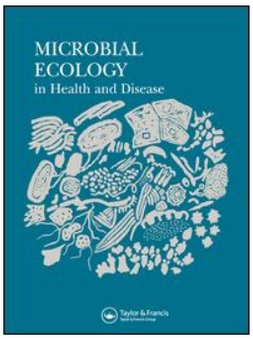

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ORIGINAL ARTICLE

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Expression of GroEL and DnaK Proteins during the Acquisition of a Transitory Resistance to Lethal Stresses by *Actinobacillus actinomycetemcomitans*

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Actinobacillus actinomycetemcomitans is a periodontopathogenic bacterium associated with active lesions of localized aggressive periodontitis. Cells of *A. actinomycetemcomitans* grown to mid-log phase, diluted or undiluted, and subjected to a sublethal heat stress demonstrated a partial protection to a subsequent lethal heat stress. On the contrary, stationary phase heat-stressed cells did not exhibit this transient thermal protection. Heat-stressed cells at sublethal temperature showed a higher expression of GroEL and DnaK proteins by immunoblotting, which was in agreement with *de novo* synthesis. *A. actinomycetemcomitans* was found to be more sensitive to a pH than to a heat stress. A transient protection to a lethal acid stress was demonstrated when cells were previously subjected to a sublethal acid stress. On the contrary, such transient protection did not occur for alkaline stresses. DnaK but not GroEL proteins made up a high proportion of the total protein synthesized following acid stress. The protection phenomenon is highly complex and heat shock proteins, which may help *A. actinomycetemcomitans* to support subsequent lethal conditions, are not expressed in the same way during the different stresses investigated.
Key words: *Actinobacillus actinomycetemcomitans*, periodontitis, heat shock protein, stress tolerance.

INTRODUCTION

Periodontal diseases, which are mixed infections dominated by Gram-negative anaerobic bacteria, are a family of soft–hard tissue diseases involving complex host–bacteria interactions. *Actinobacillus actinomycetemcomitans*, a periodontopathogenic bacterium, is strongly associated with localized aggressive periodontitis, a disease involving inflammation of the gingiva and destruction of the periodontal ligament and the alveolar bone (1). This bacterium is able to colonize subgingival sites, to evade host defences and to promote host tissue destruction. While many putative virulence factors of *A. actinomycetemcomitans* have been identified, the actual *in vivo* pathogenic mechanisms remain poorly understood (1, 2).

The oral cavity is a complex ecosystem that is subjected to a wide range of environmental changes, which present as stress factors affecting the physiology of oral microorganisms (3). During periodontal infections, environmental changes lead periodontopathogens to synthesize heat shock proteins (HSPs), acting as chaperones to protect the cells against deleterious effects of stress. HSPs are highly conserved proteins which have been classified into five families based on their molecular weight, including GroES (10 kDa), DnaJ (40 kDa), GroEL (60 kDa), DnaK (70 kDa) and HtpG (90 kDa) (4). Some HSPs may help in transporting proteins across cell membranes or assist protein folding (4–7), while others may play a key role in the assembly of cell-surface components (8).

The heat shock response in *A. actinomycetemcomitans* was originally studied by Koga et al. (9) and by Løkensgard et al. (10). Cloning and molecular characterization of the genes encoding GroEL and DnaK of *A. actinomycetemcomitans* have also been reported (9, 11, 12). By using specific antibodies directed against the purified GroEL of *A. actinomycetemcomitans*, the extracellular localization and 14-mer ultrastructure of this protein were demonstrated by electron microscopy (13). The GroEL of *A. actinomycetemcomitans* was reported to exhibit a cytotoxic effect toward epithelial cells (13).

Heat-stressed cells of different organisms can acquire a transient resistance to a secondary stress. This phenomenon, called thermotolerance, is distinct from 'adaptation', as reported earlier (14, 15). Recently, we reported a detailed analysis of the stress response in *A. actinomycetemcomitans* subjected to various temperature and pH stresses (16); 74 regulated proteins (including GroEL and DnaK) were identified and classified as general or stress-specific proteins. In this study, we aimed to evaluate, under different conditions, the transitory acquisition of resistance to a

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lethal stress following exposure of *A. actinomycetemcomitans* cells to a sublethal stress. It represents the first investigation into the tolerance phenomenon related to the expression of HSPs in a periodontopathogen.

MATERIALS AND METHODS

Bacteria and growth conditions

A. actinomycetemcomitans Y4 was grown in Todd Hewitt Broth (THB, BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 1% (w/v) yeast extract (YE) and incubated at 35°C in an anaerobic chamber (N_2:H_2:CO_2/75:10:15). The initial pH of the medium was 6.7 and did not change following growth of *A. actinomycetemcomitans* to early mid-log phase ($OD_{660nm} = 0.25$).

Analysis of stress tolerance

A. actinomycetemcomitans was grown at 35°C to early mid-log phase or to stationary phase. The culture was divided into three aliquots. The first aliquot was kept at 35°C under anaerobic conditions while the second aliquot was directly subjected to a previously determined lethal stress condition (48°C, pH 4.5 and pH 9.5). Lastly, the third aliquot was subjected to a sublethal stress for 30 min or 1 h (43°C, pH 6, 5.5 or 5, or pH 8.5), and then to the lethal stress. Lethal temperatures and pHs were defined as the conditions that lead to a decrease of more than half a log in the viable counts of *A. actinomycetemcomitans* during the first 2 h of the incubation. To determine viable counts (CFU/ml) of *A. actinomycetemcomitans* subjected to each condition, samples of the cultures were taken every 30 min for 4 h, serially diluted in culture medium and immediately spread on THB plates supplemented with 1% YE. Plates were incubated for 48 h at 35°C under anaerobic conditions.

The effect of diluting the bacterial culture on the survival of *A. actinomycetemcomitans* in response to a heat stress was also evaluated. *A. actinomycetemcomitans* was grown at 35°C to early mid-log phase, diluted 1:100 in fresh culture medium, divided into three aliquots, and treated as described above.

SDS-PAGE and immunoblotting analysis

Aliquots of cells subjected to the different stressful conditions were harvested by centrifugation (6000 *g*, 2 min), washed twice in cold phosphate buffer (50 mM, pH 7.2) and resuspended in 100 µl of the same buffer. The protein concentration was determined by the method of Lowry et al. (17). Sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (18) using 10% slab gels (Mini PROTEAN II, Bio-Rad Laboratory, Richmond, CA, USA). Proteins were stained with 0.1% Coomassie brilliant blue R-250. Immunoblotting analysis was performed as described previously (19) using either a rabbit antiserum against the purified *A. actinomycetemcomitans* GroEL or a commercial polyclonal rabbit antibody against *Escherichia coli* DnaK (Dako Corporation, Carpinteria, CA, USA).

Analysis of protein synthesis by autofluorography

De novo protein synthesis by *A. actinomycetemcomitans* subjected to different stress conditions was evaluated by autofluorography. Mid-log phase *A. actinomycetemcomitans* cells were subjected to acid, alkaline or heat stress as described above. All the above assays were performed in the presence of 100 µl of a mixture of ^{14}C-labelled amino acids (50 µCi ml^{-1}, Amersham Canada Ltd, Oakville, ON, Canada) added to 500 µl of the culture at the beginning of experiment. Following the stress, cells were harvested by centrifugation (6000 *g*, 2 min), washed twice in cold phosphate buffer (50 mM, pH 7.2) and resuspended in electrophoresis sample buffer (50 µl), and boiled for 5 min. Aliquots of 5 µl and 10 µl were counted in 5 ml of scintillator liquid using a Beckman LS7500 counter. Finally 5000 cpm of each sample were subjected to SDS-PAGE. Proteins were fixed by treating the gel in 20% (v/v) methanol, 10% (v/v) acetic acid and then an autofluorographic amplification was performed using Intensify solutions (NEN research products, DuPont, Boston, MA, USA). The film (Kodak X-OMAT) was exposed for 5 days at −80°C until revelation.

RESULTS

Thermotolerance and HSP expression

Cells of *A. actinomycetemcomitans* grown to mid-log phase and subjected to a sublethal heat stress at 43°C (30 min or 1 h) demonstrated a partial protection to a subsequent lethal stress at 48°C, particularly in the first 2 h of the incubation (Fig. 1A). However, at the end of the incubation period (4 h), bacteria subjected or not to the sublethal stresses, before the application of lethal stress, showed a comparable level of viability. Stationary phase heat-stressed cells did not exhibit this transient thermal protection (Fig. 1B). Interestingly, for an incubation up to 2 h at 48°C, diluted (1/100) mid-log cell culture pre-stressed at 43°C, demonstrated an increased viability when compared with the undiluted pre-stressed culture at 43°C (Fig. 1C vs Fig. 1A). However, the viability curve of the diluted cell culture subjected to the lethal temperature showed a rapid drop (after 2 h incubation) leading to no viable cells at 3 h of incubation. Moreover, at the same time (3 h), diluted bacteria subjected to the pre-stress prior lethal conditions maintained a better viability (Fig. 1C vs Fig. 1A).

Lanes 1 and 2 in Fig. 2 show the basal level of GroEL expressed in mid-log cells at time 0 and 1 h at 35°C, respectively. Lane 7 in Fig. 2 shows that after incubation for 1 h at lethal temperature (48°C), GroEL kept its basal concentration (lane 7 vs lane 2). Moreover, mid-log cells of

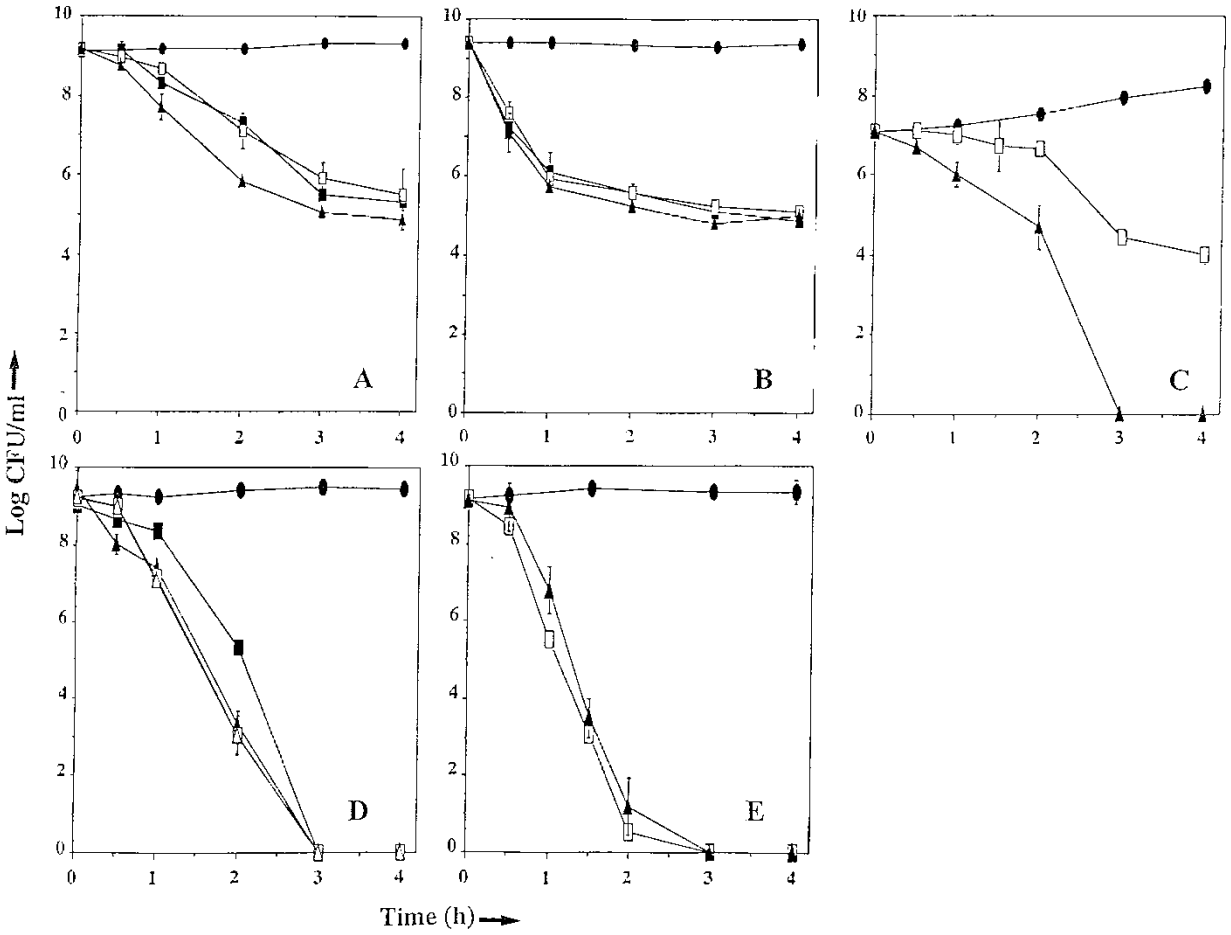

Fig. 1. Survival of *A. actinomycetemcomitans* Y4 under different stress conditions. Each point represents the mean (±SD) of two experiments performed in triplicate. In panels A, B, and C, time 0 of the pre-stressed cells at 43°C corresponds to the time they were subjected to the lethal temperature 48°C, and in panel D and E, time 0 of the pre-stressed cells at sublethal pH corresponds to the time they were subjected to the lethal pH 4.5 and 9.5 respectively. Unstressed control cells (●) were maintained at 35°C with uncontrolled pH. (A) Survival of mid-log phase grown *A. actinomycetemcomitans* following a 30 min (■) or 1 h (□) heat stress at 43°C followed by a lethal stress at 48°C, or directly subjected to a lethal stress at 48°C (▲). (B) Survival of early stationary phase grown *A. actinomycetemcomitans* following a 30 min heat stress at 43°C followed by a lethal stress at 48°C (■) or 1 h (□), or directly subjected to a lethal stress at 48°C (▲). (C) Survival of diluted (1/100) mid-log *A. actinomycetemcomitans* pre-stressed at 43°C for 30 min followed by a lethal stress at 48 °C (□), or directly subjected to a lethal stress at 48°C (▲). (D) Survival of mid-log phase grown *A. actinomycetemcomitans* following a 30 min acid shock at pH 6.0 (□) or 5.5 (△) or 5.0 (■) followed by a lethal stress at pH 4.5, or directly subjected to a lethal stress at pH 4.5 (▲). (E) Survival of mid-log phase grown *A. actinomycetemcomitans* following a 30 min alkaline stress at pH 8.5 (□) followed by a lethal stress at pH 9.5, or directly subjected to a lethal stress at pH 9.5 (▲).

A. actinomycetemcomitans overexpressed GroEL when heat-stressed for 30 min or 1 h at the sublethal temperature (43°C) (Fig. 2, lanes 3 and 5 vs lanes 1 and 2, respectively). The amount of GroEL overexpressed following a heat shock appeared to be maintained for 30 min or 1 h (Fig. 2, lanes 4 and 6) and for 4 h (data not shown) when exposed to a subsequent lethal stress at 48°C. Cells stressed at 43°C for 1 h showed a higher level of GroEL compared with those stressed for 30 min (Fig. 2, lane 5 vs lane 3). The DnaK protein of *A. actinomycetemcomitans* showed the same behaviour as the GroEL (data not shown). Stationary growth phase cells also overexpressed both GroEL and DnaK during heat stress and the level was maintained for up to 4 h (data not shown). These results are in agreement with the *de novo* synthesized GroEL and DnaK observed by autofluorography for cells stressed at 43°C (Fig. 3). The identity of these two bands was assessed by immunoblotting analysis. Autofluorography revealed a low protein synthesis

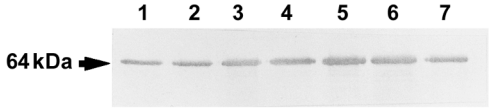

Fig. 2. Immunodetection of of *A. actinomycetemcomitans* GroEL (see Fig. 1A). Lane 1, unstressed cells at time 0; 2, unstressed cells at time 1 h; 3, 30 min stressed cells at 43°C at time 0; 4, cells stressed at 43°C (30 min) then subjected to 48°C for 30 min; 5, 1 h stressed cells at 43°C at time 0; 6, 1 h stressed cells at 43°C then subjected to 48°C for 1 h; 7, stressed cells at 48°C at 1 h.

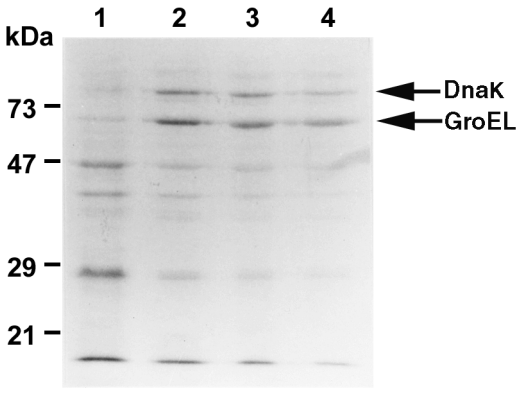

Fig. 3. Autofluorography of mid-log phase *A. actinomycetemcomitans* cells incubated in the presence of a ^{14}C-labelled amino acid mixture. Lane 1, unstressed cells; 2, cells heat-stressed for 30 min at 43°C; 3, cells heat-stressed for 30 min at 43°C then 30 min at 48°C; 4, cells heat-stressed for 30 min at 43°C and 4 h at 48°C. Arrows indicate the position of DnaK and GroEL proteins.

for cells incubated at the lethal temperature (48°C) for 30 min. No further protein synthesis was observed for a longer incubation period (Fig. 3).

Protection against acid and alkaline pHs and HSP expression

Mid-log *A. actinomycetemcomitans* cells subjected to a sublethal stress at pH 5.0 demonstrated a transient protection (up to 2 h) to a subsequent lethal stress at pH 4.5 (Fig. 1D). On the other hand, using a sublethal stress at pH 5.5 or 6.0 led to minimal protection restricted to the first 30 min of the incubation (Fig. 1D). After incubation for 3 h at the lethal pH stress, bacteria subjected or not to the sublethal stress were all dead.

A transient protection to the lethal alkaline stress (pH 9.5) could not be observed with cells pre-stressed at pH 8.5 (Fig. 1E). The slope of the two viability curves was the same. A 30 min pre-stress period at pH 8.5 did not affect the viability of *A. actinomycetemcomitans* (time 0 of Fig. 1E). Nevertheless, a lethal stress at pH 9.5 applied to pre-treated cells at pH 8.5 affected their viability more as compared with the cells directly subjected to lethal stress (Fig. 1E).

No overexpression or *de novo* synthesis of GroEL could be observed after an acid stress using immunoblotting analysis (data not shown) or autofluorography (Fig. 4). On the other hand, in response to a sublethal stress at pH 5.5, stressed *A. actinomycetemcomitans* clearly synthesized *de novo* DnaK, which highly contributes to the total *de novo* protein synthesis (Fig. 4). Immunoblotting analysis showed a comparable level of DnaK for cells stressed at pH 5.0–6.0 (data not shown). Cells subjected to a pH stress at <4.5 did not exhibit any protein synthesis. At pH 5.0, little *de novo* protein synthesis was observed compared with pH 5.5 and 6.0, suggesting that the small amount of DnaK produced rapidly reached a high proportion of the total protein. Alkaline stresses did not lead to the overexpression or *de novo* synthesis of GroEL and DnaK in *A. actinomycetemcomitans* (Fig. 4). No *de novo* protein synthesis could be observed beyond pH 9.0.

DISCUSSION

Temperature in the oral cavity is an important parameter, which can affect the survival of bacteria. The diseased periodontal sites are subjected to an increase in temperature and to alkaline and low oxydo-reduction potential conditions (20, 21). We evaluated the acquisition by *A. actinomycetemcomitans* of a transitory resistance to a lethal stress after an exposure to a sublethal pre-stress, to investigate the tolerance phenomenon related to the expression of HSPs. More specifically, our study investigated the effect of environmental changes, similar but not identical to those occurring in the periodontal pocket, on *A. actinomycetemcomitans* viability and GroEL and DnaK protein expression. We are aware that the model we have used is not ideal since it amplifies the *in vivo* conditions. We have chosen these conditions to accentuate the *A. actinomycetemcomitans* response to temperature and pH.

A thermotolerance phenomenon in which HSPs appears to play a key role, has previously been demonstrated in *Candida albicans*, the causative agent of oral stomatitis (22, 23). Indeed, cells pre-stressed at 45°C were better able to

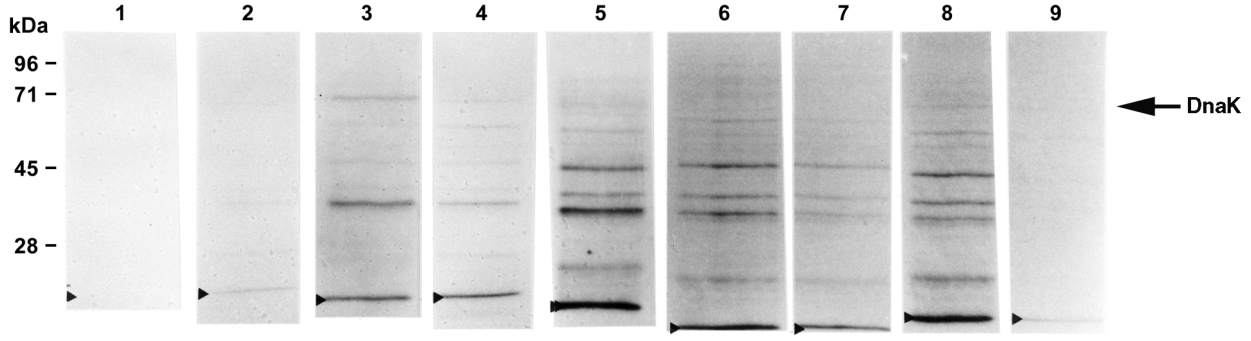

Fig. 4. Autofluorography of mid-log phase *A. actinomycetemcomitans* cells labelled with a ^{14}C amino acid mixture. Cells were subjected to different pH conditions. Lane 1, pH 4.1; 2, pH 5.0; 3, pH 5.5; 4, pH 6.0; 5, pH 6.9; 6, pH 7.5; 7, pH 8.0; 8, pH 8.5; 9, pH 9.0. Arrow on the right indicates the position of DnaK. Arrowheads at the bottom indicate the migration front of the gel.

survive a lethal shock at 55°C. Such a phenomenon involving *A. actinomycetemcomitans* was demonstrated in our study. A 30 min sublethal heat stress seems to be sufficient to protect *A. actinomycetemcomitans* cells from lethal heat-stress conditions, suggesting that proteins required for the protection are synthesized very quickly. It was also shown that GroEL and DnaK are induced during sublethal heat stress, and are maintained at a high concentration level for up to 4 h after the stress. We previously classified these two HSPs as general stress proteins (16). Other proteins were also found to be up-regulated following a 30 min heat stress (16). Thus, we should not exclude the possibility that other proteins and mechanisms may be involved in this protection phenomenon. Indeed, a diluted mid-log *A. actinomycetemcomitans* culture was found to be less sensitive, up to 2 h, to the lethal heat condition when compared with undiluted cells, suggesting that a lower cellular concentration could help cells to survive better in non-favourable conditions. These results are in accordance with those obtained with cells grown to early stationary phase, which did not exhibit this protection phenomenon. The stationary phase may be a potent stressing condition, as cells have limited nutrients and their basal expression of HSPs is elevated when compared with mid-log cell expression. These findings suggest that cells in stationary grown phase, which are already in a stressed condition, cannot afford an additional thermal stress. Thus, the cell density may play a role in the protection phenomenon.

pH is also a key element for oral bacteria, as it affects their growth and metabolism. Bacterial products influence the pH in the oral cavity, which may vary from 6.75 to 7.25 (24). Indeed, the metabolic activity of asaccharolytic bacteria (such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum* found in the periodontal pocket) is associated with a rise in pH of the gingival crevicular fluid to values up to 7.4, and to 7.8, as compared with the value of 6.9 in healthy sulcus (21). It was previously reported that *P. gingivalis* cells survived stress at pH 8.3 better than at pH 5 (48% vs 19% survivors, respectively) (25), suggesting a higher tolerance at alkaline pH, which was not observed in *A. actinomycetemcomitans* in our study. Moreover, pre-stress protection at acid pH, which has been demonstrated for non-mutans streptococci (26), was also observed in *A. actinomycetemcomitans*. We also demonstrated that *A. actinomycetemcomitans* cells subjected to a sublethal pH stress and then to the lethal pH stress are all killed at the end of the incubation period as opposed to a heat stress which allowed survivors, suggesting that *A. actinomycetemcomitans* is more sensitive to pH than to heat stresses. The optimal growth pH for *A. actinomycetemcomitans* ranges from 7.0 to 8.0 (2) and this may be the reason why *A. actinomycetemcomitans* did not induce GroEL and DnaK during alkaline stress at pH 7.5, 8.0 or 8.5. On the other hand, we previously demonstrated by 2-dimensional SDS-PAGE analysis, that, after a 30 min stress at pH 8.5, the relative contribution (spot volume) of GroEL and DnaK to the total protein is very high (16). These findings suggest that the GroEL protein is not synthesized *de novo* during alkaline and acid stresses but its relative contribution to the total protein is very important. As opposed to the heat-stress condition, GroEL was not induced by all acid stresses tested, as demonstrated by autofluorography and immunoblotting analyses. Moreover, we previously demonstrated, using 2-dimensional SDS-PAGE, that the volume of the spots identified as GroEL and DnaK proteins, after a 30 min stress at pH 5.5, was bigger in relation to the whole protein volume in the SDS-PAGE (16). An acid shock (pH 6.0) decreased the synthesis of the major HSPs in *F. nucleatum* while an alkaline shock (8.5) increased their synthesis (27). On the contrary, an increased synthesis of GroEL was observed when *P. gingivalis* was subjected to a pH drop (7.2 to 6.0) (25, 28). The pH protection phenomenon appears to be very complex, since different microorganisms respond differently. In *A. actinomycetemcomitans*, DnaK is likely to play an important role in acid tolerance, although additional studies are needed to identify other mechanisms or other proteins involved in the phenomenon.

The model we used, which emphasizes the environmental changes, indicated that *A. actinomycetemcomitans* can acquire a transitory resistance to a lethal stress following exposure to a sublethal stress. In the periodontal pocket, microorganisms have to withstand several stressing factors at the same time. The tolerance *in vivo* is probably very complex and our study represents a first step in the understanding of this phenomenon. HSPs, which may help *A. actinomycetemcomitans* to resist subsequent lethal conditions, did not play the same role in the different stresses investigated. The protection phenomenon may be highly complex and involves other mechanisms and proteins. Further proteomic studies are needed to identify the proteins that are involved in the stress tolerance response and which may be important in the cell physiology.

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