed and unstressed *P. gingivalis* strains

	ATCC 33277		A7A1-28	
	37°C	45°C	37°C	45°C
No. of cells in 20 fields[a]	153	137	168	207
% of cells labelled[b]	8%	18%	6%	23%
Mean gold particles in labelled cells	18.4 ± 4	23.7 ± 7	24.3 ± 5	28.4 ± 6
Mean gold particles in cytoplasm of labelled cells	3.6 ± 2	3.4 ± 2	2.2 ± 1	4.2 ± 3
Mean gold particles membrane-associated in labelled cells[c]	15.4 ± 3	20.4 ± 4	22.5 ± 4	24.3 ± 2
No. of vesicles in 20 fields (not cell-associated)	220	247	180	276
% of vesicles labelled[d]	6%	13%	11%	23%
Mean gold particles per labelled vesicle	23.5 ± 5	27.5 ± 6	18.7 ± 6	31.7 ± 4

[a]All cells that were in focus on the photographs were analyzed unless the cell envelope was disrupted or non-detectable.
[b]Gold particles were included which were either located within a cell or located less than a distance of two gold particles from the cell membrane. This distance corresponds to the maximal theoretical distance that a gold particle can be from the primary antibody bound to a membrane-associated antigen.
[c]Gold particles were considered membrane-associated if they were less than a two gold particle distance from the membrane.
[d]Gold particles were considered vesicle-associated if they were less than a two gold particle distance from the vesicle.

shown in Fig. 3A, cells (A7A1-28) that were highly labelled generally possessed the typical cytoplasmic staining pattern and then presented with clusters of particles associated with what appeared to be budding membrane vesicles. When labelled, these putative vesicles were always intensely decorated with gold particles (10–30 gold particles per vesicle). In limited instances, non-budding areas of the cell membrane were decorated with several individual gold particles. As shown in Fig. 3B, a limited number (5–15%) of free vesicles (not cell-associated) were also seen to be labelled. When labelled, the vesicles were always found to be highly decorated with gold particles (15–30 particles per vesicle).

As shown in Table 1, when the staining of heat-stressed cells was compared to the unstressed cells, there was a 2–4-fold increase in the mean numbers of cells that were labelled with gold particles and the numbers of gold particles that were associated with the cell membranes and vesicles increased. However, the numbers of gold particles labelling the cytoplasmic spaces did not change significantly following heat stress.

4. Discussion

Preliminary studies showed that *P. gingivalis*, a putative pathogen in the etiology of periodontal disease, expressed elevated levels of a Hsp90 homologue following heat stress. We sought to localize this protein as a first step in our investigation of the role of this protein in the etiology of periodontal disease.

Western blot analysis revealed two major immunoreactive bands at 44 and 68 kDa. A 68-kDa band is characteristic of the Hsp90 homologue of prokaryotic cells. The 44-kDa band does not demonstrate the heat-inducibility of the 68-kDa band and may represent an accumulated degradation fragment of the intact protein due to autolytic or degradative pathways described for other stress protein families [13]. This finding is also reminiscent of studies by Matthews et al. [14], who described a predominant 47-kDa fragment of the *C. albicans* Hsp90 protein.

Immunogold labelling of *P. gingivalis* cells revealed several trends. First, in the unstressed populations, only about 6–11% of the cells bound gold particles. This increased to 18–23% or about a doubling of the number of labelled cells following heat stress. Remarkably, the actual distribution of gold particles, cytoplasmic or membrane-associated, did not change significantly after stress. The most intensely gold-labelled organelles were the vesicles or 'blebs' that were either still cell-associated or completely independent of the intact cells. This membrane localization was confirmed by our cellular subfractionation studies. As shown in Table 1, the percentage of labelled vesicles increased following stress and those labelled vesicles were highly deco-

rated with gold. There are also conflicting data on localization patterns of DnaK (Hsp70 family) in *E. coli*. Cellular fractionation studies by Zylicz et al [15] and Kostyal et al. [16] reported that this stress protein co-fractionates with membrane components, while Bardwell et al. [17] reported that there were significant amounts that were found in a soluble form as well as membrane-associated. Since many of these proteins serve as chaperones, it was proposed that cell fractionation studies might be prone to artifactual association between DnaK and protein elements released during the fractionation process [18]. A more recent paper by Bukau et al. [19], using immunogold labelling to localize the DnaK protein in *E. coli*, reported that labelling was found predominantly in the cytoplasm, however, a small fraction of the gold particles was in proximity to the membranes. They also reported that heat shock did not induce a detectable relocalization of DnaK within the cell. This most closely reflects our findings in the detection of Hsp90 immunoreactivity specifically within the intact *P. gingivalis* cells. There is, however, a paucity of data regarding the presence of stress proteins and chaperones in the extracellular vesicles of other species, making additional comparisons difficult.

Several laboratories have reported an association between virulence and expression of Hsp90 homologues. The earliest reports described the association between immunity to specific epitopes of the Hsp90 protein of *C. albicans* and protection against systemic candidiasis [4,20]. Our laboratory recently reported that there was an association between periodontal health and elevated anti-Hsp90 serum antibody titers [8]. Statistical modeling indicated that colonization by *P. gingivalis* was intimately associated with this relationship. It is not clear from any of these studies to date how Hsp90 proteins contribute to the virulence of the microorganisms. This family of proteins plays a critical role in chaperoning steroid hormone receptors, protein kinases and other regulatory proteins in eukaryotic systems and perhaps expression of these proteins by microbial systems is disruptive to their normal functions. Alternatively, membrane-associated Hsp90 homologues may serve as cellular receptors or to stabilize or transport 'true' virulence components of these microorganisms.

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References

[1] Sauk, J.J. (1990) Stress proteins in development and disease. Crit. Rev. Oral Biol. Med. 1, 235–245.
[2] Johnson, K., Charles, I., Dougan, G., Pickard, D., O'Gaora, P., Costa, G., Ali, T., Miller, I. and Hormaeche, C. (1991) The role of a stress-response protein in *Salmonella typhimurium* virulence. Mol. Microbiol. 5, 401–407.
[3] Ensgraber, M. and Loos, M. (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.
[4] Matthews, R.C., Burnie, J.P., Howat, D., Rowland, T. and Walton, F. (1991) Autoantibody to heat-shock protein 90 can mediate protection against systemic candidosis. Immunology 74, 20–24.
[5] Lu, B. and McBride, B.C. (1994) Stress response of *Porphyromonas gingivalis*. Oral Microbiol. 9, 166–173.
[6] Hinode, D., Nakamura, R., Grenier, D. and Mayrand, D. (1998) Cross-reactivity of specific antibodies directed to heat shock proteins from periodontopathogenic bacteria of human origin. Oral Microbiol. 13, 55–58.
[7] Bramanti, T.E. and Holt, S.C. (1992) Localization of a *Porphyromonas gingivalis* 26-kilodalton heat-modifiable, hemin-regulated surface protein which translocates across the outer membrane. J. Bacteriol. 174, 5827–5839.
[8] Lopatin, D.E., Shelburne, C.E., Van Poperin, N., Kowalski, C.J. and Bagramian, R.A. (1999) Humoral immunity to stress proteins and periodontal disease. J. Periodontol. (in press).
[9] Nemoto, T., Sato, N., Iwanari, H., Yamashita, H. and Takagi, T. (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.
[10] Weidner, M.F., Grenier, D. and Mayrand, D. (1996) Proteolytic artifacts in SDS-PAGE analysis of selected periodontal pathogens. Oral Microbiol. Immunol. 11, 103–108.
[11] Kim, S.J., Chu, L. and Holt, S.C. (1996) Isolation and characterization of a hemin-binding cell envelope protein from *Porphyromonas gingivalis*. Microb. Pathog. 21, 65–70.
[12] Progulske-Fox, A., Tumwasorn, S. and Holt, S.C. (1989) The expression and function of a *Bacteroides gingivalis* hemagglutinin gene in *Escherichia coli*. Oral Microbiol. Immunol. 4, 121–131.
[13] Mitchell, H.K., Petersen, N.S. and Buzin, C.H. (1985) Self-degradation of heat shock proteins. Proc. Natl. Acad. Sci. USA 82, 4969–4973.
[14] Matthews, R.C., Burnie, J.P. and Tabaqchali, S. (1987) Iso-

lation of immunodominant antigens from sera of patients with systemic candidiasis and characterization of serological response to *Candida albicans*. J. Clin. Microbiol. 25, 230–237.

[15] Zylicz, M., Nieradko, J. and Taylor, K. (1983) *Escherichia coli* dnaJ- and dnaK-gene products: synthesis in minicells and membrane-affinity. Biochem. Biophys. Res. Commun. 110, 176–180.

[16] Kostyal, D.A., Farrell, M., McCabe, A., Mei, Z. and Firshein, W. (1989) Replication of an RK2 miniplasmid derivative in vitro by a DNA/membrane complex extracted from *Escherichia coli*: involvement of the dnaA but not dnaK host proteins and association of these and plasmid-encoded proteins with the inner membrane. Plasmid 21, 226–237.

[17] Bardwell, J.C., Tilly, K., Craig, E., King, J., Zylicz, M. and Georgopoulos, C. (1986) The nucleotide sequence of the *Escherichia coli* K12 dnaJ+ gene. A gene that encodes a heat shock protein. J. Biol. Chem. 261, 1782–1785.

[18] Liberek, K., Georgopoulos, C. and Zylicz, M. (1988) Role of the *Escherichia coli* DnaK and DnaJ heat shock proteins in the initiation of bacteriophage lambda DNA replication. Proc. Natl. Acad. Sci. USA 85, 6632–6636.

[19] Bukau, B., Reilly, P., McCarty, J. and Walker, G.C. (1993) Immunogold localization of the DnaK heat shock protein in *Escherichia coli* cells. J. Gen. Microbiol. 139, 95–99.

[20] al-Dughaym, A.M., Matthews, R.C. and Burnie, J.P. (1994) Epitope mapping human heat shock protein 90 with sera from infected patients. FEMS Immunol. Med. Microbiol. 8, 43–48.