STRESS RESPONSE OF *PORPHYROMONAS GINGIVALIS*

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ABSTRACT

The heat shock response of *Porphyromonas gingivalis* was examined by one- and two-dimensional polyacrylamide gel electrophoresis after metabolic labeling with (^{14}C)-amino acids. When *P. gingivalis* cells were shifted from 37°C to 42°C, elevated synthesis of four proteins with the apparent molecular weight of 92, 80, 74, and 62kDa was observed, whereas synthesis of a 50kDa protein decreased during heat shock. The 74 and 62kDa proteins were identified as homologs of *E. coli* DnaK and GroEL respectively by Western immunoblotting. On two-dimensional Western blots, two forms of DnaK and four forms of GroEL were identified due to their slightly different isoelectric points. *dna*K and *gro*EL gene homologs were identified in several *P. gingivalis* and some other black-pigmented *Bacteroides* strains by Southern hybridization. DnaK and GroEL homolog proteins and some other proteins were also induced when *P. gingivalis* cells were challenged by ethanol. Exposure to oxidative stress and an elevation or decrease in pH did not show a discernible induction of these heat shock proteins. GroEL and DnaK homolog proteins were not induced in *P. gingivalis* cells recovered from a guinea-pig infection model.

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INTRODUCTION

The heat shock response

Prokaryotic and eucaryotic cells respond to potentially damaging stimuli such as elevated temperature by increasing the synthesis of a family of proteins collectively known as stress proteins. One to two dozen proteins are induced in response to a range of different stresses, including heat shock, nutrition deprivation, oxygen radicals, and metabolic disruption. The best studied of these stresses is heat shock, in which a sudden increase in temperature induces the increased synthesis of heat shock proteins (HSPs). The heat shock response is amongst the most highly conserved genetic systems known. Because many of the heat shock proteins are also induced by other stresses, they are some times referred to as stress proteins.

Heat shock response (HSR) was originally described by Ritossa in 1962 who showed that upon a temperature shift from 20 to 37°C, as well as treatment with dinitrophenol or sodium salicylate, several new puffs appeared in the salivary gland polytene chromosomes in *Drosophila melanogaster* (90). Over the next several years it became clear that the puffs were the sites of vigorous RNA transcription and that a number of these RNAs were translated into the heat shock proteins. Discovery of the *E. coli* heat shock response helped make it evident that there is a universal cellular response to a shift up in temperature, and soon it became established that components of the response have been highly conserved in bacteria, fungi, plants, and animals. The heat shock response has been intensively investigated by numerous laboratories around the world. The continued interest in heat shock response rests on (a). the highly conserved nature of the structure and function of many HSPs. (b). the regulation of the HSR as a paradigm of gene expression. (c). the important role the HSPs play in protein folding, oligomerization, translocation and degradation. (d). the role of the major HSPs in infection and autoimmunity.

Regulation of the heat shock response

The mechanism of HSR regulation is not completely clear. The HSR is positively regulated at the transcriptional level by the σ^{32} polypeptide, the product of the *rpo*H (*htp*R) gene (23, 45). In *E. coli*, one of the most intensively studied microorganisms, heat shock gene expression requires the heat shock-specific σ subunit of RNA-polymerase, σ^{32}, which confers on core RNA polymerase the specificity to transcribe heat shock genes (20, 46). Regulation of heat shock gene expression is mediated by controlling the cellular concentration as well as the activity (109) of σ^{32}. The cellular concentration of σ^{32} is controlled by regulation of the transcription of *rpo*H and the translation of its message (31, 47, 80), and regulation of the stability of σ^{32} (47, 108-110, 117). In fact, one of the most remarkable features of σ^{32} is its extremely short half-life (about one minute) at steady state growth conditions (47, 108). Genetic evidence indicates key negative regulatory functions for the HSPs DnaK, DnaJ, and GrpE at the levels of synthesis, activity, and degradation of σ^{32} (Figure 1).

Mutations in *dna*K, *dna*J, and *grp*E cause partial stabilization of σ^{32}(116), loss of repression of heat shock gene transcription after temperature downshift(109, 116), and deficiencies in post-transcriptional regulation of σ^{32} synthesis after heat shock (47, 110). The mechanism by which DnaK, DnaJ, and GrpE regulate the activity and stability of σ^{32} is assumed to rely on their concerted activity as chaperones. This activity involves the ATP-dependent binding to substrates of DnaK and the stimulation of hydrolysis of DnaK-bound ATP by DnaJ and GrpE (58, 59, 98). It has been proposed that DnaK interacts with σ^{32} and dissociates it from RNA polymerase, thereby rendering it accessible to cellular proteases (39). In a recent report, Gamer et al. (36) presented evidence for the physical association of DnaK, DnaJ, and GrpE chaperones with σ^{32} *in vivo*.

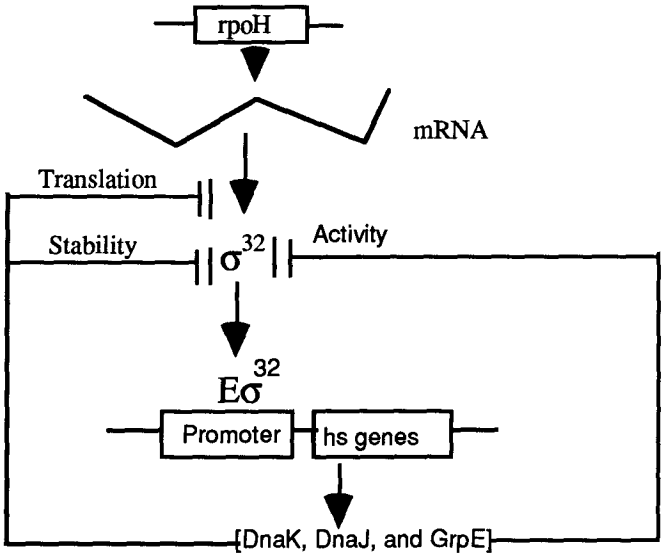


Figure 1. The speculative role of DnaK in heat shock response.

A speculative model for the role of HSP70(DnaK) in controlling expression of HSPs, showing how DnaK, along with DnaJ and GrpE, could act to control HSP expression in bacteria. Upon temperature upshift, depletion of the free pool of these HSPs relieves their negative regulatory effects. Increased synthesis, stability, and activity of σ^{32} permits increased transcription of heat shock genes (adopted from (23)).

The signal transduction pathway that converts environmental stress to specific alterations of heat shock genes expression remains unclear. There is evidence to suggest that the intracellular concentration of aberrant proteins is a major determinant of the cellular HSPs in *E. coli* as well as in eucaryotic cells (5, 35, 41, 53). Another suggested model is that by sequestering HSP70 through its binding to aberrant proteins the heat shock transcription factor σ^{32} is prevented from interacting with HSP70, which in turn allows activation of heat shock gene transcription (23).

It was recently shown that there is another set of heat shock genes which are positively regulated at the transcriptional level by the σ^{24} (σ^{E}) polypeptide (45). One of the promoters of the *rpo*H (*htp*R) gene turned out to be transcribed by the $E\sigma^{24}$ RNA polymerase holoenzyme. The gene coding for σ^{24} has not been discovered.

The function of HSPs

The importance of many HSPs is based on their capacity to associate with other proteins in a way that modifies the destiny and function of the proteins (21, 28, 60, 61, 91). Anfinsen's classic experiments on the refolding of ribonuclease *in vitro* (6) established that all the information required to determine the final conformation of a protein can reside in the polypeptide chain itself: the denatured protein can refold into its native conformation in the absence of other proteins. Such studies suggest that refolding *in vitro* may be initiated by (1). collapse of hydrophobic regions into the interior of the molecule., (2). formation of stable secondary structures that provide a framework for subsequent folding, and (3). formation of covalent interactions, such as disulfide bonds, that stabilize the polypeptide in particular conformations. However, *in vitro* experiments do not accurately reflect the process of folding of nascent proteins in the interior of a cell. Refolding *in vitro* is frequently very inefficient in comparison to folding *in vivo*, and often requires proteins and physicochemical conditions very different from those occurring intracellularly.

Under normal conditions, a polypeptide chain must be correctly folded, processed, localized, and in some cases, complexed with other polypeptides to properly perform its biological functions. This is a very complex biological process with many pitfalls along the way. For example, when the growing polypeptide chain emerges from the ribosome, it is subject to premature contact with other protein domains, either intra or inter specific, because of the high cytosolic protein concentration. Such premature interaction among protein domains must be avoided to prevent misfolding (23, 56, 78, 91). In the case of proteins crossing membranes, it is clear that only unfolded proteins can traverse biological membranes (84), again creating the opportunity for inappropriate interactions among protein domains. In both cases, the need for some sort of "chaperoning' activity to maintain the protein in an unfolded state, and to prevent undesired interactions is clear.

A large set of the protein chaperones are heat shock proteins, whose biological role is to maintain and shield the unfolded state of newly synthesized proteins. Their main functions are (1). preventing proteins from misfolding or aggregating, (2). allowing proteins to traverse biological membranes, and (3). allowing them to fold properly, thus leading to oligomerization. Under conditions of stress, chaperones protect other proteins from heat denaturation, or, once damage has occurred, disaggregate and allow them to refold back to an active form.

Immunodominance and pathogenicity of HSPs

HSPs are biologically complex proteins implicated not only in thermotolerance but also in infection and autoimmunity (29, 50, 132). Studies have shown that 60kDa GroEL homologs from bacterial pathogens are major antigens, evoking both humoral and cellular immune response in infected hosts and immunized animals. A link between stress protein synthesis and survival of bacterial pathogens within the mammalian host during infection has been suggested by studies on *Salmonella typhimurium* and *Mycobacterium tuberculosis*. Buchmeier and Heffron (14)

demonstrated that *S. typhimurium* DnaK and GroEL are among the most prominent proteins induced following entry into host macrophages in a tissue culture system, while Johnson et al. (49) reported that a periplasmic protease (HtrA) identified as a heat shock protein in *E. coli* plays an important role in *in vivo* survival and pathogenicity. Similarly, studies on the intracellular parasite *Listeria monocytogenes* have led to the suggestion that factors important in host cell interactions may be induced by heat shock (106).

Since both DnaK and GroEL have 50% homology to their eucaryotic counterpart HSP70 and HSP60 at the amino acid sequence level and the fact that HSP60 and GroEL expression is increased in cells of both the host and pathogen during the process of infection (14, 51, 131), it has been hypothesized that these HSPs are involved in the development of autoimmunity. Evidence supporting this hypothesis includes modulation of the immune response to GroEL homolog proteins from *Mycobacterium tuberculosis* and *Chlamydia trachomatis,* which resulted in immunological damage in animal models (79, 129, 131).

The HSP70/DnaK Family

This class of proteins has been universally conserved, its members being at least 50% identical to each other at the amino acid sequence level. In *E. coli* this class is represented by the single copy *dnaK* gene, whereas yeast possesses at least eight gene copies (22). Part of the reason for the abundance of gene copies in eukaryotes is the fact that some of their gene products are found exclusively in specialized organelles such as the endoplasmic reticulum, mitochondria, and chloroplasts (22, 30). The HSp70 proteins are the "workhorses" of the chaperones, not only because of their promiscuity in binding to other unfolded polypeptides (78) but also due to their relative abundance in the various cellular compartments.

The *E. coli dnaK* gene was originally discovered because mutations in it block λ DNA replication (39). subsequently it was found that DnaK performs almost

indispensable bacterial functions, since deletion of the gene can be tolerated only within a narrow temperature range, and even under this condition, extragenic suppressers accumulate very rapidly (15). HSP70 proteins have a weak ATPase activity, and the ATPase domain lies in the amino-terminal portion of the protein. DnaK can bind to other polypeptides, such as GrpE, λP, p53, staphylococcal A protein, and unfold bovine pancreatic trypsin inhibitor (19, 48, 58, 59, 135). Such binding is inhibited in the presence of ATP.

The DnaJ and GrpE proteins

The *dnaJ* and *grpE* genes of *E. coli* were originally discovered because mutations in them block bacteriophage λ growth. The *dnaJ* gene forms an operon with the *dnaK* gene, the order being promoter-*dnaK*-*dnaJ*, whereas *grpE* maps elsewhere and is monocistronic. Bacterial homologues to the *dnaJ* gene have been discovered (39). The *dnaJ* and *grpE* proteins are absolutely essential for bacteriophage λ DNA replication as well as bacteriophage P1 plasmid replication *in vivo* and *in vitro* (1, 126, 136). The presence of both GrpE and DnaJ stimulates DnaK's ATPase activity many fold. DnaJ specifically accelerates the hydrolysis step, and GrpE specifically stimulates the nucleotide release step. These results suggest that one role of DnaJ and GrpE is to facilitate the intracellular recycling of DnaK (58). It is interesting that in some organisms the three genes form an operon, *grpE*-*dnaK*-*dnaJ* (124).

The HSP60/GroEL family

The HSP60/GroEL family of proteins has been widely conserved across evolution, although its members have been found only in bacteria, chloroplasts, and mitochondria. The *groES* and *groEL* genes of *E. coli* were originally discovered because mutations in them block bacteriophage growth at the level of assembly of the dodecameric head-tail connector structure (134). The amino acid sequence and function of GroEL protein has been widely conserved, being approximately 50%

identical at the amino acid sequence level to the HSP60 proteins of eukaryotes (30). The GroES protein also appears to be universally conserved since its homologs have been found in mitochondria (65). The *gro*ES and *gro*EL genes are indispensable for growth of *E. coli* at all temperatures and under all conditions tested (39). The *gro*ES and *gro*EL genes form an operon expressed from both a σ^{32}- and a σ^{70}-dependent promoter, resulting in a substantial rate of transcription of the *gro*E operon, even in the absence of σ^{32} (45). The GroES and GroEL proteins functionally interact, leading to an inhibition of GroEL's ATPase activity (134). GroEL protein can bind various unfolded polypeptides *in vitro*. Although it is not clear how GroEL recognizes unfolded polypeptides, the binding appears to be both promiscuous and amino acid sequence-independent (56). The binding of unfolded polypeptides by GroEL promotes their correct assembly by preventing premature inter- or intramolecular interactions that can lead to aggregation. The GroEL/GroES interaction is necessary for the release of some unfolded polypeptides bound to GroEL. Figure 2 depicts a proposed model of interaction between polypeptide, GroEL and GroES (adopted from (7)).

Microbiology of Periodontal Disease

The periodontium consists of gingiva, periodontal ligament, root cementum, and alveolar bone (figure 3a). Diseases that affect the periodontium are collectively called periodontal diseases (figure 3b, 3c).

Various microorganisms can cause virtually all forms of inflammatory periodontal diseases which can be broadly grouped into gingivitis and periodontitis, and each can be further divided into subgroups according to disease activity and severity, age of onset, related systemic disorders, and other factors (figure 4).

Gingivitis is inflammation of the gingiva, but it does not affect the attachment apparatus of teeth. Periodontitis affects connective tissue attachment and adjacent alveolar bone. The classic progression of inflammatory periodontitis is characterized

Figure 2. A model for the action of the GroEL/GroES chaperonins.

The GroEL chaperonin binds to many, but perhaps not all, unfolded polypeptides, some of which may still be nascent. The hydrolysis of ATP allows the release of some polypeptides. ATP hydrolysis coupled with the "cogwheel" action of GroES results in release of the rest.

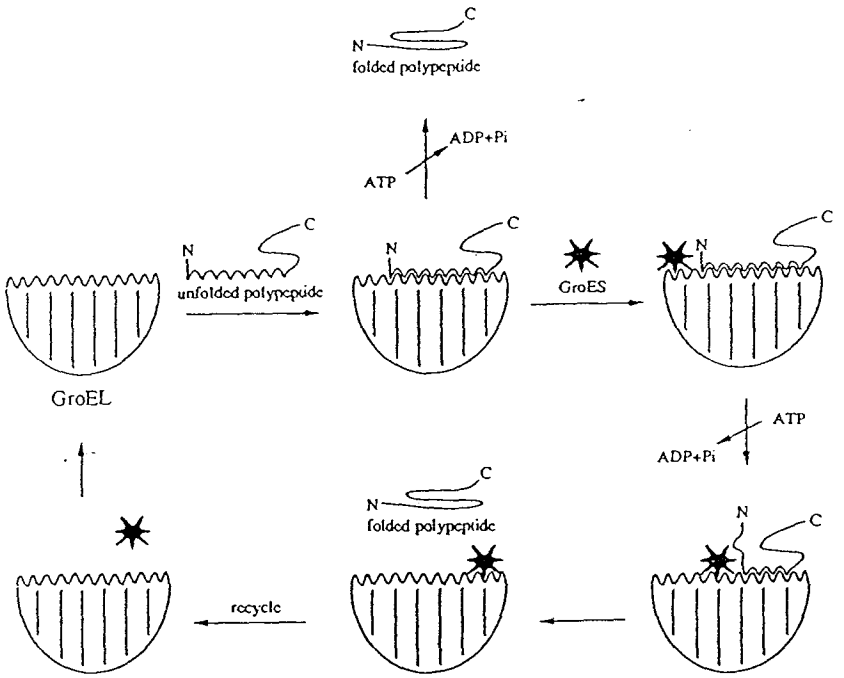


Figure 3a. The periodontium in health. A, periodontal ligament; B, alveolar bone; C, cementum; D, oral epithelium; E, sulcular epithelium; F, junctional epithelium; G, gingival sulcus; H, cementoenamel junction; I, tooth enamel; J, dental plaque microflora.

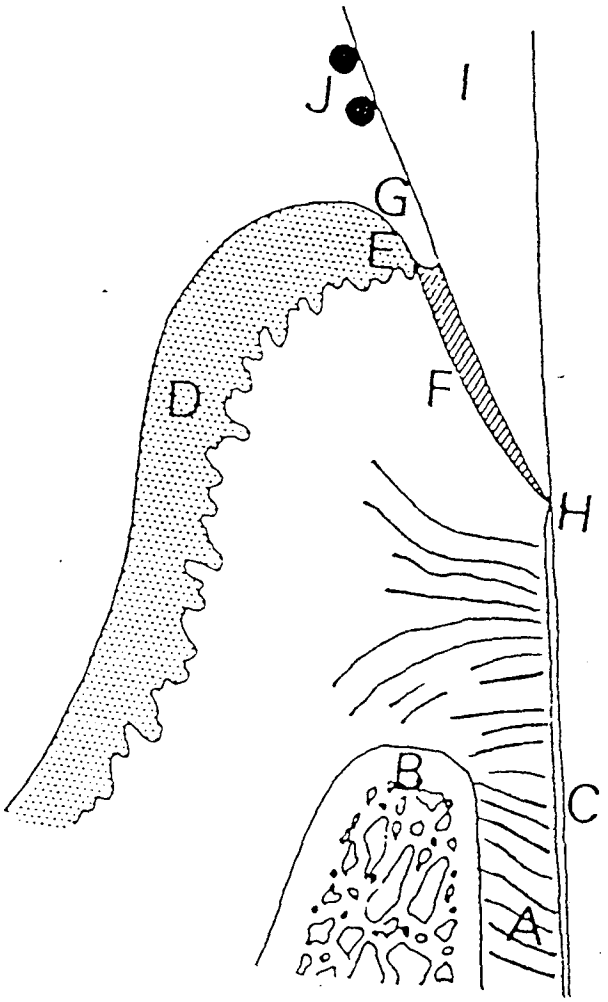


Figure 3b. Established gingivitis lesion. A, inflammatory cell infiltrates in gingival connective tissues, sulcular epithelium, and junctional epithelium; B, gingival tissue swelling leading to increased gingival sulcus depth; C, junctional epithelium at cementoenamel junction; D, dental plaque microflora.

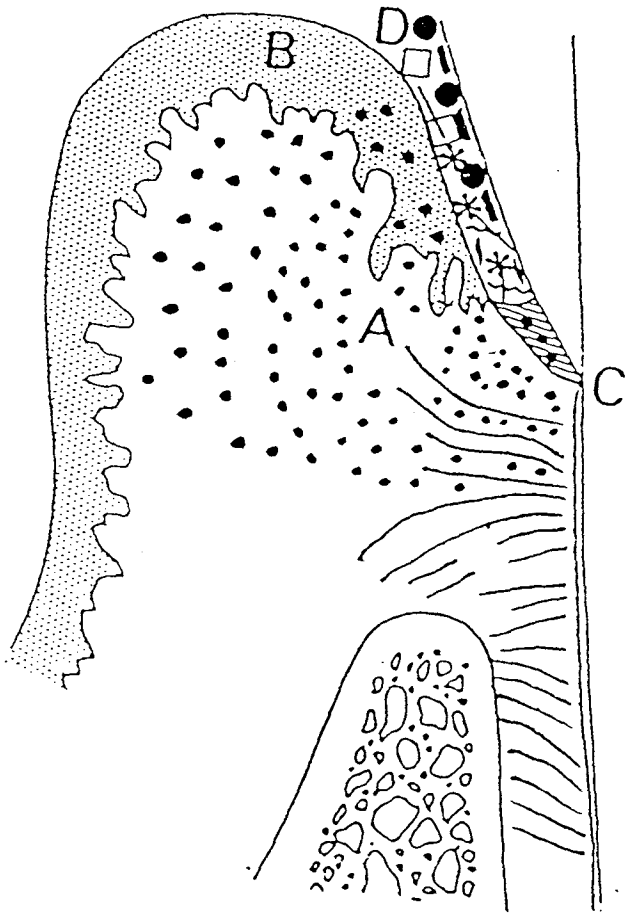
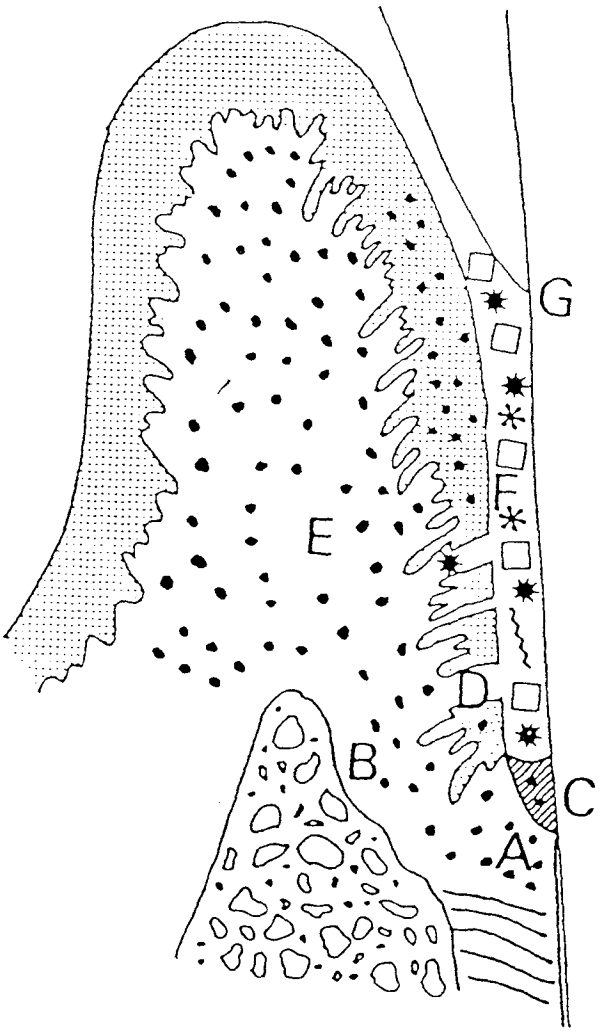


Figure 3c. Periodontitis lesion. A, loss of connective tissue attachment; B, loss of crestal alveolar bone; C, apical migration of junctional epithelium; D, ulceration of periodontal pocket epithelium; E, inflammatory cell infiltrates in gingival connective tissues, sulcular epithelium, and junctional epithelium; F, deepened periodontal pocket and pathogenic microbial flora; G, cementoenamel junction.



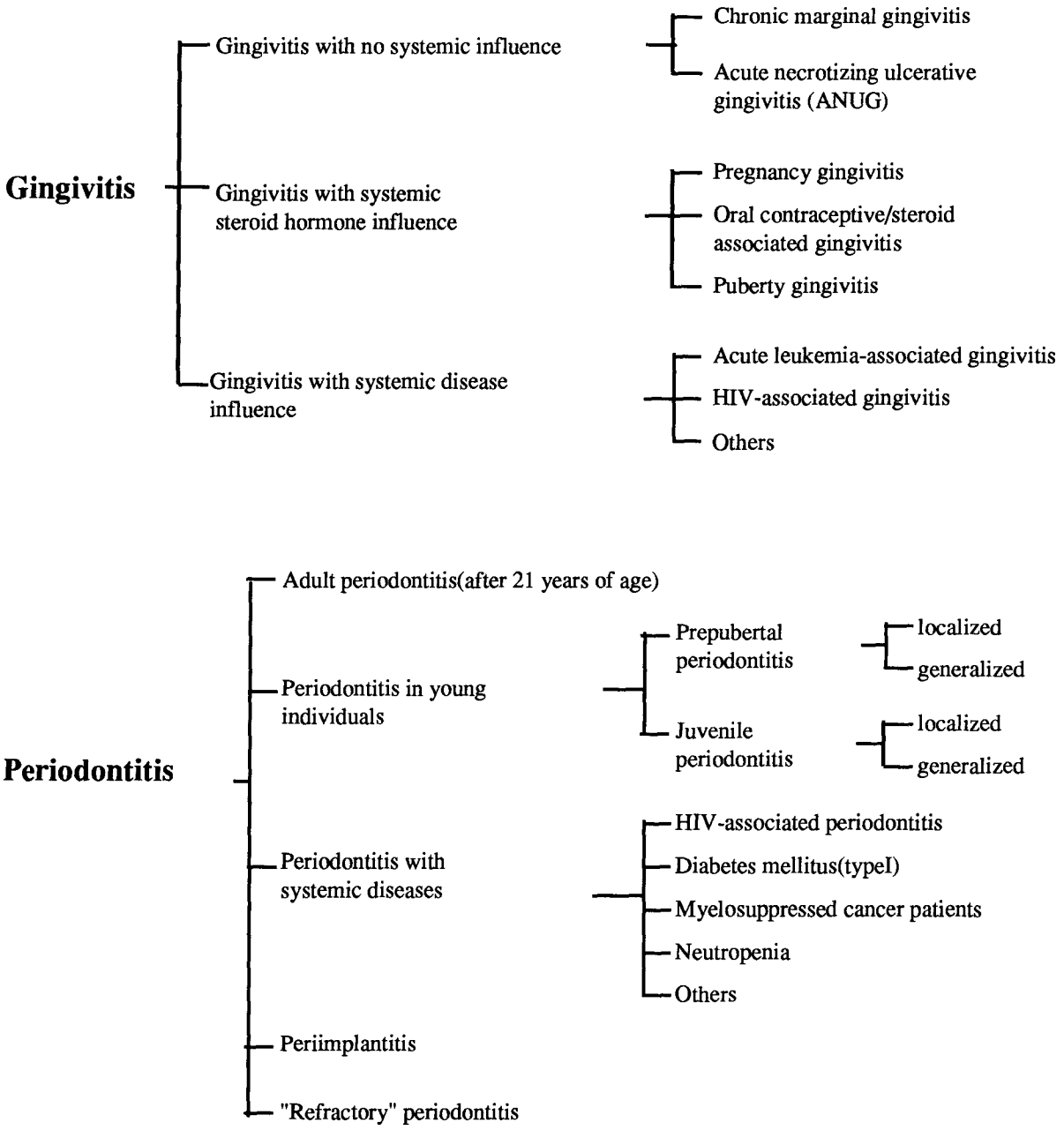


Figure 4. Microbe-associated periodontal diseases

by a highly reproducible microbiological transition of the subgingival microflora from a mainly facultative Gram-positive microbiota to highly pathogenic Gram-negative rods and motile organisms. Although at least 300 different microbial species have been identified from the oral region, it appears that only a very limited number of microbial species are involved in the destruction of periodontal tissues. Among these putative periodontal pathogens are members of the genera *Porphyromonas, Fusobacterium, Wolinella, Actinobacillus, Capnocytophaga,* and *Eikenella*. For example, *Porphyromonas gingivalis* has been implicated in chronic and advanced adult periodontitis (100), *Actinobacillus actinomycetemcomitans* in localized juvenile periodontitis (104, 133), *Prevotella intermedius* and *Treponema denticola* are involved in acute necrotizing ulcerative gingivitis (18, 63). *Capnocytophaga spp.* appear to play an important role in advanced periodontitis in juvenile diabetes (69). There is a growing body of evidence that *Wolinella recta*, *Eikenella corrodens*, and *Bacteroides forsythus* may play a role in the progression of periodontal disease (25, 66, 67, 93, 112).

The subgingival plaque microbiota is of such complexity that, at present, it is not clear whether these periodontal diseases are the result of a pathogenic synergy, or are the result of a monoinfection by an invading or opportunistic member of the resident oral microbiota. It is more likely that the development of periodontal disease involves a consortium of the plaque microbiota that interacts in a cooperative or synergistic manner (42, 70, 114).

Classification of black-pigmented oral anaerobic rods:

Studies have shown that anaerobic, black-pigmented gram-negative rods formerly known as black-pigmented Bacteroides species are associated with destructive periodontitis (reviews by (100, 103, 122)). Since Oliver and Wherry (87) described a small anaerobic gram-negative rod isolated from various parts of human body and named them as "Bacterium melaninogenicum", there have been many changes in the

taxonomy of this group of bacteria. These taxonomic changes have been extensively reviewed (71, 103, 121, 122). Since this group of black-pigmented gram-negative anaerobic rods is very heterogeneous, comprising both saccharolytic and asaccharolytic species, new genera have been proposed: genus *Porphyromonas* for the asaccharolytic species(94) and *Prevotella* for the saccharolytic species (95) , see Table 1 (adopted from (120)).

Pathogenicity of *Porphyromonas gingivalis*

P. gingivalis has been repeatedly implicated in the establishment and progression of periodontal diseases (100, 103, 122). White and Mayrand (125) demonstrated that *P. gingivalis* was present in the gingival sulcus of patients with severe inflammation, but absent from healthy sites. *P. gingivalis* has been recovered not only from subgingival cultures of adults diagnosed as having generalized advanced periodontitis (62, 99, 125), but also from adult patients who have actively progressing periodontitis (101, 113). Di Murro and co-workers (24) found *P. gingivalis* to be consistently associated with the subgingival microflora in patients with rapidly progressive periodontitis. *P. gingivalis* has also been suggested to be involved in severe, recurrent adult periodontitis (118) and both generalized and localized juvenile periodontitis (52, 62, 77, 127). Immunological studies provide additional evidence of the involvement of *P gingivalis* in periodontal diseases. Patients diagnosed with adult periodontitis and generalized juvenile periodontitis have higher serum antibody levels against *P. gingivalis* than other groups of individuals (27, 32). Studies also showed elevated antibody levels to *P. gingivalis* in gingival crevicular fluid of patients with periodontitis (26, 115). The pathogenicity of *P. gingivalis* has also been demonstrated in animal model infection studies (37, 38, 43, 44, 111, 119).

Table 1. Nomenclature of black-pigmented anaerobic rods

Former designation	New designation
Black-pigmented *Bacteroides*	Black-pigmented anaerobic rods
B. gingivalis	*Porphyromonas gingivalis*
B. asaccharolyticus	*Porphyromonas asaccharolyticus*
B. endodontalis	*Porphyromonas endodontalis*
B. salivosus	Possibly related to *Porphyromonas*
B. macacae	Possibly related to *Porphyromonas*
B. levii	Possibly related to *Porphyromonas*
B. intermedius	*Prevotella intermedia*
B. corporis	*Prevotella corporis*
B. melaninogenicus	*Prevotella melaninogenica*
B. denticola	*Prevotella denticola*
B. loescheii	*Prevotella loescheii*

Virulence factors of *P. gingivalis*

A variety of putative pathogenic factors of *P. gingivalis* has been identified which may contribute to the colonization and virulence of this oral pathogen. These factors have been shown to have wide-ranging effects on tissue comprising the periodontium as well as on host immune mechanisms. These determinants include the ability to adhere to other bacterial species or epithelial surfaces, the ability to invade host tissues, the production of toxins or enzymes which are destructive to the host tissue, as well as the bacterial surface components such as lipopolysaccharides or capsular material which can induce infection or protect the bacteria from host immune responses, see Table 2 (adapted from (120)). These studies have usually been conducted *in vitro* and the possible virulence remains to be determined *in vivo*.

Aim of this study

Porphyromonas gingivalis has been implicated in the etiology of adult periodontitis (102, 105). The virulence of *P. gingivalis* is associated with its ability to evade host defenses and produce a range of cytotoxins and enzymes with tissue damaging potential (102). The expression of such virulence components may be greatly influenced by the environmental conditions in the periodontal pocket. Studies have shown that expression of many virulence factors of *P. gingivalis* such as outer membrane proteins (11-13, 88), fimbriae, capsule, outer membrane vesicles (44, 74), and proteolytic activity (68, 73, 92) are regulated by the environmental conditions such as pH change, hemin-limitation and *in vivo* infections. It has been shown that in periodontitis, the subgingival temperature was in general higher at diseased sites than at healthy sites (54), the pH in the periodontal pocket increases with its depth and also with the severity of the inflammatory host response (10). Phagocytosis of bacteria by polymorphonuclear leukocytes (PMNLs) is accompanied by enhancement of PMNL oxidative metabolism (2, 76). All these

Table 2. Virulence factors of *P. gingivalis*

Factors	Possible effect
fimbriae	colonization activate host immune response
hemagglutinin	colonization
other surface binding molecules	colonization
capsule	resistance to phagocytosis
lipopolysaccharide	inhibits growth of fibroblasts, induces bone resorption, inhibits bone collagen formation, activates inflammatory response
outer membrane vesicles	proteolytic and collagenolytic activities, hemagglutinate erythrocytes, promote adherence between noncoaggregating species, impede host immune defence
collagenase	degradation of host tissue
proteases	collaborate with collagenase in tissue destruction, degrade complement, immunoglobulin and serum Fe-binding proteins
butyric acid	cytotoxic
proprionic acid	cytotoxic
indole and ammonia	inhibition of matrix formation cytotoxic
volatile sulphur compounds	cytotoxic

situations could inflict a heat shock and/or other stress on *P. gingivalis.* Very little is known about the regulation of the heat shock response in obligate anaerobic bacteria. The heat shock response studies conducted in *Clostridium acetobulylicum* (81, 82) and several *Spirochetes* (107) have suggested the regulation of the expression of some major heat shock proteins in strict anaerobes is different from that of *E. coli.* I therefore investigated the heat shock response and some other stress responses of *P. gingivalis* as a convenient model for studying environmentally regulated gene expression and anticipate that amongst the proteins induced by heat shock or other stresses we will be able to find some stress responding proteins which are also involved in the process of infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions

P. gingivalis W50 and W83 were grown in peptone-yeast medium which contains 1.7% trypticase peptone, 0.3% yeast extract, 0.25% K_2HPO_4, 0.5% NaCl, 1μg/ml vitamin K and 5μg/ml hemin. The cultures were incubated in a Coy anaerobic chamber in a 5%CO_2-10%H_2-85%N_2 atmosphere at 37°C. Recombinant *E. coli* strain MC4100 (kindly provided by Dr. McCarty (72)) contains plasmid pBB1 that has been constructed by inserting a *Bam*HI fragment containing *E. coli dnaK/J* genes into pBR322. Recombinant *E. coli* strain with plasmid pOF39 which contains an *E. coli groEL/ES* gene insert was kindly provided by Dr. Fayet (33). All recombinant *E. coli* strains were maintained in LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl, pH7.2) with 100μg/ml ampicillin.

Purification of *E. coli* recombinant GroEL protein

A single colony of the recombinant *E. coli* strain containing plasmid pOF39 was inoculated into a 10ml LB broth with 100μg/ml ampicillin. The culture was incubated at 37°C overnight with vigorous shaking. 10ml of this culture was inoculated into 1 liter of LB broth with 200μg/ml ampicillin and incubated at 37°C with vigorous shaking for 24 hours. The bacterial culture was centrifuged in a Beckman JA-10 rotor at 7,000rpm for 10 minutes at 0°C, the cell pellet was washed twice in 100ml of 10mMTris-HCl, pH7.5, resuspended in 50ml 10mMTris-HCl (pH7.5) with 0.02mg/ml DNAse A, 0.02mg/ml RNAse I, 10mM$MgCl_2$. The cell suspension was then sonicated (discontinuous sonication, five 3-minute periods, with cooling in an ice bath, and 2-minute resting periods between each sonication; 40% duty cycle, output 3, Sonifier, Cell Disrupter 350; Branson Sonic Power Co.). The sonicated mixture was centrifuged for 20 minutes at 8,000xg. Unbroken cells and large cell debris were discarded and the supernatant was further centrifuged for 2 hours at 100,000xg.

The semi-transparent, brownish colored pellet containing most of membrane fraction was discarded and the supernatant was subjected to ammonium sulfate precipitation. Ammonium sulfate precipitates were taken for 0-20%, 20-30%, 30-40%, 40-50% and 50-60% saturation. Precipitates were resuspended in 10mM Tris-HCl, pH7.5 and the protein profile of each sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing most of the GroEL protein were pooled, dialyzed against 4 liters of 10mM Tris-HCl (pH7.5), 50mM NaCl for 48 hours. The dialyzed solution containing GroEL and other proteins was loaded onto a 25ml DEAE-Sepharose column equilibrated with the same buffer. The column was first washed with 5 bed-volumes of 10mM Tris-HCl, 50mM NaCl (pH7.5) and then a salt gradient of 50mM to 0.5M NaCl. All fractions were collected by an Automated Econo System (Bio-Rad) and the protein profile of all fractions was determined on a SDS-PAGE gel. The fractions containing most of the GroEL protein were pooled, dialyzed against 4 liters of 10mM Tris-HCl (pH7.5), and 1/3 was loaded onto a 1ml pre-equilibrated MonoQ ion-exchange column connected to an FPLC unit (Pharmacia Fine Chemicals AB.). The column was washed with 10mM Tris-HCl (pH7.5) and a 50mM to 0.5M NaCl salt gradient. Fractions were collected and protein contents were checked on SDS-PAGE. Fractions containing GroEL were pooled, dialyzed against 4 liters of 10mM Tris-HCl (pH7.5) and prepared for immunization (see Immunological procedures).

Purification of *E. coli* recombinant DnaK protein

Ammonium sulfate precipitates of recombinant *E. coli* strain MC4100 were prepared the same way as described above. The precipitate fractions containing most of the DnaK protein were pooled, dialyzed against 4 liters of 20mM Tris-HCl, pH7.5 for 48 hours. The dialyzed sample was then loaded onto a 5 ml ATP-agarose affinity column (C-8 linkage, Sigma A-2767) equilibrated with the same buffer. The column was washed with 20mM Tris-HCl and 0.5M NaCl. After re-equilibration of the column

with 20mM Tris-HCl (pH7.5), the DnaK protein was eluted with 10mM ATP in 20mM Tris-HCl (pH7.5). Sample fractions were checked on SDS-PAGE and the fractions containing DnaK were pooled and dialyzed against 10mM Tris-HCl (pH7.5).

Protein determination

The amount of protein in the purified fractions and in the whole cell lysates was determined according to the Bradford (Bio-Rad) protein assay. A standard protein concentration curve was prepared by using bovine serum albumin (BSA) as the standard. Aliquots of diluted standard solution of BSA, containing 0 to 20μg of protein, were reacted with 0.2ml of the Bio-Rad color reagent and the absorbance measured at A_{595}.

Immunological procedures

Rabbit antiserum was raised against purified GroEL as described below: 40μl (40μg) of purified GroEL protein was mixed with equal volume of Hunter's TiterMax™ Research Adjuvant (CytRX® Co.) and injected intramuscularly into a New Zealand white rabbit. The rabbit was given a 1 mg purified GroEL injection every two week for four weeks. One week before collecting blood, the rabbit was given a booster of 1mg purified GroEL protein.

Rabbit anti-E. coli DnaK polyclonal antibodies provided by Dr. McCarty (Massachusetts Institute of Technology, Boston) were used in all the immunoblot studies.

Affinity purification of anti-GroEL antibodies

Affi-Gel 10 affinity support (Bio-Rad) was cross-linked to purified GroEL protein according to the procedures described by Formosa et al. (34). Briefly, 20mg of purified GroEL protein was dialyzed against deionized water to remove Tris-HCl (Tris interferes with ligand coupling reactions) and then mixed with 2ml of Affi-Gel 10 with gentle agitation of the gel slurry for four hours at 4°C. 0.2ml of 1M ethanolamine-HCl (pH8) was added to block any active esters which might remain. The GroEL protein

was bound with an efficiency of approximately 85%. Serum from the rabbit immunized with GroEL protein was passed through the affinity column which had been equilibrated with deionized water. The column was washed with 5 bed volumes of water and 5 bed volumes of 2M urea, and then the anti-GroEL antibody bound to the column was eluted with 8M urea. The specific activity of the purified anti-GroEL antibodies was checked by an immunoblot dot assay on an Easy-Titer™ ELIFA System (PIERCE) as directed by the manufacturer. Anti-GroEL activity was determined by measuring the absorbance of the colored reaction products at 495nm in a microplate reader (Model 3550, Bio-Rad).

Heat shock and other stress response

For heat shock experiments, *P. gingivalis* cultures were incubated in a Coy anaerobic chamber in a 5%CO_2-10%H_2-85%N_2 atmosphere at 37°C until they reached the mid-exponential phase of growth. Sets of 1 ml samples were transferred to sterile glass tubes under anaerobic conditions and the tubes sealed with rubber stoppers. The samples were incubated in a water bath at 37°C for 10 minutes before they were shifted to 42°C. A control sample remained at 37°C. 1 min after the temperature shift, 20μCi of (^{14}C)-amino acid mix (Amersham, 273mCi/mMol) was injected into each of the tubes and the incubation was continued for 4 hours. For the time course study of the heat shock response, 20μCi of (^{14}C)-amino acids was injected into each culture tubes and incubated at 1-5 minute, 5-10 minute, 10-20 minute, and 20-60 minutes periods after the temperature shift to 42°C. The samples were then chilled in ice and the cells were pelleted by centrifugation, washed 3 times in ice-cold phosphate-buffered saline, resuspended in protease inhibitor TLCK (final concentration 20mM) and stored at -20°C.

P. gingivalis response to other stresses such as oxidative stress (H_2O_2), KNO_3, ethanol and high or low pH was similarly analyzed by radio-labeling for 4 hours after mid-exponential growth phase cultures were exposed to the stress conditions. For

pH changes, 1N HCl or 1N NaOH were added to the cultures (pH7.3) until the pH reached 5.0 or 8.3 respectively. After each experiment, viable cells were counted by plating samples on blood agar plates. All heat shock and other stress experiments were repeated three times. The amount of radio-labeling incorporated in each sample was determined by a Beckman LS7500 Liquid Scintillation Counter (Beckman Instruments, Inc.)

Electrophoretic analysis

For SDS-PAGE, bacterial extracts or purified protein samples were mixed with an equal volume of sample buffer (55) and incubated in a boiling water bath for 10 minutes. Proteins were separated on 12% (wt/vol.) polyacrylamide resolving gels with 4% (wt/vol.) polyacrylamide stacking gels by the method of Ames (4). Electrophoresis was carried out at room temperature at a constant 200V until the dye front reached the bottom of the gels. The gels were stained with Coomassie Brilliant Blue or silver nitrate (86). For radio-labeled samples, the gels were stained in Coomassie Brilliant Blue, treated with fluorography amplification reagent (Amersham) and dried at 80°C under vacuum before exposure to Kodak X-Omat film at -70°C.

For two-dimensional PAGE, bacterial pellets were boiled in 5% Nonidet P-40 for 5 minutes before solubilization in lysis buffer containing urea and Nonidet P-40 as described by O'Farrell (85). Samples were separated initially by isoelectric focusing in mini-tube gels containing 4% ampholytes in the pH range of 5 to 8 and 1% ampholytes in the pH range of 3 to 10, and then by SDS-PAGE using 12% separation gels as described in the Bio-Rad mini-2D instruction manual.

Western Immunoblotting

P. gingivalis W50 whole cell lysate proteins separated by SDS-PAGE or two-dimensional PAGE were transferred to nitrocellulose membranes in 25mM Tris-HCl, 192mM glycine and 20% methanol buffer (pH8.3). Transfer was carried out first at

25V overnight then at 60V for 2 hours in a Bio-Rad Trans-Blot Cell. After blocking the unreactive sites with bovine serum albumin, the sheets were washed twice with TTBS (20mM Tris, 500mM NaCl, 0.05% Tween-20) and then incubated with rabbit antibodies against *E. coli* DnaK and GroEL. The unbound antibodies were removed by washing with TTBS. The sheets were then incubated with goat anti-rabbit IgG coupled to alkaline phosphatase for one hour. Antigen-antibody reaction bands were visualized by following the procedures described in the Bio-Rad technical bulletin supplied with the Bio-Rad Immuno-Blot (GAR-AP) assay kit.

Preparation of gene probes

Alkaline lysis miniprep of plasmid pBB1 and pOF39 was conducted as described (9). 5µl (2µg) of plasmid pBB1 DNA was mixed with 10U of *Bam*HI and the reaction mixture was incubated at 37°C for 1 hour. Digested DNA fragments were separated on a 0.8% agarose gel at 60 volts. The DNA bands were visualized by staining the gel in 0.5µg/ml ethidium bromide. pBB1 was digested by *Bam*HI to 4 and 8kb fragments. The 8kb band which contained *dnaK*/J genes was cut out in a narrow strip of gel and the DNA was extracted from the gel following the QIAEX agarose gel extraction protocol (QIAGEN, Germany). The purified 8kb *Bam*HI-*Bam*HI internal fragment of pBB1 was labeled with biotin-14-dATP in the presence of DNA Polymerase I and DNAse I as described in the standard nick translation protocols (BluGENE™, Nonradioactive Nucleic Detection System, BRL). The 2kb *Eco*RI-*Sma*I internal fragment of plasmid pOF39 which contained the *gro*EL/ES genes was similarly isolated and labeled with biotin-14-dATP.

Southern hybridization

Chromosomal DNA from *P. gingivalis* W50, W83, ATCC33277, W12, *P. asaccharolyticus, Prevotella corporis, Prevotella denticola, Prevotella intermedius, Bacteroides levii, Prevotella loescheii*, and *Prevotella melaninogenicus* was isolated by the method of Ausubel (9). *EcoRI*-digested chromosomal DNA from the above

strains was separated by 0.8% agarose gel electrophoresis and transferred to nitrocellulose filters. The filters were baked at 80°C for 2 hours. After prehybridization for four hours at 42°C in 6X SSC (0.9M sodium acetate, 0.09M sodium citrate, pH7.0) containing 100μg/ml denatured sperm DNA, 0.5% SDS, 0.2% Denhardt's solution, the bound DNA was hybridized to the labeled probes at 55°C in 6X SSC, 100μg/ml denatured sperm DNA, 0.5% SDS, 0.2% Denhardt's solution, 25μg/ml probe DNA for 18 hours.

Probes used were biotin-dATP-labeled *Bam*HI-*Bam*HI internal fragment of plasmid pBB1 which contained the cloned *E. coli dnaK/J* genes or biotin-dATP-labeled *Eco*RI-*Sma*I internal fragment of plasmid pOF39 which contained the cloned *E. coli gro*EL/ES genes. The membranes were washed twice at room temperature in 2X SSC buffer containing 0.1% SDS for 3 minutes each followed by washing twice in 0.16X SSC-0.1% SDS at 55°C for 15 minutes each. The membranes were incubated for 1 hour at 55°C in 3% BSA in 0.1M Tris-HCl, 0.15M NaCl (pH7.5) and then reacted with streptavidin-alkaline phosphatase (SA-AP) conjugate for 20 minutes. After washing in 0.1M Tris-HCl buffer (pH7.5) for 30 min, the membranes were incubated in nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP). The incubations were carried out at room temperature until reactive bands appeared. Hybridization bands were detected by the BluGENE Nonradioactive Nucleic Acid Detection System (BluGENE™, BRL, Gaithersburg, MD). The reactions were stopped by washing the membranes in 20mM Tris-0.5mM Na_2EDTA , pH7.5.

In vivo infection studies

P. gingivalis W50 infections of guinea-pigs were conducted as described by Grenier and McBride (44). Briefly, *P. gingivalis* cells were cultivated to late exponential growth phase and the cells were harvested by centrifugation. The bacteria were resuspended in 0.05% sodium thioglycolate to a concentration of

$2x10^{11}$ cells per ml. The bacterial suspension (0.5ml) was then injected subcutaneously into the abdomen region of each of two Hartley guinea-pigs. After the development of a spreading infection, exudates (approximately 45 mls) from both animals were collected, 0.5ml was injected into a second set of animals and exudates from the second animals were also collected. Exudates from the first and second infections were centrifuged twice at low speed (1,000xg for 5 min) to remove red blood cells and leukocytes. The bacterial cells were then pelleted at 8,000xg for 10 min, washed once in ice cold saline, resuspended in 20mM TLCK and stored at -20°C.

RESULTS

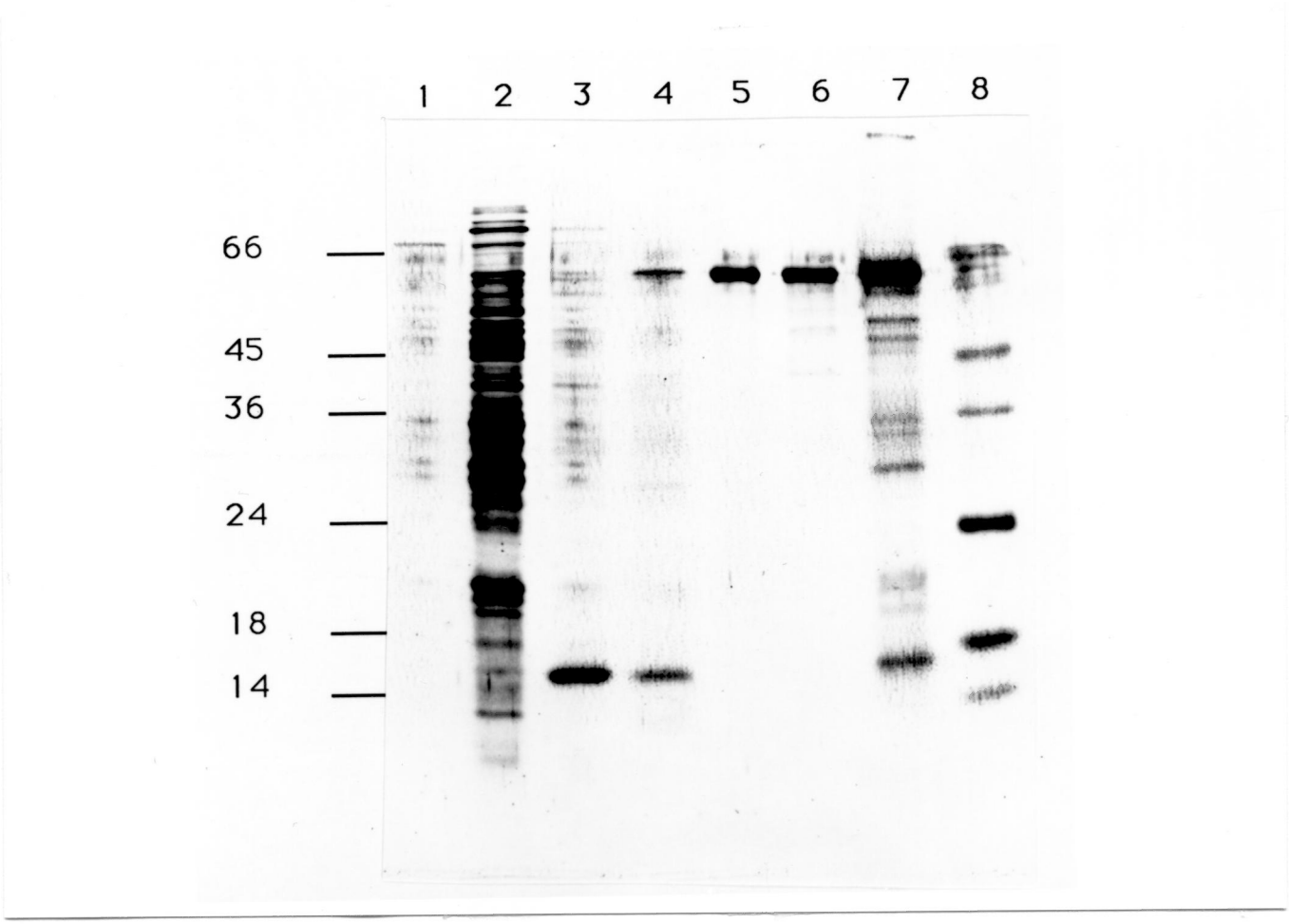
Purification of GroEL and DnaK proteins

The recombinant *E. coli* strain containing plasmid pOF39 overexpresses GroEL (apparent molecular weight 58kDa) and GroES (apparent molecular weight 16kDa) proteins at 37°C (Figure 5, lane 7). High concentrations of ampicillin (200μg/ml) which prevents the cells from losing plasmid pOF39 also enhances the yield of GroEL protein. After ammonium sulfate precipitation, most of the GroEL protein was found in the 20-30% and 30-40% precipitates. These two fractions were pooled, dialyzed, and loaded on a pre-equilibrated DEAE-Sepharose column and eluted with a gradient of 50mM-0.5M NaCl. Figure 6 shows several peaks were eluted from the DEAE-Sepharose column. SDS-PAGE analysis of the fractions revealed that fractions eluting between 0.2-0.3M NaCl contained most of the GroEL protein. The fractions containing most of the GroEL protein were pooled (Figure 5, lane 6), dialyzed, and 1/3 of the material was chromatographed on a MonoQ column. One major peak was eluted from the column as shown in Figure 7. SDS-PAGE analysis revealed that this peak fraction contained highly purified GroEL protein (Figure 5, lane 5).

Recombinant *E. coli* strain MC4100 expresses DnaK protein at a low level in the presence of 200μg/ml ampicillin (Figure 5, lane 2), and heat shock does not enhance the production of DnaK very much (data not shown). Most of the DnaK protein was present in the 0-20% and 20-30% ammonium sulfate precipitates. After dialysis, the samples were chromatographed on an ATP-agarose column. Figure 8 shows that several peaks were washed off the column by salt, the 69kDa DnaK protein was eluted by 10mM ATP as determined on SDS-PAGE (Figure 5. Lane 1). Because some other proteins also have affinity for ATP, we did not obtain a high purity DnaK sample. In a separate experiment, the DnaK fraction eluted by ATP was dialysed and chromatographed on a DEAE-Sepharose column. The DnaK protein was separated

Figure 5. Purification of GroEL and DnaK proteins.

Protein samples were separated by SDS-PAGE and silver stained. Lanes: 1, partially purified DnaK (69kDa) protein after elution from an ATP-agarose column; 2, whole cell lysate of recombinant *E. coli* strain MC4100; 3, partially purified GroES protein after elution from a MonoQ ion-exchange column; 4, partially purified GroES protein after elution from a DEAE-Sepharose column; 5, purified GroEL protein eluted from a MonoQ ion-exchange column; 6, partially purified GroEL protein eluted from a DEAE-Sepharose column; 7, whole cell lysate of recombinant *E. coli* strain over-expressing GroEL, GroES proteins; 8, molecular weight markers. Molecular weight standards are indicated on the left in kilodaltons.



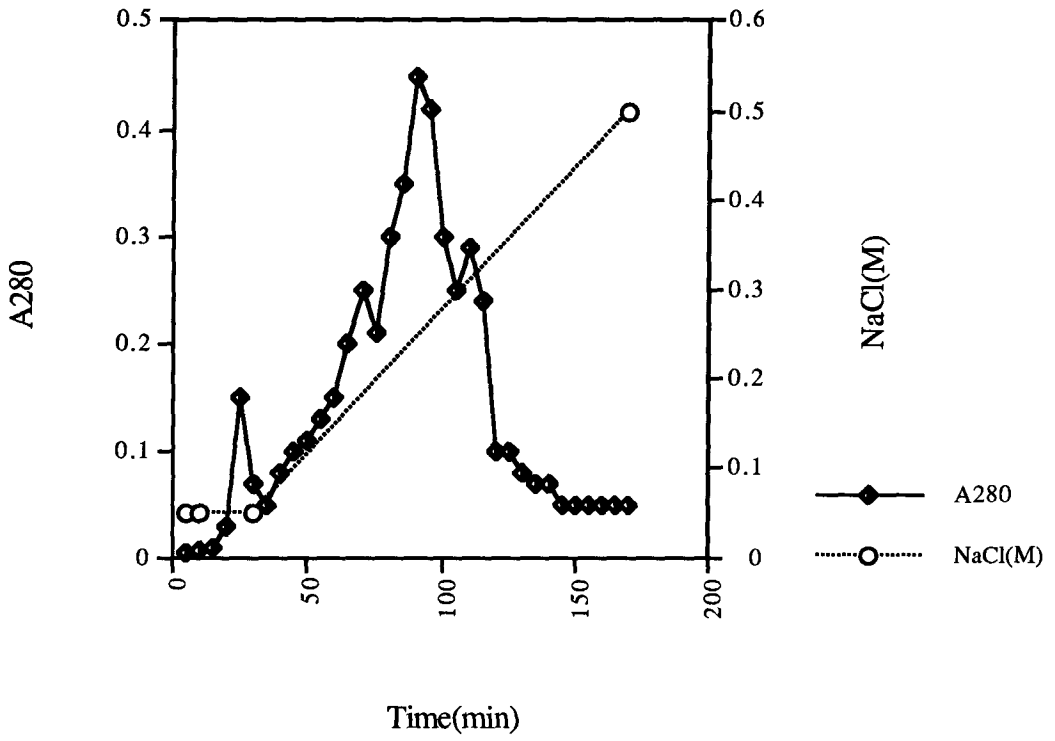


Figure 6. Chromatography of GroEL on DEAE-Sepharose. The column was eluted with a linear gradient of 0.05-0.5M NaCl.

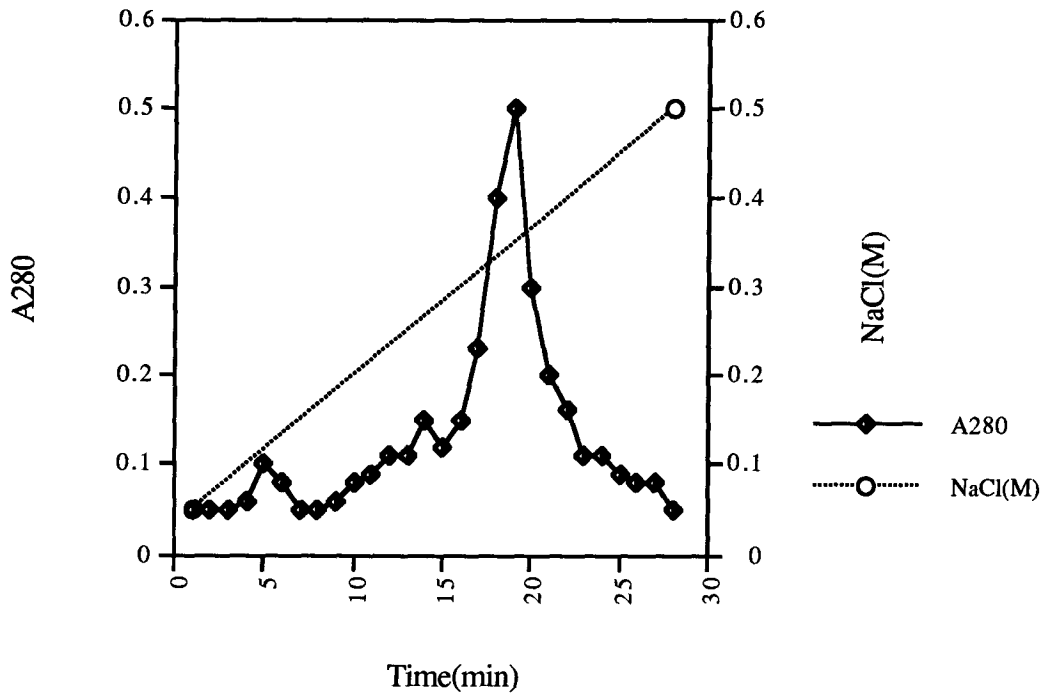


Figure 7. Chromatography of GroEL protein on a MonoQ column. Pooled and dialysed GroEL containing fractions from DEAE-Sepharose chromatography were applied to a MonoQ column and eluted with a gradient of 0.05-0.5M NaCl.

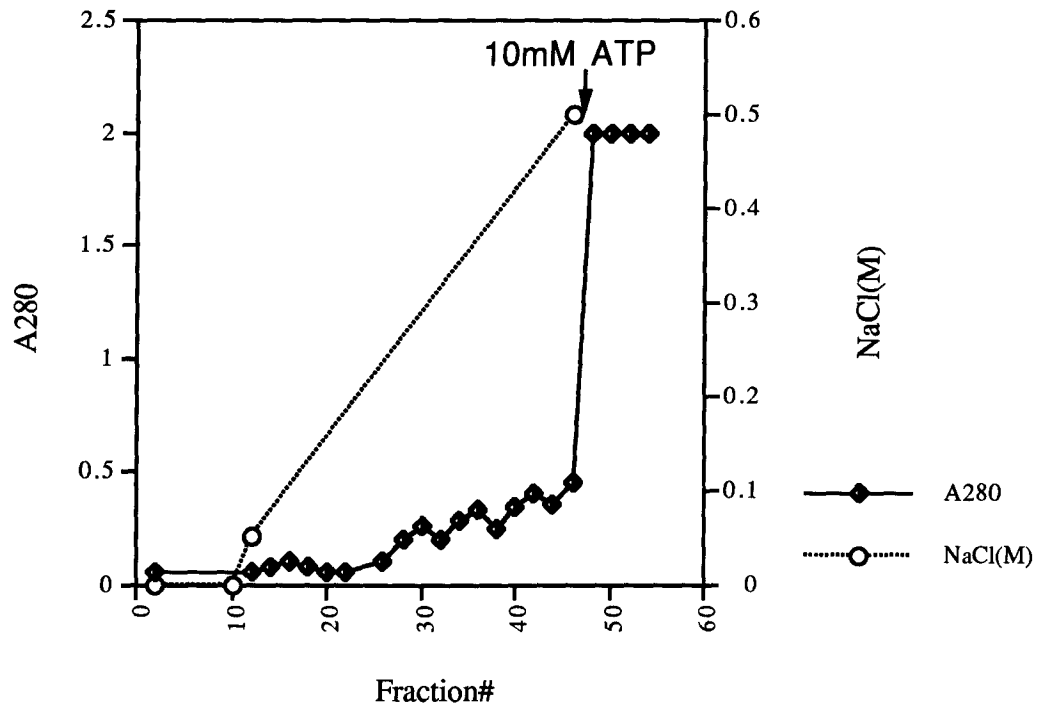


Figure 8. Affinity chromatography of DnaK on an ATP-agarose column. The 0-20% and 20-30% ammonium sulphate precipitates of recombinant *E. coli* MC4100 were dialysed and loaded on an ATP-agarose column. The column was first washed with a gradient of 0.05-0.5M NaCl. The DnaK protein bound to the column was eluted with 10mM ATP. The high value of A280 after adding 10mM ATP is due to the high absorbance of ATP at 280nm.

from most of the other contaminating proteins. Due to the low yield of the DnaK protein, we did not use this DnaK preparation to generate antibodies in animals.

Affinity purification of anti-GroEL antibodies

Serum obtained from the rabbit immunized with purified *E. coli* GroEL protein had a high reactivity against a large number of *P. gingivalis* proteins. The serum was affinity purified by passing through an Affi-gel 10 column coupled with purified GroEL protein (Figure 9). Most of the anti-GroEL activity was found in the 8M urea eluate as determined by an immunoblot dot assay.

Heat shock response of *P. gingivalis*

When *P. gingivalis* W50 cells were shifted from 37°C to 42°C and metabolically labeled with (^{14}C)-amino acids, SDS-PAGE autoradiographic analysis of whole cell lysates revealed elevated synthesis of five proteins with the apparent molecular weights of 92, 80, 74, 62, and 45kDa, a 50 and a 19 kDa protein diminished during heat shock (Figure 10). Two-dimensional PAGE analysis of labeled proteins allowed better resolution of these heat responding proteins. Elevated synthesis of the 92, 80, 74 and 62kDa proteins and decreased synthesis of the 50kDa protein were also observed on 2D-PAGE (Figure 11, 37°C and 42°C). A 12kDa protein was prominently induced and some other low-molecular weight proteins increased or decreased during heat shock.

Time course study of the heat shock response revealed that the 62kDa protein was induced within 5 minutes of temperature upshift and other heat shock proteins could be seen within one hour of heat shock (Figure 12).

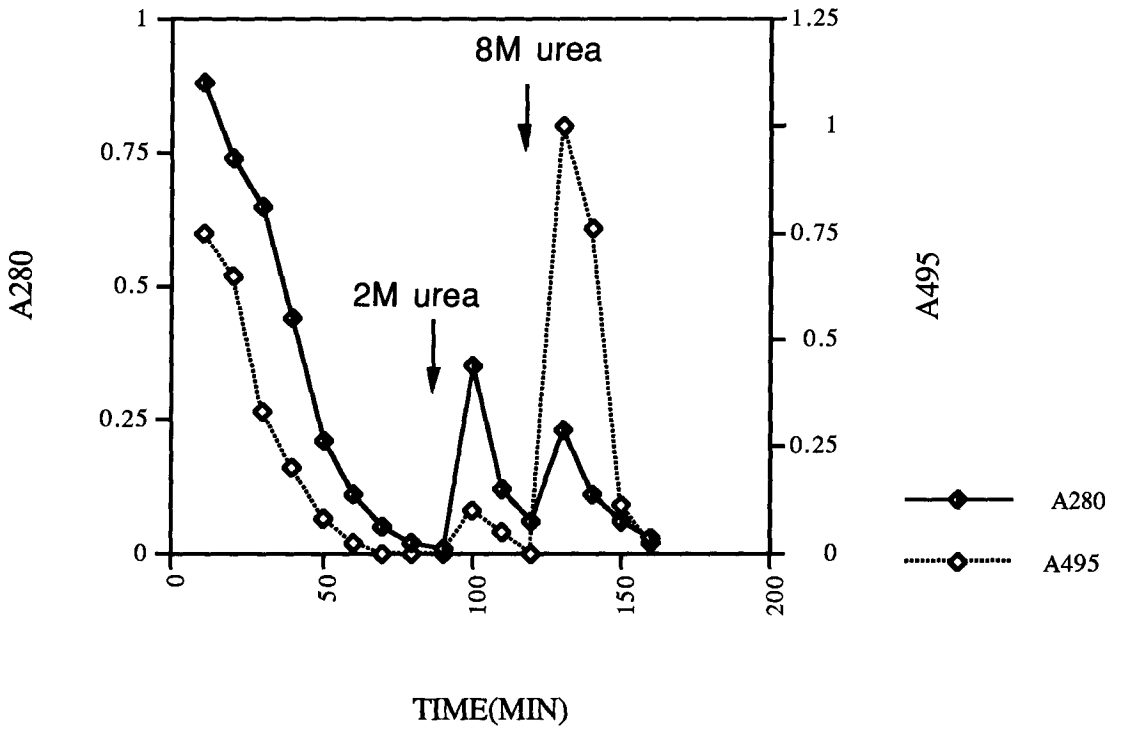


Figure 9. Affinity purification of anti-GroEL antibody on an Affi-gel 10 column coupled with purified GroEL.
The column was washed with deionized water and 2M and 8M urea. Protein concentration was measured at A280. Anti-GroEL activity was determined by an ELIFA immunoblot dot assay and the absorbance of the colored reaction product was measured at A495.

Figure 10. SDS-PAGE autoradiogram of the heat shock response of *P. gingivalis*.

Mid-log phase *P. gingivalis* W50 cells were shifted from 37°C to 42°C and metabolically labeled with (^{14}C)-amino acids followed by SDS-PAGE and autoradiography. Lanes: 1. *P. gingivalis* cells labeled at 37°C; 2. *P. gingivalis* cells labeled at 42°C. Heat responding protein bands are indicated by arrows on the right. Molecular weight standards are indicated on the left in kilodaltons.

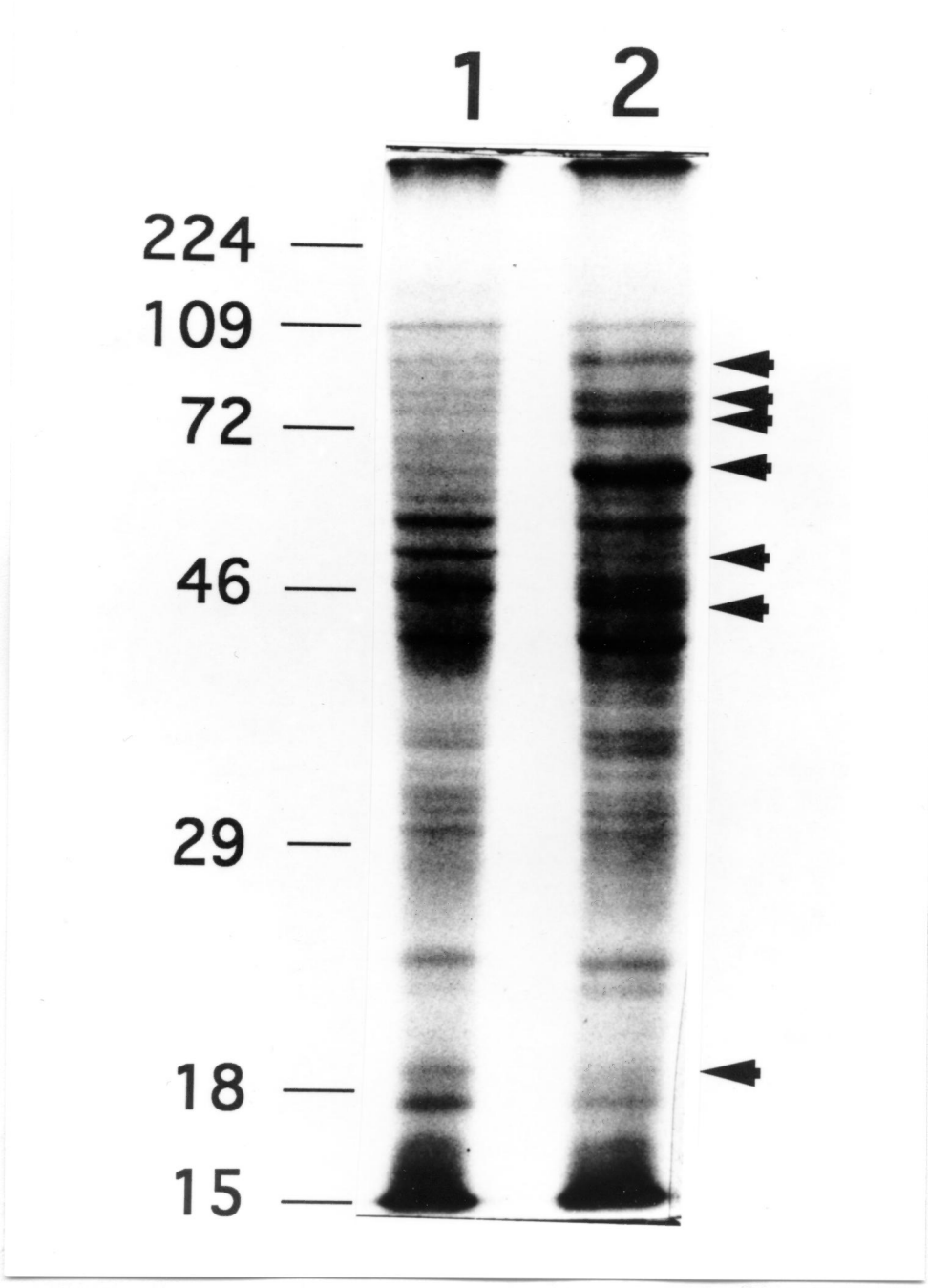
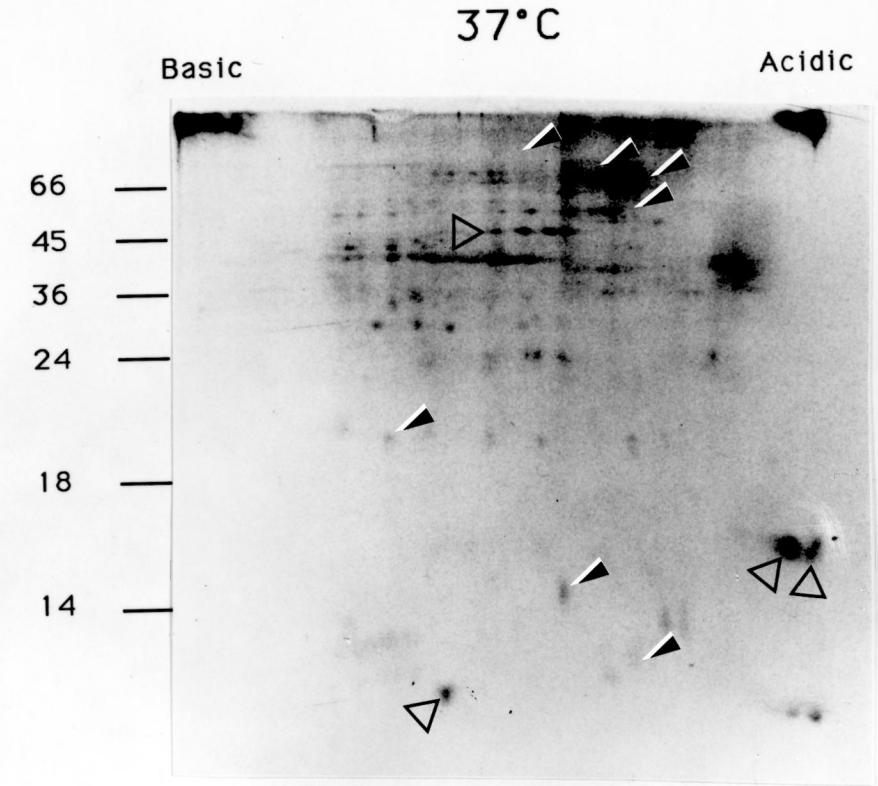


Figure 11. Two-dimensional PAGE autoradiogram of the heat shock response of *P. gingivalis*.

Cell extracts from *P. gingivalis* W50 labeled with (^{14}C)-amino acids at 37°C or 42°C were analyzed by 2D-PAGE and autoradiography. The heat inducible proteins are indicated by (◄), proteins decreased during heat shock are indicated by (◁). Molecular weight standards are indicated on the left in kilodaltons.


37°C
Basic
Acidic
66
45
36
24
18
14

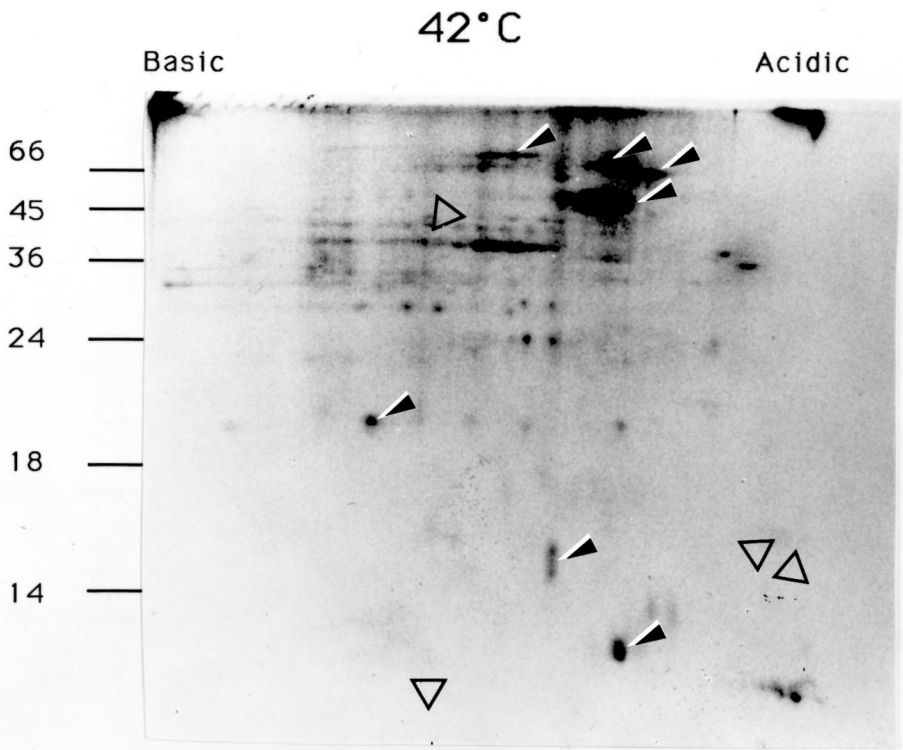
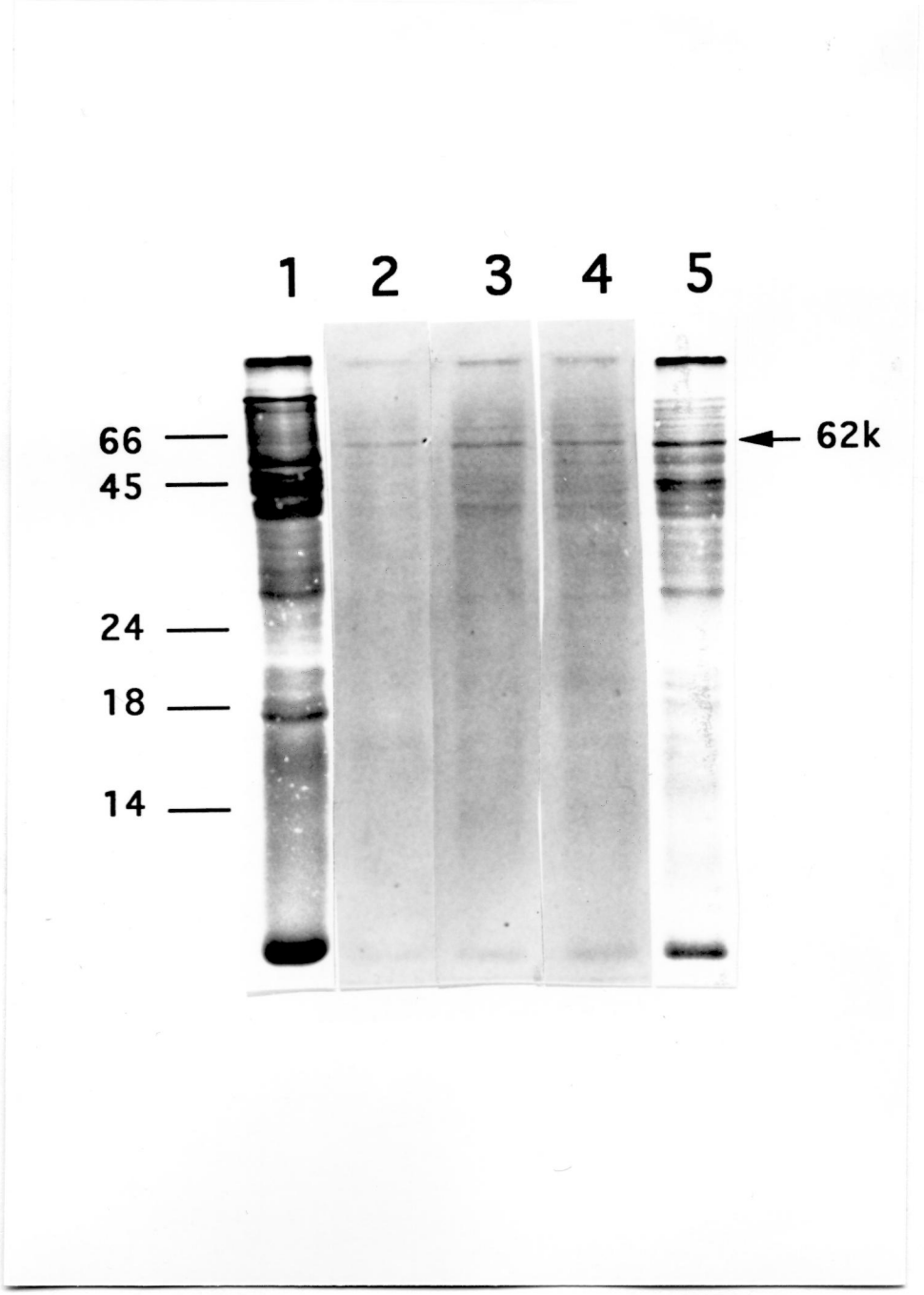

42°C
Basic
Acidic
66
45
36
24
18
14

Figure 12. Time course study of the heat shock response of *P. gingivalis*.

P. gingivalis W83 cells were labeled with (14C)-amino acids for different time intervals after heat shock, cell extracts were analyzed by SDS-PAGE autoradiography. Lanes: 1. control cells grown at 37°C radio-labeled for 4 hours; 2. cells radio-labeled at 1-5 min after heat shock at 42°C; 3. cells radio-labeled at 5-10 min after heat shock; 4. cells labeled at 10-20 min after heat shock; 5. cells radio-labeled at 20-60 min after heat shock.



In order to identify some of the heat shock proteins, antibodies against *E. coli* DnaK and GroEL were applied in Western blots of heat shocked and control *P. gingivalis* whole cell lysates transferred from SDS-PAGE onto nitrocellulose filters. The 74kDa and the 62kDa proteins were recognized by anti-DnaK and anti-GroEL antibodies respectively, therefore, they were identified as homologs of DnaK and GroEL (Figure 13, lane 1 and 2). On two-dimensional Western blots, two protein spots with the apparent molecular weight of 74kDa reacted with anti-DnaK antibody and at least four adjacent protein spots of 62kDa reacted with anti-GroEL antibody both before and after heat shock (Figure 14).

Pulse-chase labeling study of the 50 kDa protein

In order to determine whether the decreased radio-labeling of the 50 kDa protein during heat shock was due to specific degradation by proteases or due to decreased synthesis of this protein after heat shock, we pulse radio-labeled *P. gingivalis* W50 cells at 37°C for 4 hours and then chased without labeling at 42°C for 2 hours (Figure 15, lane 2). The level of radio-labeled 50kDa protein was similar to the control which had not been subjected to heat shock (Figure 15, lane 1). When *P. gingivalis* cells were radio-labeled for 2 hours at 37°C and then shifted to 42°C and labeled for another 2 hours, the intensity of radio-labeling of this 50 kDa protein band decreased (Figure 15, lane 3) when compared to the control. These results suggested that the 50kDa protein synthesis was down-regulated by heat shock.

Southern hybridization with heat shock gene probes

To determine whether *dna*K and *gro*EL gene homologs were conserved, *EcoRI*-digested genomic DNA from *P. gingivalis* W50, W83, ATCC33277, W12, *P. asaccharolyticus*, *Prevotella corporis, Prevotella denticola, Prevotella intermedius, Bacteroides levii, Prevotella loescheii*, and *Prevotella melaninogenicus* were separated on agarose gels, transferred to nitrocellulose filters and hybridized with biotin-dATP-labeled *E. coli dna*K/J or *gro*EL/ES gene probes under conditions of low

Figure 13. Western blot of *P. gingivalis* cells treated with different stress stimuli.

Solubilized cellular extracts from equal numbers of *P. gingivalis* W50 cells grown under different stress conditions were separated by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with a mixture of anti-DnaK and anti-GroEL antibodies. Lanes: 1. control cells grown at 37°C; 2. cells heat shocked at 42°C; 3. cells treated with 30μM H_2O_2; 4. cells treated with 4% ethanol; 5. cells treated with 10mM KNO_3; 6. cells grown at pH5; 7. cells grown at pH8.3. The position of DnaK and GroEL homolog proteins are indicated by arrows on the right. Molecular weight standards are indicated on the left in kilodaltons.

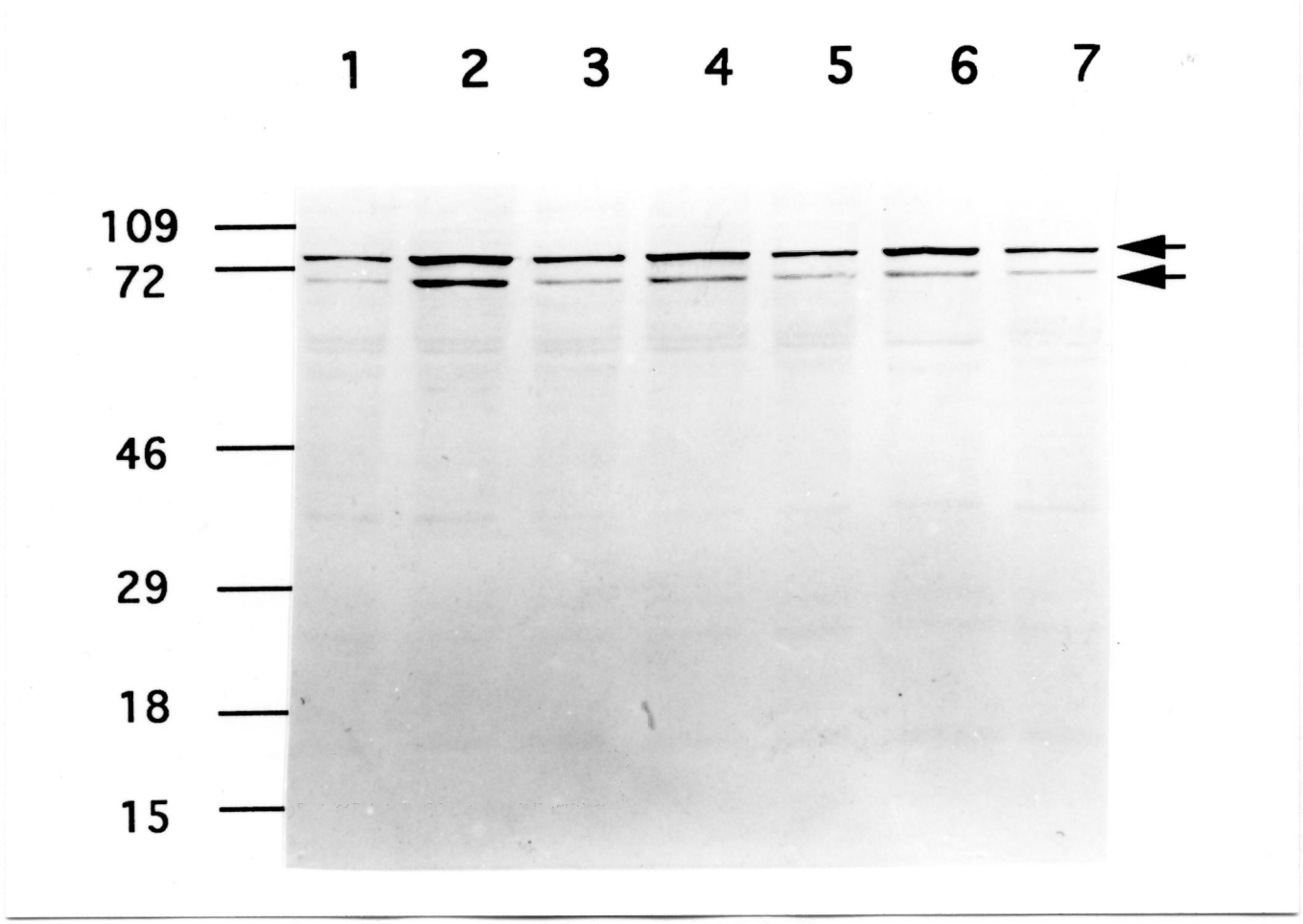
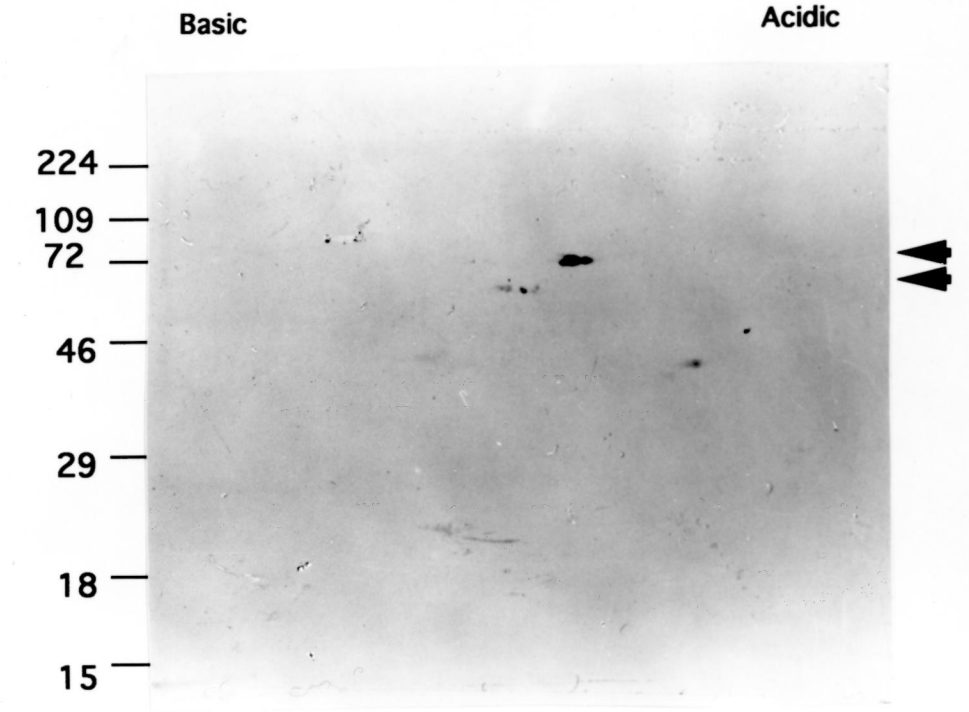


Figure 14. Two-dimensional Western blots *P. gingivalis* before and after heat shock.

Cellular extracts from equal numbers of *P. gingivalis* W50 cells grown at 37°C or heat shocked at 42°C were separated by 2D-PAGE, transferred to nitrocellulose membranes and incubated with a mixture of anti-DnaK and anti-GroEL antibodies. The position of DnaK and GroEL homolog proteins are indicated by arrows on the right. Molecular weight standards are indicated on the left in kilodaltons.

37°C


Basic
Acidic
224
109
72
46
29
18
15

42°C

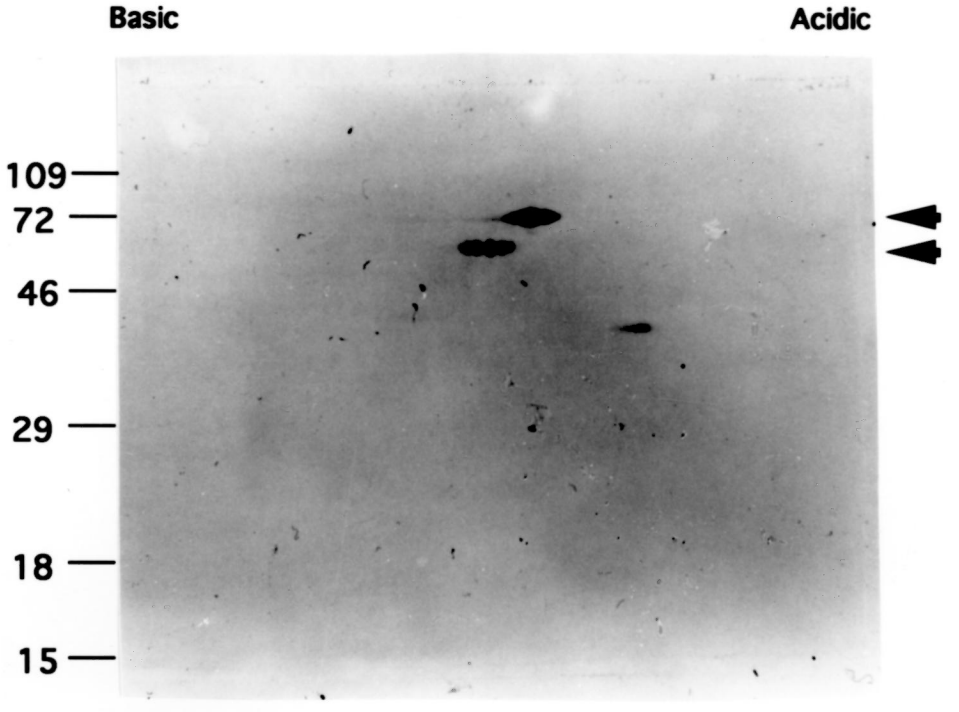
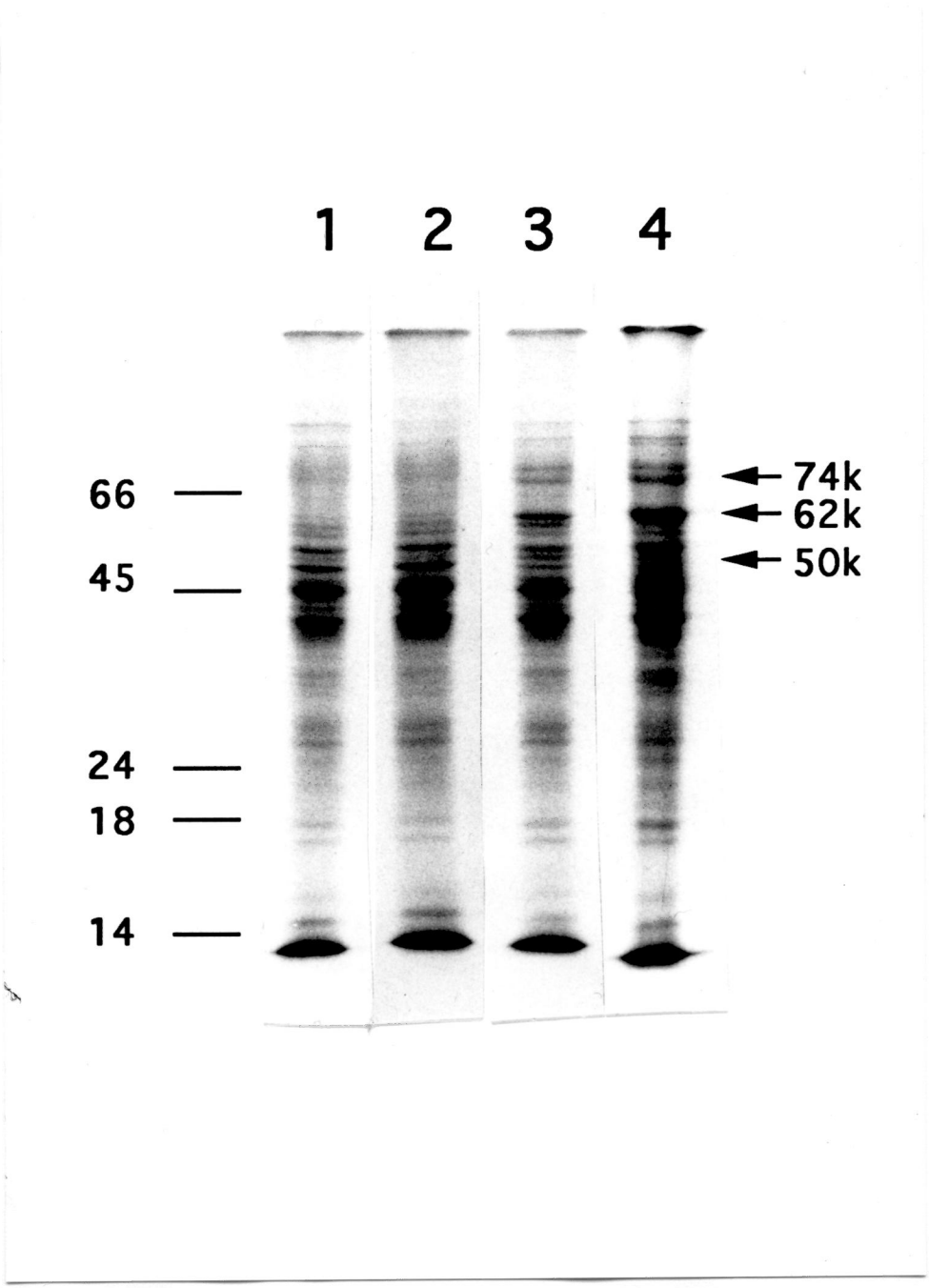

Basic
Acidic
109
72
46
29
18
15

Figure 15. Pulse-chase labeling study of the 50 kDa protein.

Lanes: 1. *P. gingivalis* W50 cells radio-labeled at 37°C for 4 hours; 2. Cells were radio-labeled at 37°C for 4 hours and then chased without labeling at 42°C for 2 hours; 3. Cells were radio-labeled at 37°C for 2 hours then shifted to 42°C and radio-labeled for 2 hours; 4. Cells radio-labeled at 42°C for 4 hours.



stringency. The *E. coli dnaK/J* probe hybridized to a discrete fragment in each of the *P. gingivalis* strains and all the black-pigmented *Bacteroides* strains tested(Figure 16). The *E. coli groEL/ES* probe hybridized to a discrete DNA band in all the *P. gingivalis* strains and most of the black-pigmented *Bacteroides* strains except *P. asaccharolyticus* and *Prevotella denticola*(Figure 17). Among *P. gingivalis* strains, both *dnaK/J* and *groEL/ES* probes hybridized to similar size DNA fragments in strains W50, W83 and W12, but hybridized to much larger DNA fragments in strain ATCC33277. Hybridization to heterogeneous DNA fragments was also observed among the other black-pigmented *Bacteroides* strains.

Effect of different stress stimuli on *P. gingivalis*

Since we are interested in the heat shock proteins that may be induced under *in vivo* conditions, we studied *P. gingivalis* response to stress stimuli such as H_2O_2, KNO_3, high or low pH, and ethanol. The effect of stress stimuli on the viability of *P. gingivalis* W50 cells was studied by incubating *P. gingivalis* cells in different stress conditions for 4 hours and viable cells were determined by plating on blood-agar plates. The results are shown in Table 3. Growth at 42°C did not have any effect on the viability of *P. gingivalis* cells. When the temperature increased to 45°C, more than half of the cells lost their viability. 45% and 39% of *P. gingivalis* cells survived in 4% ethanol and 30mM H_2O_2 respectively, but incubation of cells in 8% ethanol or 60μM H_2O_2 resulted in lost of most of the viable cells. 90% and 57% of the cells survived in 10mM and 20mM KNO_3 respectively. About 20% and 50% of the cells retained their viability when the culture pH was changed to pH5 and pH8.3 respectively.

Figure 16. Southern hybridization of *P. gingivalis* and other black-pigmented *Bacteroides* chromosomal DNA with an *E. coli dna*K/J probe.

Chromosomal DNA was digested to completion with *Eco*RI, electrophoresed on an agarose gel, and transferred to a nitrocellulose filter. Hybridization was performed at low stringency with a biotin-dATP-labeled *Bam*HI-*Bam*HI internal fragment of plasmid pBB1 which contained the cloned *dna*K/J genes. Lanes: 1. *P. gingivalis* W50; 2. *P. gingivalis* W83; 3. *P. gingivalis* ATCC33277; 4. *P. gingivalis* W12; 5. *P. asaccharolyticus*; 6. *Prevotella corporis*; 7. *Prevotella denticola*; 8. *Prevotella intermedius*; 9. *Bacteroides levii*; 10. *Prevotella loescheii*; 11. *Prevotella melaninogenicus*; 12. *E. coli* JM83; 13. plasmid pBB1.

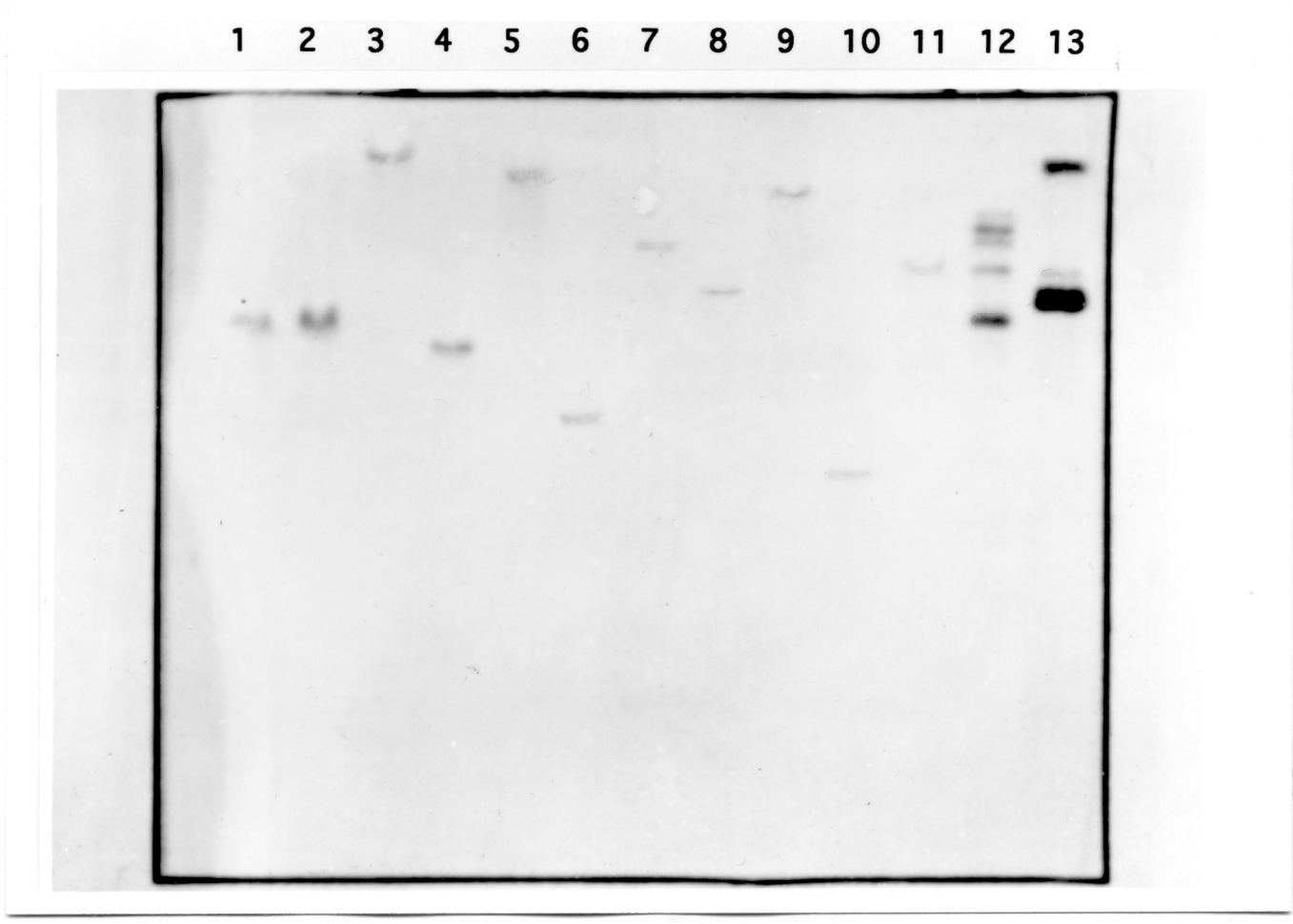


Figure 17. Southern hybridization of *P. gingivalis* and a number of black-pigmented *Bacteroides* chromosomal DNA with an *E. coli gro*EL/ES probe.

Chromosomal DNA was digested to completion with *Eco*RI, electrophoresed on an agarose gel, and transferred to a nitrocellulose filter. Hybridization was performed at low stringency with a biotin-dATP-labeled *Eco*RI-*Sma*I internal fragment of plasmid pOF39 which contained the cloned *gro*EL/ES genes. Lanes: 1. *P. gingivalis* W50; 2. *P. gingivalis* W83; 3. *P. gingivalis* ATCC33277; 4. *P. gingivalis* W12; 5. *P. asaccharolyticus*; 6. *Prevotella corporis*; 7. *Prevotella denticola*; 8. *Prevotella intermedius*; 9. *Bacteroides levii*; 10. *Prevotella loescheii*; 11. *Prevotella melaninogenicus*; 12. *E. coli* JM83; 13. plasmid pOF39.

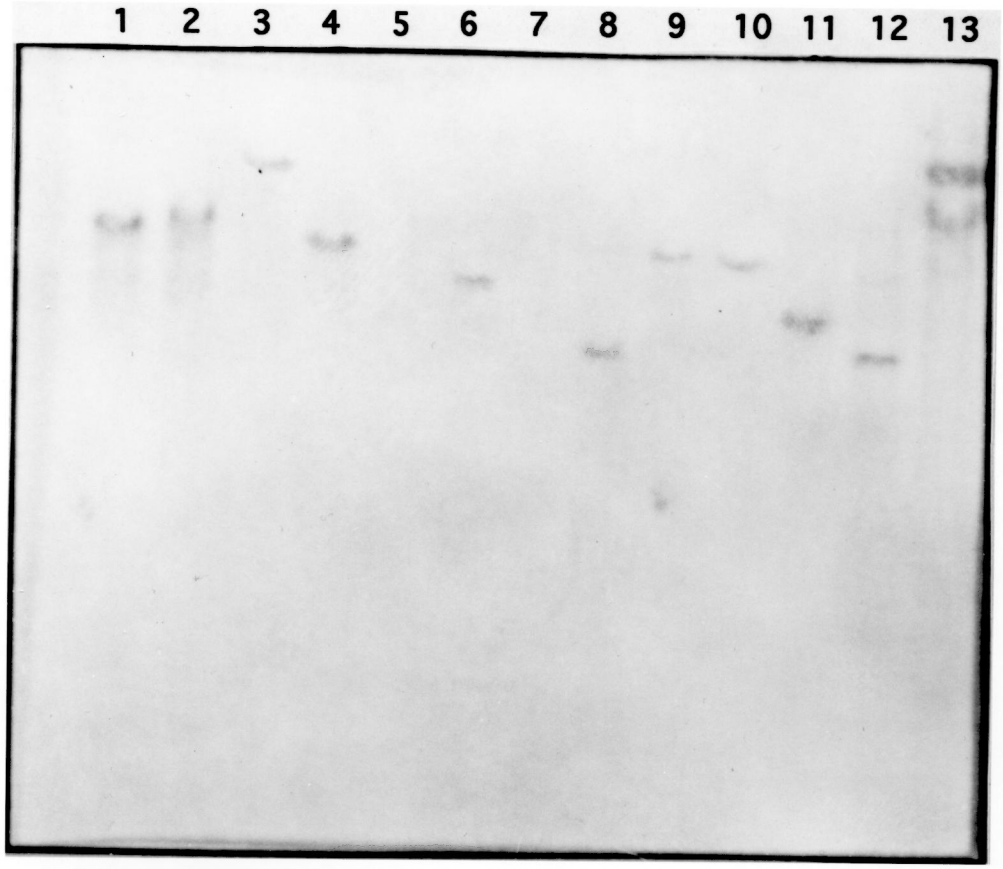


Table 3. Viability of *P. gingivalis* W50 cells exposed to different stress conditions.

Stress condition	Average number of viable cells/ml[a] (% viability)[b]
Control, no stress	2.30×10^9 (100)
Heat shock at 42°C	2.50×10^9 (109)
Heat shock at 45°C	1.03×10^9 (45)
4% ethanol	1.02×10^9 (45)
8% ethanol	0.06×10^9 (2.8)
30 µM H_2O_2	0.90×10^9 (39)
60 µM H_2O_2	0.05×10^9 (2.2)
10 mM KNO_3	2.10×10^9 (90)
20 mM KNO_3	1.30×10^9 (57)
pH 5	0.43×10^9 (19)
pH8.3	1.10×10^9 (48)

[a]. Average after three independent experiments. [b]. Control cells viability is expressed as 100%.

Western immunoblots of *P. gingivalis* whole cell lysates with anti-DnaK and anti-GroEL antibodies have shown that both DnaK and GroEL homolog proteins are induced by 4% ethanol (Figure 13, lane 4). Whereas 30μM H_2O_2 (Figure 13, lane 3), 10mM KNO_3 (Figure 13, lane 5) and pH8.3 (Figure 13, lane 7) did not induce GroEL or DnaK. Because these HSPs are expressed under all growth conditions, three independent stress response experiments were conducted and the same results were obtained except variations of the expression of GroEL and DnaK homolog proteins were observed in *P. gingivalis* cells grown at pH5 as checked by Western blots. Two experiments showed these heat shock proteins were likely to be induced by pH5 (Figure 13, lane 6), the other experiment did not show a discernible induction of DnaK or GroEL homolog proteins (data not shown).

SDS-PAGE autoradiographic analysis of the stress responses of *P. gingivalis* is shown in Figure 18 and Figure 19. The extent of radio-label incorporated into DnaK and GroEL homolog proteins was measured by densitometry as shown in Table 4. From the autoradiograph and densitometry analysis, it is clear that both DnaK and GroEL homolog proteins are induced by 4% ethanol (Figure 18, lane 4), no discernible induction of these HSPs was observed in 30μM H_2O_2 (Figure 18, lane 3), 10mM KNO_3 (Figure 18, lane 5), pH5 (Figure 19, lane 2) and pH8.3 (Figure 19, lane 3). A 68kDa and a 60kDa protein was induced by 4% ethanol (Figure 18, lane 4).

One interesting observation was that when *P. gingivalis* cells were grown at pH5, the cell pellet had a much darker color than the control cells. SDS-PAGE analysis revealed that two new proteins with the apparent molecular weight of 31 and 26kDa were induced at pH5 (Figure 19, lane 2).

Figure 18. SDS-PAGE autoradiogram of *P gingivalis* grown under different stress conditions.

Solubilized cellular extracts from equal numbers of *P. gingivalis* W50 cells grown under different stress conditions and labeled with (^{14}C)-amino acids were separated by SDS-PAGE, and autoradiographed. Lanes: 1. control cells grown at 37°C; 2. cells heat shocked at 42°C; 3. cells treated with 30μM H_2O_2; 4. cells treated with 4% ethanol; 5. cells treated with 10mM KNO_3. The position of DnaK and GroEL homolog proteins of 74 and 62kDa respectively are indicated by arrows on the right. Molecular weight standards are indicated on the left in kilodaltons.

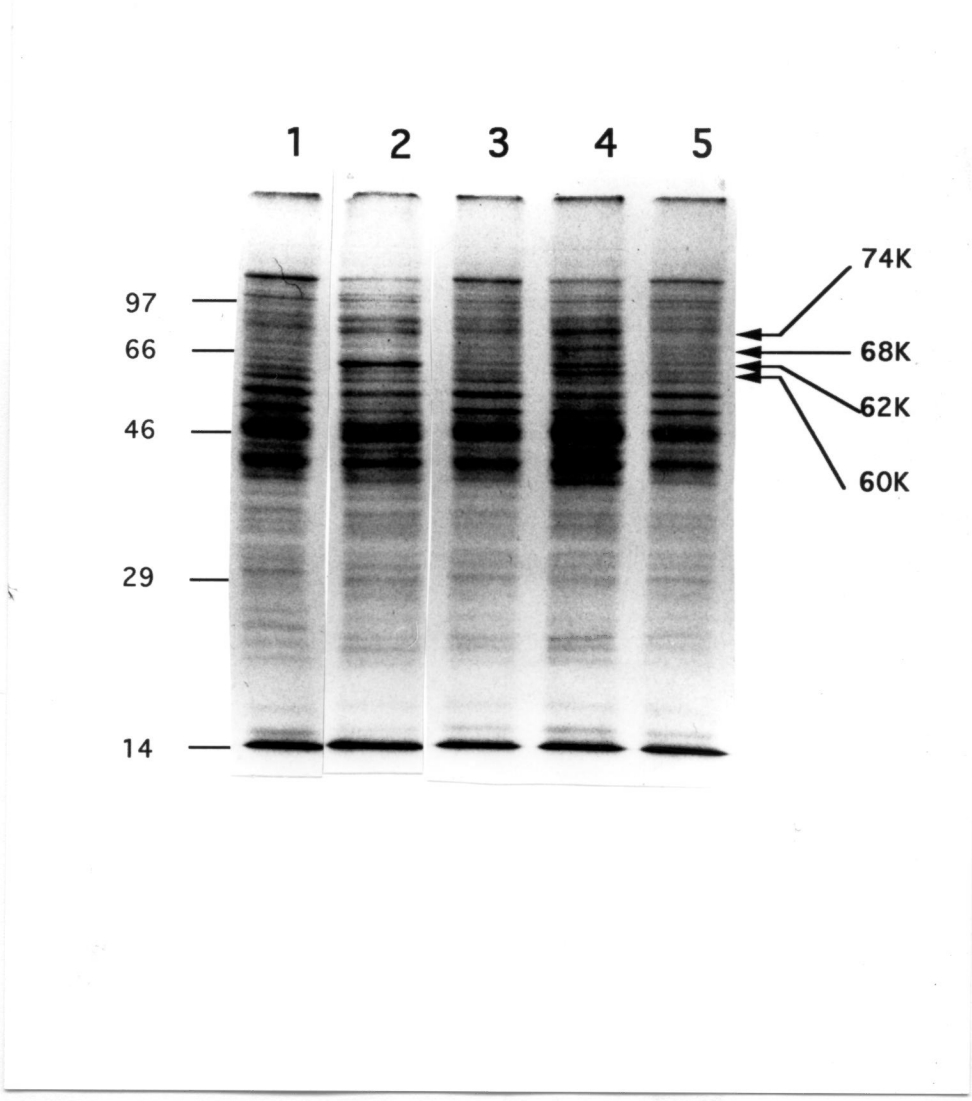


Figure 19. SDS-PAGE autoradiogram of *P. gingivalis* grown at different pH values.

Lanes: 1. control cells grown at 37°C, pH7; 2. cells grown at pH5; 3. cells grown at pH8.3. The position of DnaK and GroEL homolog proteins of 74 and 62kDa respectively are indicated by arrows on the right. Molecular weight standards are indicated on the left in kilodaltons.

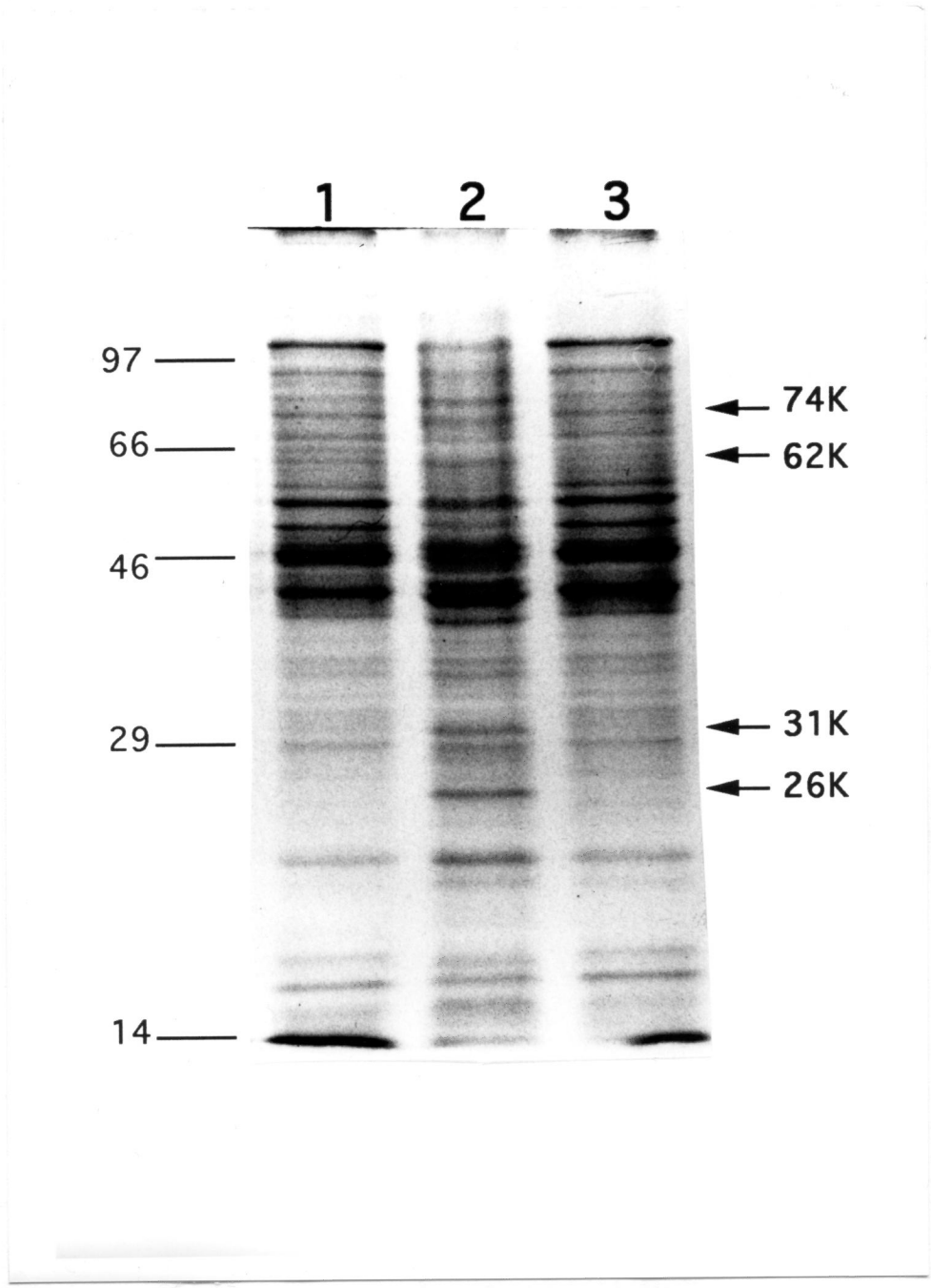


Table 4. Induction of GroEL and Dnak homolog proteins by different stress stimuli[a]

	% incorporation in test sample/% incorporation in control	
stimulus	62 kDa (GroEL)	74 kDa (DnaK)
42°C	7.8	2.3
30μM H_2O_2	1.2	1.3
4% EtOH	3.5	3.1
10mM KNO3	0.85	0.9
pH5	1.6	1.4
pH8.3	1.1	0.8

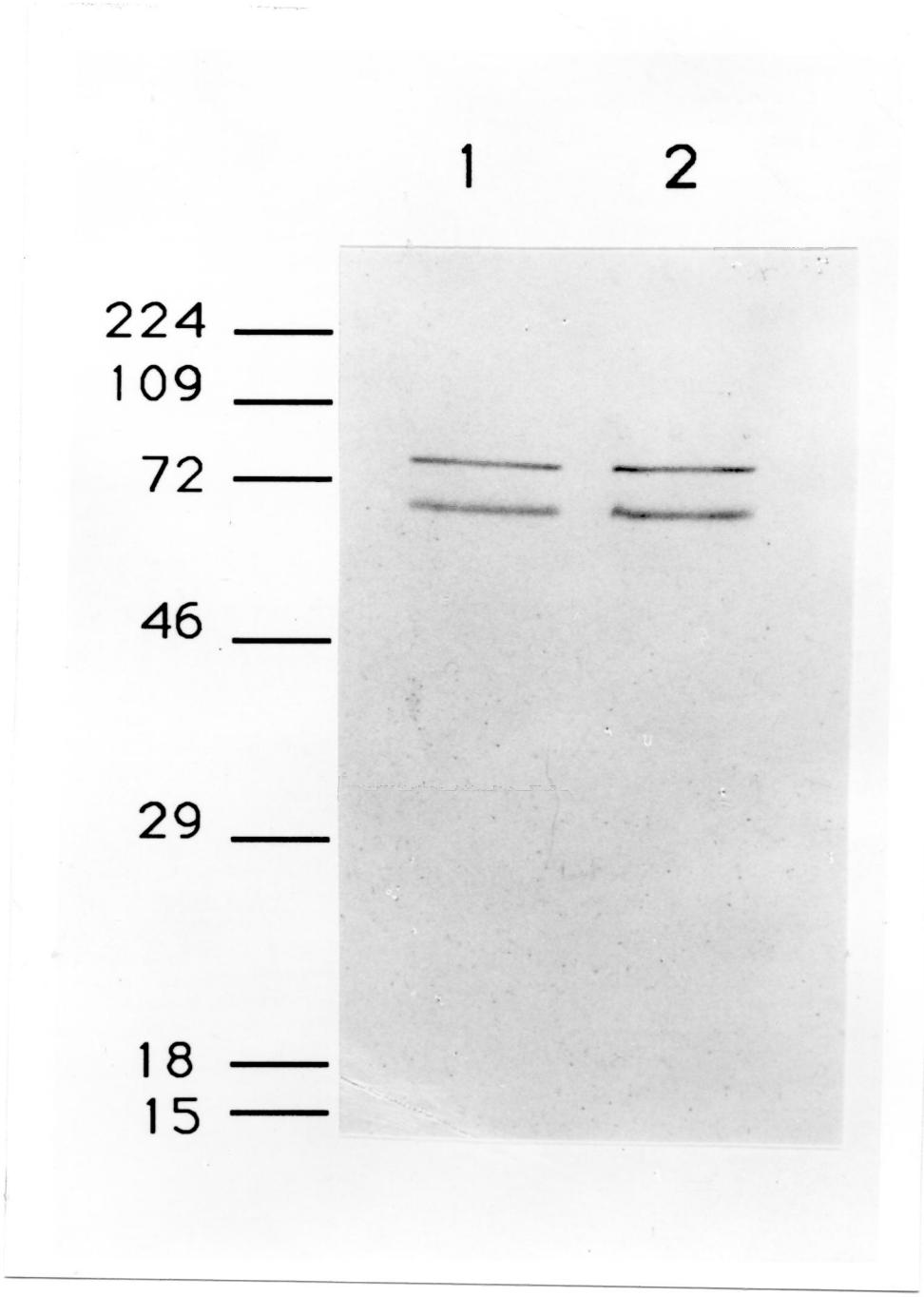
[a] Labeling of DnaK and GroEL homolog proteins was estimated as a percentage of incorporation into total protein by densitometer scanning of SDS-PAGE as shown in Figure 18 and 19. Results are expressed as the percentage of incorporation in the test sample divided by the percentage of incorporation into the same proteins in a control without addition of stress stimuli.

In vivo infection

The subcutaneous injection of *P. gingivalis* W50 in guinea-pigs resulted in an acute infection throughout the abdominal region within 36 hours. The guinea-pigs lost their appetite and movement. From each animal, about 25ml of exudate was recovered. The injection of 0.5ml of exudate into a second animal resulted in massive infection within 24 hours. The pH of the exudates was between pH8.0-pH8.5. The first infection showed a 5 times increase in bacterial numbers (from 10^{11} to $5X10^{11}$) and the second infection indicated that the bacterial number increased about 50 times (from 10^{10} to $5X10^{11}$). When observed under a microscope, the exudates contained a large number of *P. gingivalis* cells, a few red blood cells and very few leukocytes and macrophages. Western immunoblotting studies of DnaK and GroEL homolog proteins showed that these HSPs were not induced during *in vivo* infection of guinea-pigs (Figure 20).

Figure 20. Western blot of *P. gingivalis* W50 grown *in vitro* and *in vivo.*

Cellular extracts from equal numbers of cells grown in the test tubes or recovered from infected guinea-pig exudates were separated on SDS-PAGE, transferred to a nitrocellulose membrane and incubated with a mixture of anti-DnaK and anti-GroEL antibodies. Lanes; 1. cells grown *in vitro*; 2. cells from the second infection in guinea-pig. Molecular weight standards are indicated on the left in kilodaltons.



DISCUSSION

Although the heat shock response in many microorganisms has been studied, very little is known about the regulation of heat shock response in obligate anaerobic bacteria. In the study of heat shock response of *Spirochetes*, Stamm et al. (107) reported that *Treponema pallidum* failed to exhibit a heat shock response and the GroEL and DnaK homolog proteins in *Treponema pallidum* and *Treponema denticola* were not thermoinducible. Because *T. pallidum* was cultured in rabbit tissues, they suspected either *T. pallidum* HSP homologs were maximally expressed *in vivo* and could not be further induced *in vitro* or that *T. pallidum* lacks a regulated stress response. Anzola et al. (8) provided evidence that the DnaK homolog protein in *Borrelia burgdorferi* is an immunologically important *Spirochetal* antigen. Recent studies by Narberhaus and co-workers (81, 82) found the *dnaK* locus of *Clostridium acetobutylicum* contained four heat shock genes organized in an operon in the order of *orf*A-*grp*E-*dnaK*-*dnaJ*. Analysis of the transcription start sites of these heat shock genes suggested σ^{32} factor was not involved in heat shock regulation. Therefore, they suggested that the chromosomal organization as well as the regulatory mechanism for the expression of major heat shock genes in *C. acetobutylicum* is different from that in *E. coli*. There has been no report on heat shock response of *P. gingivalis*, the putative pathogen of adult periodontitis. In this study, I utilized the highly conserved nature of the major heat shock proteins by using antibodies against *E. coli* DnaK or GroEL and *E. coli dnaK* and *gro*EL gene probes to study the heat shock proteins and genes in *P. gingivalis*.

I have demonstrated a heat shock response in *P. gingivalis*. A 74kDa and a 62kDa heat shock protein was identified as major HSPs DnaK and GroEL homologs respectively by reacting with anti-*E. coli* DnaK and anti-*E. coli* GroEL antibodies. These are the most prominent proteins induced by heat shock in *P. gingivalis*. By analogy with other bacteria, the prominent high-molecular-weight 92kDa heat shock

protein may be related to the ATP-dependent protease encoded by the *lon* gene in *E. coli* (41). The 45kDa heat shock protein observed by SDS-PAGE may be related to the *E. coli* DnaJ protein (41kDa) which is coexpressed with DnaK. The low-molecular-weight 12kDa heat shock protein found on 2D-PAGE was suspected to be the homolog of *E. coli* GroES protein.

High affinity binding to ATP is a characteristic of the HSP70 family which has been used for purification of the DnaK protein (123). But due to the ability of ATP to bind various proteins including some heat shock proteins (i.e. DnaJ and GrpE), my one-step purification of DnaK protein on the ATP-agarose column was not very successful. In a separate experiment, further chromatography of the DnaK fraction on a DEAE-Sepharose column separated the DnaK protein from most of the other contaminating proteins.

During the immunization of rabbits with HSPs, I observed high immunity against *P. gingivalis* in some of the animals. The serum from the rabbit immunized with purified *E. coli* GroEL protein had a high reactivity against a large number of *P. gingivalis* proteins. Interestingly, the preimmune serum from the same rabbit had little reactivity to *P. gingivalis* proteins. At the same time, several other rabbits exhibited high immune responses to *P. gingivalis* proteins in their preimmune or immune sera even though they had never been subjected to laboratory immunological procedures. Anaerobic cultures of mouth and fecal samples from these rabbits did not show black-pigmented colonies on blood-agar plates, but the possibility of an infection by a bacterial strain close to *P. gingivalis* could not be excluded.

My attempt to radio-label *P. gingivalis* with (^{35}S)-methionine was not very successful. This may due to the poor incorporation of methionine by *P. gingivalis* and also to the fact that some heat shock proteins may not contain methionine residues, an example is the GroES-like protein in *Mycobacterium tuberculosis* (130). Better success was achieved when (^{14}C)-amino acids were used. However, the relatively

low level of incorporation required a long radio-labeling time in order to obtain enough radio-activity to expose the X-ray film. The poor incorporation of amino acids by *P. gingivalis* could be due to: (1). preference of *P. gingivalis* for peptides rather than amino acids (40, 96); (2). large pool of amino acids in the complex medium used to culture *P. gingivalis;* (3). use of amino acids as a source of energy by *P. gingivalis* leading to degradation of many amino acids rather than incorporate them into proteins.

The time course study of the heat shock response of *P. gingivalis* showed that the 62kDa GroEL homolog protein was induced within 5 minutes of an upshift in temperature. This heat shock response continued to exist throughout the one-hour heat shock experiment period. In *E. coli*, heat shock resulted in a rapid transient increase in heat shock gene expression (maximal induction is about 5 minutes) followed by a rapid decrease that lead to a higher steady-state level of HSPs (110). The relatively slow kinetics of the heat shock response in *P. gingivalis* may reflect the slower rate of protein synthesis in this organism. The doubling time of *P. gingivalis* is approximately 8 times longer than that of *E. coli*.

Two-dimensional PAGE and 2D-Western blot analysis revealed that there were at least two different isoelectric forms of DnaK and four isoelectric forms of GroEL homologs existing both at 37°C and 42°C. There have been reports that in *E. coli*, DnaK and GroEL are phosphorylated in the process of protein folding and oligomerization (57, 97), different levels of phosphorylation could result in different isoelectric points of these HSPs on two-dimensional PAGE. The different forms of DnaK and GroEL homologs found in *P. gingivalis* may represent different phosphorylated forms of the same proteins or this microheterogeneity could occur due to deamidation, acetylation or other modifications that result in an alteration of charge of these proteins.

In agreement with the finding that DnaK and GroEL homologs are conserved in *P. gingivalis,* we have identified a single copy of the *dna*K gene homolog in all the *P. gingivalis* and black-pigmented *Bacteroides* strains we studied. We also found a single copy of *gro*EL gene homolog in all the *P. gingivalis* strains and most of the black-pigmented *Bacteroides* strains except *P. asaccharolyticus* and *Prevotella denticola.* A possible reason for this is that the *gro*EL gene homologs in these two strains have lower homology to *E. coli gro*EL gene probe than the rest of the strains we studied, therefore, they could not be detected by the low stringency hybridization conditions we used. It was noticed that both *dna*K and *gro*EL gene probes hybridized to similar sizes of DNA fragments in *P. gingivalis* W50, W83 and W12 strains but to much larger DNA fragments in the avirulent *P. gingivalis* strain ATCC33277. When studying a protease gene cloned from *P. gingivalis*, Park and McBride (89) reported that the gene probe hybridized to a 5-kb DNA fragment in *P. gingivalis* ATCC33277 but in *P. gingivalis* W50, W83, and W12, the gene probe hybridized to a 3.2-kb DNA fragment. Restriction endonuclease typing of genomic DNA from different *P. gingivalis* isolates has revealed extensive genetic heterogeneity within the species (64). This observation was supported by heterogeneous DNA fingerprints obtained from the application of the polymerase chain reaction with arbitrary primer (AP-PCR) to different strains of *P. gingivalis* (75). This genetic heterogeneity may reflect the different virulence levels of different *P. gingivalis* strains.

In addition to the limited group of proteins induced by heat shock, we also observed decreased radio-labeling of a prominent 50kDa protein and several minor low-molecular-weight proteins during heat shock. Although in *E. coli* and many eucaryotic cells, heat shock resulted in a slower rate of synthesis of a large number of proteins, the specific decreased radio-labeling of the 50kDa protein is quite unique. In order to determine whether this protein was specifically degraded due to elevated protease activity such as Lon (or La) or clpP proteases (16, 17), or due to decreased

synthesis of this protein, we conducted a pulse-chase labeling study of the expression of the 50 kDa protein upon heat shock. The results suggested that the 50kDa protein synthesis was down-regulated by heat shock. The functional significance of this protein during heat shock is not known.

In *E. coli*, HSPs are not only induced by increased temperature, but also by a variety of stress insults, many of which affect protein structure and conformation (83). Therefore, we studied the expression of GroEL and DnaK homolog proteins in *P. gingivalis* grown under different stress conditions. Because of their important intracellular functions, GroEL and DnaK homolog proteins are expressed under all growth conditions. This makes it difficult to determine the induction of these heat shock proteins under stress conditions that may have minor impact on the expression of heat shock genes. Therefore, all the stress response experiments were conducted independently three times and Western immunoblotting and (^{14}C)-amino acids labeling were performed to evaluate the expression of stress proteins. Our results show the major heat shock protein homologs of DnaK and GroEL are significantly induced by ethanol, but not by the other stress stimuli. Ethanol also induced two proteins of the molecular weight of 68 kDa and 60 kDa, these two proteins were not induced by heat shock.

When *P. gingivalis* was cultured at pH5, the cells had a much darker color than the control cells. Since *P. gingivalis* stores hemin for growth, this black-pigmentation suggests it was storing more hemin than normal. At the same time, two new proteins of 26 kDa and 31 kDa were induced in these cells suggesting the function of these proteins may be related to hemin uptake or storage. Bramanti and Holt (13) have reported a heat-modifiable, hemin-regulated 26 kDa surface protein of *P. gingivalis* which changes its location on the outer membrane in response to different stress stimuli. At this time the relationship between these two 26 kDa proteins is not known. The location of the stress proteins induced by pH5 has not been determined.

The finding that in *P. gingivalis,* DnaK and GroEL homolog proteins and some other stress proteins were induced by ethanol and low pH raised the question whether these stress proteins could be induced during infection. There has been great interest in the roles of some HSPs as immunodominant antigens in *Mycobacteria* spp. (128, 129, 131, 132). Although our studies of *P. gingivalis* recovered from a guinea-pig infection model did not show that DnaK or GroEL homolog proteins were induced, caution must be taken when interpreting these results. The guinea-pig infection model is quite different from what could be happening in human periodontitis. In the animal model, the infection is rapid instead of chronic, the exudates recovered from the animal contained mostly of *P. gingivalis* cells with very few macrophages and leukocytes, showing that very little host immune response was involved.

In adult periodontitis, large numbers of polymorphonuclear leukocytes (PMNLs) accumulate in the area of inflammation. PMNLs produce oxygen metabolites such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radical ($^\cdot OH$) which could serve as stress protein inducing agents (2, 3). It is possible that *P. gingivalis* expresses elevated levels of stress proteins in order to survive in the hostile environment, or *P. gingivalis* may utilize protection mechanisms other than heat shock response as suggested by our oxidative stress (H_2O_2) studies which did not induce the major heat shock proteins in *P. gingivalis*. The pH of *P. gingivalis* infectious exudates recovered from guinea-pigs was about pH8.5 (44), which is very similar to the pH in deep periodontal pocket, but this pH did not induce major heat shock proteins in our study. The lack of host immune response suggests that the guinea-pig infection model may not be appropriate for studying *P. gingivalis* stress proteins expression under *in vivo* situations. Further research is needed to elucidate the roles of *P. gingivalis* stress proteins in *in vivo* bacterial survival and pathogenesis.

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