Multiple stress responses in *Streptococcus mutans* and the induction of general and stress-specific proteins

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The authors have previously demonstrated that *Streptococcus mutans* shows an exponential-phase acid-tolerance response following an acid shock from pH 7.5 to 5.5 that enhances survival at pH 3.0. In this study the response of *S. mutans* H7 to acid shock was compared with the responses generated by salt, heat, oxidation and starvation. Prior induction of the acid-tolerance response did not cross-protect the cells from a subsequent challenge by the other stresses; however, prior adaptation to the other stresses, except heat (42 °C), protected the cells during a subsequent acid challenge at pH 3.5. Starvation by fivefold dilution of the basal medium (BM) plus fivefold reduction of its glucose content increased the numbers of survivors 12-fold, whereas elimination of glucose from fivefold-diluted BM led to a sevenfold enhancement compared to the control cells; this indicated a relationship between the acid and starvation responses. The stress responses were further characterized by comparing the 2D electrophoretic protein profiles of exponential-phase cells subjected to the various stress conditions. Cells were grown to exponential phase at pH 7.5 (37 °C) and then incubated for 30 min under the various stress conditions in the presence of ¹⁴C-labelled amino acids followed by cell extraction, protein separation by 2D gel electrophoresis and image analysis of the resulting autoradiograms. Using consistent twofold or greater changes in IOD % as a measure, oxidative stress resulted in the upregulation of 69 proteins, 15 of which were oxidation-specific, and in the downregulation of 24 proteins, when compared to the control cells. An acid shock from pH 7.5 to 5.5 enhanced synthesis of 64 proteins, 25 of them acid-specific, while 49 proteins exhibited diminished synthesis. The dilution of BM resulted in the increased formation of 58 proteins, with 11 starvation-specific proteins and 20 showing decreased synthesis. Some 52 and 40 proteins were enhanced by salt and heat stress, with 10 and 6 of these proteins, respectively, specific to the stress. The synthesis of a significant number of proteins was increased by more than one, but not all stress conditions; six proteins were enhanced by all five stress conditions and could be classified as general stress proteins. Clearly, the response of *S. mutans* to adverse environmental conditions results in complex and diverse alterations in protein synthesis to further cell survival.

Keywords: stress, stress proteins, cross-protection, *Streptococcus mutans*

INTRODUCTION

Bacteria in the biofilms on teeth (dental plaque) are subjected to cycles of acid shock resulting from the rapid

formation of acid end-products generated during the metabolism of dietary carbohydrate by the acidogenic oral microflora. The rate of acid formation in human plaque, as measured by *in vivo* pH telemetry, shows that

Abbreviations: 2DE, two-dimensional polyacrylamide electrophoresis; BM, basal medium (the salts, vitamins and amino acids of MM4 medium); IOD, integrated optical density.

the intake of carbohydrates can lower the plaque pH from 7 to 4 in as little as 3 min depending on the age of the biofilm and the concentration of the carbohydrate (Imfeld & Lutz, 1980; Yamada *et al.*, 1980; Jensen *et al.*, 1982). Furthermore, the frequent ingestion of sugar is associated with a lowering of the 'resting' plaque pH and an increase in dental caries. Early studies (Stephan, 1944) demonstrated that the microflora associated with high caries activity was not only capable of rapid plaque acidification following the ingestion of sugar, but was also tolerant to the low resting plaque pH. Thus, it is not surprising that acid tolerance is an important 'virulence' property associated with cariogenic bacteria, such as *Streptococcus mutans*, *Lactobacillus* species and certain non-mutans streptococci (Bowden, 1991; Sansone *et al.*, 1993; van Houte *et al.*, 1996).

During short-term exposure to acidic environments, *S. mutans* maintains pH homeostasis by proton extrusion from the cell via the membrane-associated, proton-translocating ATPase (H^+ /ATPase) (Bender *et al.*, 1986; Hamilton & Buckley, 1991), and by acid end-product efflux (Carlsson & Hamilton, 1996; Dashper & Reynolds, 1996). Sustained growth at pH 5.5, however, results in increased H^+ /ATPase (Bender *et al.*, 1986; Belli & Marquis, 1991; Hamilton & Buckley, 1991) and glycolytic activity (Hamilton & Ellwood, 1978), which is accompanied by a lowering of the pH optimum for sugar transport and glycolysis (Hamilton & Buckley, 1991), and increased lactic acid formation (Hamilton, 1987). Moreover, unlike the enteric bacteria (Padan *et al.*, 1981), *S. mutans* does not maintain a constant intracellular pH during a falling external pH, but exhibits a relatively consistent transmembrane pH gradient (~ 1.0 unit) sustained by a carbon source (Hamilton, 1986, 1990; Hamilton & Buckley, 1991; Dashper & Reynolds, 1992). Thus, growth at pH 5.5 results in the induction of metabolic changes that permit the organism to maintain the transmembrane pH gradient at lower pH values (Hamilton, 1986; Hamilton & Buckley, 1991; Dashper & Reynolds, 1992).

Recently, we have demonstrated that exposure of exponential-phase cells of *S. mutans* LT11 to a pH change from 7.5 to 5.5 resulted in the induction of an acid-tolerance response over a 2 h period that increased cell survival at pH 3.0 (Svensäter *et al.*, 1997). The pH change resulted in significant alterations in protein synthesis: pulsing the cells with ^{14}C -labelled amino acids at intervals during the 2 h period, followed by extraction and one-dimensional polyacrylamide gel electrophoresis, revealed the upregulation of 36 proteins, with 25 of these being acid-responsive proteins appearing within the first 30 min of the pH change (Hamilton & Svensäter, 1998). The synthesis of all but two of the proteins was transient during the 2 h adaptation period. The identity of the proteins is not known, but molecular mass comparisons suggested that both acid-specific proteins (i.e. components of the H^+ /ATPase) and general stress proteins (i.e. heat-shock proteins) are present in extracts of the acid-induced cells.

The coordinated induction of general and specific proteins by a variety of stress conditions is relatively well characterized in *Escherichia coli* and *Bacillus subtilis*, particularly with respect to the heat-shock proteins (Hecker *et al.*, 1996; Yura *et al.*, 1993). A number of stress responses have been identified in *Lactococcus lactis* and it is clear that there is an overlap in the protective mechanisms induced by various conditions (Rallu *et al.*, 1996). Carbohydrate-starved stationary-phase cells of *L. lactis* IL1403 exhibited increased resistance to acid, heat, ethanol and osmotic and oxidative stress (Hartke *et al.*, 1994), while UV-irradiated cells of the same strain showed enhanced survival against acid, ethanol, hydrogen peroxide and heat (Hartke *et al.*, 1995). An overlap between the heat-shock and salt responses has also been reported for *L. lactis* MG1363, with all salt-stress-induced proteins also subject to induction by heat stress (Kilstrup *et al.*, 1997).

In the current study, we were interested in the relationship between the acid-tolerance response generated by *S. mutans* and the response of the organism to other adverse conditions, such as, salt, heat, starvation and oxidative stress. Two approaches were taken: an examination of whether reciprocal cross-protection exists between these various stresses, and a comparison of the stress-induced proteins formed under the various conditions. For the latter study, cells were exposed to stress in the presence of ^{14}C -labelled amino acids and the protein extracts subjected to two-dimensional polyacrylamide electrophoresis (2DE) and autoradiography. Comparative analysis revealed three groups of proteins: general stress proteins enhanced under all conditions, those specific to the particular stress, and those induced by more than one, but not all, stress conditions.

METHODS

Bacterial strain and media. *S. mutans* H7 was isolated from an approximal caries lesion at pH 5.0 and maintained anaerobically in complex liquid medium containing (per litre): tryptone (10 g), yeast extract (5 g) and 20 mM glucose buffered with 40 mM phosphate/citrate buffer as previously described (Svensäter *et al.*, 1997). Comparative studies on cross-protection and ^{14}C -labelled amino acid incorporation into cellular protein during stress were carried out with cells growing anaerobically in a minimal medium (MM4) containing six amino acids (glutamate, serine, cysteine, valine, leucine, asparagine) to which was added various amount of glucose (Hamilton & Svensäter, 1998). In the context of this paper, the basal medium (BM) refers to the salts, vitamins and amino acids of MM4 medium.

Adaptation conditions. Cells were grown to mid-exponential phase in MM4 with 20 mM glucose at pH 7.5; the cells were rapidly concentrated by centrifugation (5000 g for 5 min) and washed in sterile saline (0.16 M NaCl), and aliquots ($\sim 10^8$ cells ml^{-1}) were inoculated into 5 ml fresh MM4 medium with 20 mM glucose buffered with 40 mM phosphate/citrate buffer under the following conditions: (a) pH 5.5, (b) 42 °C, (c) +0.2 M NaCl, and (d) +2 mM H_2O_2 . Also, three 'starvation' conditions were tested: S-1, 20% BM + 20 mM

glucose; S-2, 20% BM+4 mM glucose; and S-3, 20% BM without glucose. Both unadapted control and test cells were incubated at pH 7.5 and 37 °C, except (a) and (b), respectively. Unless otherwise specified, incubation was for 2 h and the pH of the suspensions did not change by more than ± 0.2 pH units.

Challenge conditions. Adapted and unadapted exponential-phase cells were harvested and rapidly washed in sterile saline by centrifugation (5000 g for 5 min), then added at a constant cell concentration ($\sim 10^8$ cells ml⁻¹) to 5 ml fresh MM4 medium and incubated for 2 h under the following conditions: (a) pH 3.5, (b) 50 °C, (c) +2 M NaCl, (d) +15 mM H₂O₂, and the three 'starvation' conditions listed above. Both unadapted control and test cells were incubated at pH 7.5 and 37 °C, except (a) and (b), respectively. Incubation was for 2 h and the pH of the suspensions did not change by more than ± 0.2 pH units.

Cross-protection protocols. Cross-over experiments were undertaken to determine whether (i) the prior induction of the acid-tolerance response by exponential-phase cells of *S. mutans* H7, achieved by incubating pH 7.5-grown cells at pH 5.5 for 2 h, would affect survival under challenge with the other stresses, and (ii) the prior adaptation to the other stresses would affect the ability of the cells to survive a 2 h exposure in MM4 medium buffered at pH 3.5. Following exposure to the stress, cell samples were diluted and plated on trypticase agar (g l⁻¹: trypticase, 10; yeast extract, 2; Na₂CO₃, 2; NaCl, 5; glucose, 2; agar, 10; buffered at pH 7.2). The percentage of cell survivors was calculated by comparison with viable cell counts of the culture suspension just prior to challenge. The data shown are the means of at least three determinations $\pm$ standard error.

¹⁴C labelling of stress proteins. The assays for the induction of the various stress responses followed the 'adaptation' conditions above with the exception that only starvation condition S-1 (20% BM+20 mM glucose) was tested. Cells were grown to mid-exponential phase (OD₆₀₀ 0.6; the stationary phase OD₆₀₀ was 1.2) in MM4 with 20 mM glucose at pH 7.5, centrifuged and resuspended in glucose- and buffer-free MM4. The cell suspension was added to the different adaptation media to give a concentration of 5×10^8 cells ml⁻¹ in a total volume of 2.5 ml. Immediately after addition of the cell suspension, 50 μ Ci (1850 kBq) of a ¹⁴C-labelled amino acid mixture (Amersham) was added to the adaptation medium and incubation carried out for 30 min. Then 1 mg chloramphenicol was added to stop protein synthesis, and the cell suspension was rapidly cooled in ice. The suspensions were centrifuged at 15000 g for 10 min; the cells were washed twice and resuspended in 0.5 ml 10 mM Tris/HCl, pH 6.8, with 1 mM EDTA and 5 mM MgSO₄. The cells were stored at -80 °C until used. The frozen cells were thawed, centrifuged and resuspended in 0.5 ml lysis buffer containing 8 M urea, 2% (v/v) Nonidet P-40, 62 mM DTT and 2% (v/v) Pharmalyte, pH range 4-7 (Pharmacia Amersham Biotech). Proteins were extracted by vortexing 0.5 ml cell samples in lysis buffer with 0.2 mm glass beads (1:1, v/v) six times for 30 s with cooling between vortexing. The samples were centrifuged at 2500 g for 5 min to remove the beads. The bead-free supernatant was centrifuged at 15000 g for 15 min at 4 °C and the resultant cell-free extracts were frozen until used.

2DE. The first-dimension isoelectric focusing was performed using the Multiphor II horizontal electrophoresis apparatus connected to an EPS 3500 XL Power Supply and Multitemp II thermostatic circulator (Amersham Pharmacia Biotech). Immobiline DryStrip linear immobilized pH gradient (IPG) gel strips (18 cm) with a pH range of 4-7 were rehydrated in

330 μ l sample buffer (SB) containing 8 M urea, 2% (v/v) Nonidet P-40, 10 mM DTT and 2% (v/v) Pharmalyte, pH range 4-7 (Pharmacia Amersham Biotech). Rehydration was carried out overnight at room temperature in a reswelling cassette with strips covered with silicone oil to avoid evaporation. The reswollen strips were rinsed with deionized water and carefully blotted with wet filter paper to remove excess rehydration solution. The strips were placed into the DryStrip kit on the Multiphor II and electrode strips loaded with 300 μ l deionized water. The ¹⁴C-labelled samples were diluted in the above SB buffer and applied in sample cups under silicone oil at the anodic end of the gel strip. The same amount of sample radioactivity (500000 c.p.m.) was loaded on each gel, corresponding to 30-60 μ g protein. The proteins were focused overnight at 15 °C under a protective layer of silicone oil using 150 V for 1 h, 300 V for 3 h, 600 V for 3 h, 1200 V for 12 h and 3500 V for 18 h. After focusing, the IPG gel strips were immediately frozen between plastic films at -80 °C.

The second-dimension electrophoresis was carried out essentially as described by Laemmli (1970) with 12-14% polyacrylamide gradient gels (185 $\times$ 200 $\times$ 1.0 mm). Immediately before SDS-PAGE, the IPG gel strips were shaken gently in 10 ml 50 mM Tris/HCl (pH 6.8), 2% (w/v) SDS and 26% (v/v) glycerol for 2 $\times$ 15 min. DTT (25 mg) was added to the first equilibration solution, and iodoacetamide (450 mg) and bromophenol blue to the second. The IPG strips were quickly loaded on top of the gel and overlaid with 1% (w/v) molten agarose (Bio-Rad). The separation was performed at 10 °C overnight at 40 mA until the bromophenol blue dye front reached the bottom of the gels. The gels were fixed in 40% methanol and 7.5% acetic acid for 45 min, vacuum-dried at 60 °C for 3 h and then subjected to autoradiography.

The apparent isoelectric point and molecular mass of protein spots were determined relative to those of known protein standards. The pI standards were purchased from Amersham Pharmacia Biotech (carbamylated carbonic anhydrase and glyceraldehyde-3-phosphate dehydrogenase) and co-electrophoresed with bacterial proteins in some of the first-dimension gels. The gels containing pI standards were silver stained according to the procedure recommended by the manufacturer before autoradiography. ¹⁴C-labelled molecular mass standards were obtained from Amersham (myosin, 220 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 66 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa; and lysozyme, 14.3 kDa) and run alongside the second-dimension gels.

Autoradiography. The dried gels were exposed to X-ray film (Hyperfilm b-max, Amersham) for 14 d and films developed with Kodak D19.

Image analysis. Analysis of digitized images, including spot finding, quantification and matching, was done by using Visage 2-D Electrophoresis Image Analysis software version 4.6 J (Millipore) on a Sun Sparc work station. A reference autoradiogram was chosen and each of the other autoradiograms was matched to it using the BiImage software. The matching procedure consisted of manually selecting 20 tiepoints or 'anchor proteins' on the images and then allowing the machine to automatically match the images. Proteins of known molecular mass were then used as standards to generate pI and molecular mass values for all the spots in the matched gels by interpolation and extrapolation. The integrated optical density (IOD) of each protein, expressed as a percentage of the total area of blackening of the film attributed to proteins (IOD%), was calculated along with pI and molecular mass for each individual spot. Each stress ex-

periment was carried out at least twice with different cell cultures, and only spots showing a consistent change in IOD % of at least twofold were taken into account.

Analyses. Protein was determined by the method of Bradford (1976) with the appropriate concentration of lysis buffer in the standards as described by Fey *et al.* (1997).

RESULTS

Cross-protection

An adaptive response to one stress can often lead to cross-protection against another stress (Hartke *et al.*, 1994; Leyer & Johnson, 1993); hence we were interested

Fig. 1. Effect of prior adaptation to other stresses on the survival of *S. mutans* cells following a subsequent challenge at pH 3.5 for 2 h. With the exception of the control cells (Con), maintained at pH 7.5 in 100% BM + 20 mM glucose, cells were exposed to the other stresses for 2 h prior to the acid challenge. Stress conditions: Acid, pH 5.5; Salt, 0.2 M NaCl; Oxid, 2 mM H₂O₂; Heat, 42 °C. Starvation conditions: S-1, 20% BM + 20 mM glucose; S-2, 20% BM + 4 mM glucose; S-3, 20% BM without glucose. All incubations except that for the heat stress were at 37 °C.

in the relationship of the acid response in *S. mutans* H7 to other stress responses. We therefore tested whether prior adaptation to acid (pH 7.5 to 5.5) would affect survival under challenge with other stresses. Incubation of pH 7.5-grown exponential-phase cells at pH 5.5 for 2 h resulted in the expected induction of the exponential-phase acid-tolerance response, with fourfold more survivors at pH 3.5 than for control cells maintained at pH 7.5 (data not shown). However, with exception of heating at 50 °C, which significantly reduced cell numbers at pH 3.5, adaptation to acid stress had no significant effect on survival when cells were challenged with 2 M NaCl or 15 mM H₂O₂, or with fivefold diluted BM with 20, 4 or 0 mM glucose (data not shown).

The converse of the above experiment was also undertaken to determine whether prior adaptation to the other stresses would augment or diminish cell survival after a subsequent 2 h acid challenge at pH 3.5. As seen in Fig. 1, as expected the pH 5.5-adapted cells (Acid) showed increased survival over the pH 7.5 control cells. Also, prior exposure to the other stresses tested, except a temperature increase to 42 °C (Heat) and the addition of 2 mM H₂O₂ (Oxid), stimulated survival at pH 3.5 compared to the control cells. Particularly noticeable was the effect of starvation conditions with cells pre-incubated in 20% BM containing 4 mM glucose (S-2) showing a 12-fold stimulation of survival. Even cells incubated in 20% BM without glucose (S-3) showed a sevenfold increase in survivors over the control cells incubated in 100% BM with 20 mM glucose (Con). This demonstrates a clear relationship between starvation and acid tolerance.

2DE analysis

The stress responses in *S. mutans* H7 were further characterized by comparing the expression of proteins in exponential-phase cells during the initial 30 min period immediately following the application of stress by acid, salt, H₂O₂, heat and a fivefold dilution of BM (starvation condition S-1). Just prior to the application

Table 1. Analysis of the proteins in the 2DE gels in Fig. 2 enhanced or diminished twofold or more by oxidative, acid, starvation, salt or heat stress

Data from Fig. 2 representing those proteins from two separate experiments whose synthesis was enhanced or diminished at least twofold. Designations (B) to (F) refer to the individual gels in Fig. 2.

	Stress				
	Oxidation (B)†	Acid (C)	Starvation (D)	Salt (E)	Heat (F)
Total no. of proteins	510	515	510	443	573
Proteins with enhanced expression	69	64	58	52	40
Stress-specific proteins enhanced	15	25	11	10	6
Proteins with diminished expression	24	49	20	47	56

Fig. 2. Autoradiograms of 2DE protein profiles of exponential-phase cells of *S. mutans* H7 growing at pH 7.5 with 20 mM glucose and rapidly subjected to various stress conditions for 30 min in the presence of ^{14}C -labelled amino acids followed by extraction, separation by 2DE and autoradiography. Stress conditions: A, control (cells maintained at pH 7.5 and 37 °C); B, oxidative (2 mM H_2O_2); C, acid (pH 7.5 to 5.5); D, starvation (fivefold dilution of BM); E, salt (0.2 M NaCl); F, heat (37 °C to 42 °C). All triangles indicate proteins enhanced twofold or more; solid triangles (▲) are general stress proteins and divided triangles (◁) indicate stress-specific proteins. pI scales are given at the top of each panel and molecular mass (kDa) on the left.

of each stress condition, the growth medium was supplemented with ^{14}C -labelled amino acids and incubation continued for 30 min before protein synthesis was arrested with chloramphenicol, and the cells ex-

tracted and the proteins separated by 2DE. The resultant gels were then subjected to autoradiography and image analysis. Duplicate experiments were compared and proteins whose expression was enhanced at least two-

Table 2. Number of proteins in Fig. 2 enhanced twofold or more by one stress condition which are also enhanced by a second condition

Data from Fig. 2 and Table 1 representing those proteins from two separate experiments whose synthesis was enhanced at least twofold. Designations (B) to (F) refer to the individual gels in Fig. 2.

	Total no. of proteins	Oxidation (B)	Acid (C)	Starvation (D)	Salt (E)	Heat (F)
Oxidation	69	–	23	32	32	19
Acid	64	23	–	25	17	16
Starvation	58	32	25	–	19	18
Salt	52	32	17	19	–	21
Heat	40	19	16	18	21	–

fold were identified for each of the conditions. As seen in Fig. 2B and Table 1, exposure of cells to 2 mM H₂O₂ resulted in the enhanced synthesis of 69 proteins compared to the control cells maintained at pH 7.5 (37 °C) (Fig. 2A), 15 of which were specific to oxidative stress. Acid stress, induced by a pH change from 7.5 to 5.5, resulted in the enhanced synthesis of 64 proteins, 25 of which were acid-specific (Fig. 2C), while fivefold dilution of BM resulted in the enhanced synthesis of 58 proteins, including 11 specific to starvation (Fig. 2D). Salt stress, generated by the addition of 0.2 M NaCl, enhanced the synthesis of 10 salt-specific proteins out of a total of 52 proteins (Fig. 2E), while increasing the temperature to 42 °C enhanced the synthesis of 40 proteins, 6 of which were heat-specific (Fig. 2F).

Comparative analysis of the 2DE gels in Fig. 2 indicated that the synthesis of six proteins was upregulated by all five stress conditions; these could be considered 'general' stress proteins. In addition, there were a considerable number of proteins (16–32) enhanced by two, three or four of the stress conditions (Table 2). In order to focus on the most significant protein changes, further analysis was restricted to only those proteins demonstrating fivefold or greater increases in labelling under the stress condition when compared to the same spot in the non-stressed control cells using the IOD % analysis (see Methods) (Table 3). Using this threshold, more proteins (30) were enhanced fivefold or more by acidification than by the other stress conditions, with 19 proteins (63%) specific to acid alone (ASPs). Of the ASPs, the most significant increases in synthesis occurred with proteins 510, 501 and 502, which showed 43-, 36- and 21-fold increases, respectively, in labelling during the 30 min incubation. H₂O₂-induced oxidative stress generated 25 proteins at this threshold, including 12 specific proteins (OxSPs), with the greatest incorporation of ¹⁴C in proteins 200, 106 and 210. Starvation and salt stress each enhanced the synthesis of 13 proteins; however, only one protein was specific to starvation (171) and one to salt (209). The temperature increase to 42 °C resulted in 15 upregulated proteins, with four being specific to heat (HSPs); the most significant

increase (54-fold) was with protein 210. Interestingly, protein 210 was also enhanced by oxidative (25-fold) and salt (13-fold) stress.

Further analysis of the 2DE gels revealed 18 proteins whose expression was reduced fivefold or more by the various stress conditions, with the majority (14) specific to the particular stress condition (Table 4). Eight proteins were downregulated by salt and heat stress, while oxidation, acid and starvation stress resulted in the diminished synthesis of three proteins, two proteins and one protein, respectively. Notable changes were shown by the specifically repressed proteins 43 (HSRP), 83 (OxSRP) and 108 (SaSRP), with reductions of 21-, 43- and 29-fold, respectively.

DISCUSSION

Our previous results with *S. mutans* LT11 demonstrated that an acid shock from pH 7.5 to 5.5 induced a response in exponential-phase cells that resulted in enhanced survival following incubation at a pH (3.0) that killed control cells maintained at pH 7.5 (Svensäter *et al.*, 1997). This acid-tolerance response required 2 h to complete. Subsequent pulse-chase experiments with ¹⁴C-labelled amino acids, followed by one-dimensional polyacrylamide electrophoresis and autoradiography, revealed the enhanced formation of 36 proteins following acidification when compared to control cells maintained at pH 7.5 (Hamilton & Svensäter, 1998). Some 25 proteins appeared within the first 30 min and all but two of the 36 proteins were synthesized transiently, a response similar to that seen with *Salmonella typhimurium* (Foster, 1993). Preliminary experiments employing 2DE with *S. mutans* LT11 indicated that the protein profiles for control and acid-stressed cells were similar to those seen in Fig. 2 for *S. mutans* H7 in this study (data not shown), suggesting that the ability to give complex inducible responses to various stresses is a common characteristic of this species. *S. mutans* H7 was selected for these studies because it was a fresh strain isolated from a caries lesion at low pH (unpublished

results) and, unlike *S. mutans* LT11, had no previous history of genetic manipulation (Tao *et al.*, 1993).

In this study, the reproducibility of the gels, as determined by the repeated analysis (more than six times) of control and acid-stressed cell extracts, was substantially improved by the use of the precast Immobiline pH strips and by making the extraction buffer compatible with that used in the first-dimension isoelectric focusing separation. The gels in Fig. 2 were generated with a pH range of 4–7 in the first dimension and routinely resulted in the separation of 420–575 proteins with the various stress conditions. Preliminary studies with first-dimension separation over the pH range 6–10 revealed only a total of 15–20 basic proteins, none of which were enhanced during acid shock from pH 7.5 to 5.5. This indicates that the 69 proteins enhanced twofold or more by acid in Fig. 2 constitute the major acid-responsive proteins in the organism. Although the cell extracts from the other stress conditions were not subjected to first-dimension separation in the pH 6–10 range, the relative paucity of basic proteins in the organism suggests that the vast majority were observed in this study.

In examining the protein profiles in Fig. 2, one is struck by relatively few ‘general’ stress proteins (six) enhanced twofold or more by all five stress conditions, although the number of stress-specific proteins ranged from 15% to 39% of the enhanced proteins and a significant number were induced by two to four conditions. Raising the threshold to only those proteins enhanced fivefold or more (Table 3) eliminated 66% of the proteins in the twofold enhanced group and reduced the general stress proteins from six to two (proteins 118b and 141). A higher proportion of stress-specific proteins (37 of 67, or 55%) survived this analysis, the highest number being associated with acid (19 ASPs) and oxidative stress (12 OxSPs). Furthermore, analysis of the 18 proteins whose synthesis was diminished fivefold or more (Table 4) revealed that most of these proteins (14) were specific to the stress applied. These experiments permit the analysis of alterations in the relative rate of synthesis of particular proteins compared to all other proteins and do not attempt to assess the absolute synthetic rate of these proteins. Furthermore, we are assuming that this approach identifies proteins (enzymes) whose upregulation is indicative of increased metabolic activity in the cells under the particular stress and which may, therefore, be more important than the downregulated proteins (Bloomberg, 1997). This, of course, may be an incorrect assumption.

Currently, the identity of the various proteins upregulated under the various stress conditions is not known; however, the nature of the stress does point to a number of possible proteins associated with physiological responses. For example, prolonged acidification of *S. mutans* cells results in the increased specific activity of the membrane $F_1 H^+$ /ATPase involved in proton efflux during pH homeostasis (Belli & Marquis, 1991; Hamilton & Buckley, 1991). It is conceivable that the

synthesis of the membrane F_1 ATPase subunits, previously isolated by Sutton & Marquis (1987), could be upregulated during the initial acid-shock period. Examination of Table 3 suggests that the α subunit (58 kDa) might be protein 357, enhanced 8-fold, while the β subunit (52 kDa) might be protein 503 or protein 504, upregulated 13- and 8-fold, respectively. The γ subunit (41 kDa) could be associated with either protein 135 or protein 511, while the δ (27 kDa) and ϵ (18 kDa) subunits might be associated with proteins 120 and 88, respectively.

The cross-protection experiments with *S. mutans* H7 demonstrated that a shift in pH from 7.5 to 5.5 did not protect cells against challenges by salt, oxidation, heat and starvation. Interestingly, the reverse situation, in which the cells were subjected to a 2 h adaptation to the five stress conditions followed by exposure to a challenge pH of 3.5 for 2 h, resulted in varying levels of cross-protection, except for heat shock and oxidative stress (Fig. 1). The other stresses, however, promoted survival at pH 3.5, with particularly notable protection (12-fold) afforded by diluting the basal medium (BM) fivefold and decreasing the glucose from 20 to 4 mM (starvation condition S-2). Even in the absence of a carbon source (starvation condition S-3), a sevenfold increase in survivors was observed. Since BM was comprised of salts, vitamins and amino acids, it is likely that, in addition to the carbon source, the concentration of key peptides and/or amino acids may be an important factor in the acid-tolerance response in *S. mutans* (Gale & Epps, 1942; Hersh *et al.*, 1996).

The overlap between acid and starvation responses is well known and considerable information is now available on the responses to various nutrients by *E. coli* (Nyström, 1993; VanBogelen *et al.*, 1990), *Sal. typhimurium* (Spector & Foster, 1993), *B. subtilis* (Bernhardt *et al.*, 1997) and *L. lactis* (Hartke *et al.*, 1994, 1995, 1996). The acid protection afforded to *S. mutans* by the starvation response (Fig. 1) has particular significance in the dental plaque environment. Depending on the age of plaque biofilm, the ingestion of dietary carbohydrate can result in a rapid reduction in pH to values near pH 4, and this pH can be sustained for some time at sugar concentrations above 1% (Yamada *et al.*, 1980). Thus, one can speculate that as the plaque pH and carbohydrate concentration decrease, the cells receive at least two signals to induce the acid response: one triggered by acid and the other by the concentration of the carbon source. The combined effect would generate a level of acid tolerance greater than that achieved by either signal alone. The response to reductions in the available peptide/amino acid supply is less clear. The reduction in the amino acid pool is probably responsible for the starvation response seen in Fig. 2C since amino acids are known to influence pH homeostasis (Gale & Epps, 1942; Hersh *et al.*, 1996). Furthermore, it is not yet known whether carbon and amino acid deprivation induce separate responses, although work with other bacteria suggests that the induction of specific proteins is

Table 3. Proteins upregulated fivefold or more in exponential-phase cells of *S. mutans* H7 under conditions of stress

Data obtained from the analysis of Fig. 2 and calculated as the IOD % of the protein spot under the stress condition vs the same spot in the control.

Protein ID	Mol. mass (kDa)	pI	Increase in synthesis under stress condition*					Stress protein†
			Oxid	Acid	Starved	Salt	Heat	
26	15.0	5.07		6				ASP
28	82.8	5.10					6	HSP
37a	57.3	5.64	7					OxSP
50	55.5	4.77		14			13	
57b	65.6	5.58	8					OxSP
60	66.5	6.02	6					OxSP
62a	54.6	6.04	6					OxSP
62b	55.2	6.02	14	5				
68b	49.6	5.75			8	5		
88	15.9	5.21		6				ASP
103	69.4	4.76				21	31	
106	62.5	5.17	28		6		9	
109	45.3	6.14			14		23	
118b	60.1	4.51	16	9	10	9	10	GSP
120	19.7	4.90		8	10			
121	21.7	5.38		9				ASP
135	26.6	4.84		5				ASP
136	27.5	4.66					8	HSP
141	29.1	6.40	10	16	13	17	7	GSP
156	34.6	4.81		5				ASP
158	50.6	5.99	6					OxSP
161	42.3	5.73			6		6	
171	54.8	5.68			10			SSP
172	70.6	5.70	6					OxSP
173	70.2	5.77	6					OxSP
179	64.5	4.94		6				ASP
185	52.2	4.95	5					OxSP
190	56.9	4.94	7	9		7		
191	55.7	4.85		14				ASP
198	65.3	4.92	14			7	9	
199	53.6	4.96	12			6		
200	56.4	4.98	29	6	5			
202	48.1	4.95	12	5		6	10	
203	50.0	4.99	6		6	7	8	
205	48.4	5.05		8		5		
209	60.9	5.37				5		SaSP
210	69.3	4.70	25			13	54	
342	42.5	6.44	10					OxSP
347	49.2	6.05	5					OxSP
350	63.8	5.79	7		6			
354	78.1	5.45					7	HSP
355	54.7	6.39	17					OxSP
356	59.0	6.39	6					OxSP
357	50.3	6.12		8	19			
358	28.1	6.25	5		7			
362	29.2	5.91		7				ASP
370	43.7	4.88					5	HSP
371	31.2	4.87		16				ASP
501	47.2	4.64		36				ASP

Table 3 (cont.)

Protein ID	Mol. mass (kDa)	pI	Increase in synthesis under stress condition*					Stress protein†
			Oxid	Acid	Starved	Salt	Heat	
502	43.5	4.63		21				ASP
503	41.0	4.66		13				ASP
504	41.9	4.73		8				ASP
506	24.5	4.79		17				ASP
508a	61.4	4.76		11		43		
509	59.1	4.68		5				ASP
510	60.5	5.94		43				ASP
511	29.3	5.07		8				ASP
513	54.7	4.74		7				ASP
514	47.4	4.74		7				ASP

* Stress conditions: Oxid, +2 mM H₂O₂; Acid, pH 7.5 to 5.5; Starved, 100 to 20 % BM; Salt, +0.2 M NaCl; Heat, 37 °C to 42 °C.

† Stress proteins: OxSP, oxidation-specific protein; ASP, acid-specific protein; SSP, starvation-specific protein; SaSP, salt-specific protein; HSP, heat-specific protein; GSP, general stress protein.

Table 4. Proteins downregulated fivefold or more in exponential-phase cells of *S. mutans* H7 under conditions of stress

Data obtained from the analysis of Fig. 2 and calculated as the IOD % of the protein spot under the stress condition vs the same spot in the control.

Protein ID	Mol mass (kDa)	pI	Decrease in synthesis under stress condition*					Stress protein†
			Oxid	Acid	Starved	Salt	Heat	
1a	83.6	5.38		6			8	
1b	87.3	5.40					5	HSRP
1c	87.5	5.45					5	HSRP
1d	87.3	5.35					5	HSRP
4a	40.4	5.33	5			9		
30	22.9	5.83		6				ASRP
43	18.3	4.90					21	HSRP
75	43.7	6.14				6		SaSRP
83	21.7	5.22	43					OxSRP
85	40.7	6.39				6	6	
99	35.5	6.12				9	5	
102a	74.3	4.70			6			SSRP
108	63.5	5.22				29		SaSRP
112	42.5	5.58				9		SaSRP
113	40.5	5.39				5		SaSRP
164	42.1	6.24					6	HSRP
174	48.1	5.25	6					OxSRP
335	20.4	5.25				5		SaSRP

* Stress conditions: Oxid, +2 mM H₂O₂; Acid, pH 7.5 to 5.5; Starved, 100 to 20 % BM; Salt, +0.2 M NaCl; Heat, 37 °C to 42 °C.

† Stress proteins: OxSRP, oxidation-specific repressed protein; ASRP, acid-specific repressed protein; SSRP, starvation-specific repressed protein; SaSRP, salt-specific repressed protein; HSRP, heat-specific repressed protein.

regulated by individual nutrient components (Nyström, 1993; Spector & Foster, 1993).

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