Effect of carbon starvation and proteolytic activity on stationary-phase acid tolerance of *Streptococcus mutans*

G. Svensäter,¹ Olof Björnsson¹ and I. R. Hamilton²

Author for correspondence: I. R. Hamilton. Tel: +1 204 789 3615. Fax: +1 204 789 3948.
e-mail: ihamilt@cc.umanitoba.ca

¹ Department of Oral Biology, Centre for Oral Health Sciences, Malmö University, Malmö, Sweden

² Department of Oral Biology, Faculty of Dentistry, University of Manitoba, 780 Bannatyne Ave, Winnipeg, Manitoba, Canada R3E 0W2

Previous research with *Streptococcus mutans* and other oral streptococci has demonstrated that the acid shock of exponential-phase cells (pH 7.5 to 5.5) resulted in the induction of an acid tolerance response (ATR) increasing survival at low pH (3.5–3.0). The current study was designed to determine whether two fresh isolates, H7 and BM71, and two laboratory strains, Ingbritt and LT11, were capable of a stationary-phase ATR as estimated by a survival test at pH 3.5 for 3 h. All four strains were unable to generate a stationary-phase ATR under control conditions at pH 7.5, with the exception of a burst of survivors in the transition between the exponential and stationary phases when the carbon source (glucose) was depleted. Adaptation at pH 5.5 resulted in the expected pH-dependent exponential-phase ATR, but only the fresh isolates exhibited a stationary-phase ATR at this pH. Glucose starvation of cells in complex medium was shown to enhance acid tolerance for the fresh isolates, but not the laboratory strains. This tolerance was, however, greatly diminished for all strains in a defined medium with a low concentration of amino acids. Growth of strain H7 in complex medium resulted in the formation of at least 56 extracellular proteins, nine of which were degraded in the early stationary phase following the induction of proteolytic activity during the transition period. No proteolytic activity was observed with strain LT11 and only 19 extracellular proteins/peptides were apparent in the medium with only one being degraded in the early stationary phase. Strain H7 was also shown to have two- to fourfold higher levels of intracellular glycogen in the stationary phase than strain LT11. These results suggest that *S. mutans* H7 possessed the required endogenous metabolism to support amino acid/peptide uptake in the early-stationary phase, which resulted in the formation of basic end products that, in turn, contributed to enhanced intracellular pH homeostasis.

Keywords: acidurance, starvation, protein secretion, oral streptococci

INTRODUCTION

The bacteria in dental plaque are subjected to continual cycles of 'acid shock' created by the rapid accumulation of metabolic acid end products generated during the metabolism of dietary carbohydrate by the acidogenic plaque microflora. Studies using *in vivo* pH telemetry have shown reductions in plaque pH from 7 to below 4 in as little as 3 min with the pH returning to neutral values at a much slower rate (Imfeld & Lutz, 1980;

Yamada *et al.*, 1980; Jensen *et al.*, 1982). The frequent and consistent intake of sugar over time will result in the prolonged suppression of plaque pH, a process that can lead to dental caries. Thus the organisms associated with the disease, such as *Streptococcus mutans*, *Lactobacillus* species and certain non-mutans streptococci (Bowden, 1991; van Houte *et al.*, 1996), are not only able to generate acid from carbohydrate substrates, but also possess the capacity to tolerate acidic environments for prolonged periods of time.

Previously, we have demonstrated the induction of an acid tolerance response (ATR) by exponential-phase

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Abbreviation: ATR, acid tolerance response.

cultures of *S. mutans* in response to an acid shock from pH 7.5 to 5.5 that resulted in enhanced survival at low pH, 3.5–3.0 (Svensäter *et al.*, 1997). This 'exponential-phase' ATR required protein synthesis, since survival was abolished in the presence of chloramphenicol. The incubation of cells with ^{14}C -labelled amino acids during the acid shock, followed by protein extraction and PAGE analysis, demonstrated the transient up-regulation of acid-responsive proteins over a 2 h period (Hamilton & Svensäter, 1998). More recently, 2D electrophoresis has demonstrated the up-regulation of 64 proteins within the first 30 min of a pH change from 7.5 to 5.5, with 49 proteins down-regulated during the same period (Svensäter *et al.*, 2000). Of the up-regulated proteins, 25 were specific to the acid response, while other proteins were also influenced by alternative stress conditions. These proteins are undoubtedly related to the variety of physiological changes observed with cells of *S. mutans* following a shift in pH from 7.5 to 5.5, while growing in continuous culture with a glucose limitation (Hamilton & Buckley, 1991).

Enteric bacteria possess a variety of acid survival systems with the responses differing depending on the growth medium, the stage of growth and other factors (Foster, 1995; Lin *et al.*, 1995; Castanie-Cornet *et al.*, 1999). The earlier known acid response of *Salmonella typhimurium*, now known as the 'pH-dependent exponential-phase ATR' (Lee *et al.*, 1994), is supplemented by at least two other strategies: a pH-independent general stress resistance dependent on the alternative sigma factor RpoS (σ^s), and an additional pH-dependent stationary-phase ATR. Comparisons between the acid-survival strategies in *Sal. typhimurium*, *Escherichia coli* and *Shigella flexneri* have indicated that all these organisms possessed the RpoS-dependent resistance system, while the latter two organisms possess several 'acid-resistance' systems not present in *Sal. typhimurium* and requiring components of complex medium, such as glutamate and arginine (Lin *et al.*, 1995). Recent work with *E. coli* has shown that cells actually possess three overlapping acid-resistance systems to protect stationary-phase cells in acid environments (Castanie-Cornet *et al.*, 1999).

Although current evidence indicates that most oral streptococci generate a pH-dependent exponential-phase ATR (Svensäter *et al.*, 1997; Hamilton & Svensäter, 1998), little information is available on the acid tolerance of oral streptococci during very slow growth or in the stationary phase, conditions frequently encountered by bacteria in dental plaque (Brecx *et al.*, 1983). Unlike enteric bacteria, oral streptococci are relatively inactive metabolically in the stationary phase unless they have synthesized endogenous energy reserves, such as glycogen, in the presence of exogenous carbohydrate. In addition, upon depletion of the energy reserves, the transmembrane pH gradient will dissipate with the intracellular pH assuming the same value as the external pH, which in acidic environments will result in cessation of cellular activity (Hamilton, 1990). As a consequence, we were interested in whether *S. mutans* could generate an ATR in the stationary phase and, if so,

what factors influenced such a response. For this, we compared the acid tolerance of two freshly isolated and two laboratory strains of *S. mutans* growing in complex medium at pH 7.5 and 5.5, using survival at pH 3.5 for 3 h as a measure of acid resistance. Unlike the laboratory strains, the freshly isolated strains were shown to possess a pH-dependent stationary-phase ATR and acid resistance was increased by carbon starvation in complex medium. Using the fresh isolate *S. mutans* H7 as a model system, it was demonstrated that stationary-phase acid tolerance appears to be related to enhanced protein secretion and degradation in the early-stationary phase.

METHODS

Bacterial strains and media. The organisms in this study included two established laboratory strains: *S. mutans* LT11, provided by R. R. B. Russell, Newcastle upon Tyne, UK, and *S. mutans* Ingbritt, obtained from J. Sandham, University of Toronto, Canada. The two fresh isolates included *S. mutans* H7, isolated from an approximal caries lesion at pH 5.0, and *S. mutans* BM71, isolated from human dental plaque and obtained from G. Bowden, University of Manitoba, Canada. The laboratory transfer of the fresh strains used in this study was restricted to the four to five subcultures necessary for isolation and purification of the culture. Growth was carried out anaerobically (9% H_2 , 5% CO_2 in nitrogen) with both complex and minimal media: the basal complex medium comprised (g l $^{-1}$) tryptone (10) and yeast extract (5) buffered with 40 mM phosphate/citrate buffer (TYE) supplemented with 20 mM glucose (TYEG), while the defined medium (MADM) was that previously described by Bowden *et al.* (1976) with the Casamino acid content adjusted to 200 mg l $^{-1}$. Plate counts of cells surviving an acid challenge at pH 3.5 were carried out with trypticase agar comprising (g l $^{-1}$): trypticase (10), yeast extract (2), sodium carbonate (2), glucose (2), NaCl (5) and agar (10) with the pH adjusted to 7.2.

Acid tolerance during growth. To test for pH-dependent and pH-independent stationary-phase ATRs, the acid resistance of cells was tested during normal 'batch' growth. Cells were grown anaerobically in TYEG at pH 7.5 or 5.5 with the culture pH maintained by the addition of KOH. The pH varied by less than ± 0.3 units throughout the growth period. Periodically, duplicate culture samples were removed and the cells were subjected to an acid challenge at pH 3.5 for 3 h followed by plating for survivors on trypticase agar. This latter pH is 0.2–0.5 units above the pH which kills 100% of exponential-phase cells grown at pH 7.5 (Svensäter *et al.*, 1997). Rapid acidification was achieved by centrifuging 1.0 ml of the culture suspension in a microfuge at 15000 g for 3 min, washing the cells twice in pre-warmed sterile TYEG buffered at pH 3.5 and resuspending the cells in the same medium prior to incubation at 37 °C. All dilutions were plated in triplicate with the plates incubated at 37 °C for a minimum of 3 d. The percentage of cell survivors at each time point was calculated by comparing the numbers of cells surviving the pH 3.5 challenge and the number of cells in the original culture sample just prior to acidification. The data presented represent the mean of at least three separate determinations with the standard errors calculated by the Statview program for the Macintosh.

Glucose-depleted 'stationary phase'. To assess the effect of glucose depletion on acid tolerance, exponential-phase cells grown at pH 7.5 in either TYEG or MADM were rapidly washed and resuspended in the same medium at pH 7.5 and

5.5 without glucose. Following a 2 h adaptation period at 37 °C, duplicate cell samples were removed for plate counts on trypticase agar prior to rapid acidification of the culture to pH 3.5 as described above. In order to determine the rate of acid killing, duplicate samples were removed each hour over a 3 h period and the cultures were diluted and plated for survivors on trypticase agar. As above, the percentage survival was calculated from the cell counts obtained during exposure to pH 3.5 and compared to those of the same samples prior to acidification to pH 3.5. Control cells were incubated in the same medium supplemented with 20 mM glucose. The pH in all experiments varied less than ± 0.2 units during any incubation period and the data presented represent the mean of at least three separate determinations.

Intracellular glycogen analysis. The glycogen content of cells was determined during the growth of the test strains in TYE containing 10 mM glucose. Culture samples (10 ml) were removed periodically to a boiling water bath for 10 min followed by centrifugation at 15000 *g* for 15 min. The boiled cells were washed twice in cold distilled water and resuspended at 0.4 mg dry weight ml⁻¹ and then frozen (-70 °C) until analysed for glycogen. Glycogen was assayed by the method of DiPersio *et al.* (1974).

2D gel electrophoresis. Culture supernatant fractions were filter-sterilized (0.22 µm) and concentrated 10-fold (Ultrafree-MC, 5000 NMWL; Millipore) and the proteins/peptides were separated by 2D electrophoresis essentially as previously described by Svensäter *et al.* (2000). The first dimension isoelectric focusing was run on linear 7 cm immobilized pH gradient (IPG) strips (Amersham Pharmacia Biotech) in the pH range 4–7 and the proteins were separated by 150 V for 1 h, 300 V for 1 h, 600 V for 1 h and 3500 V for 13 h. Following separation, the strips were immediately frozen at -80 °C until the second dimension, SDS-PAGE, could be carried out with 10% polyacrylamide gels using the Mini Protean II system (Bio-Rad). The gels were then silver-stained according to the manufacturer (Amersham Pharmacia Biotech) and scanned with a calibrated UMAX transmission scanner. Spot volumes were determined with BioImage software (version 1.6) on a Sun UltraSparc workstation (Genomic Solutions) and were defined as the sum of the pixel values comprising the protein minus the sum of the background pixel values. A reference gel was chosen and each of the other gels was matched to it selecting anchor proteins on the images and allowing the BioImage software to automatically match the images. Proteins of known molecular mass were used as standards to generate molecular mass values and pI values were deduced from the linearity of the IPG strips.

Zymography. Proteins in filtered culture supernatants were concentrated (100-fold) by centrifugal filtration (Amicon) and separated on 10% SDS-PAGE gels containing covalently bound gelatin or 12% SDS-PAGE gels containing covalently bound casein (Bio-Rad). Electrophoresis was carried out at 100 V for 2 h at room temperature. The gels were then incubated at room temperature in 2.5% Triton X-100 for 30 min and then placed in a developing buffer (50 mM Tris-base, pH 7.5; 0.2 M NaCl; 5 mM CaCl₂; 0.02% Brij-35) overnight at 37 °C. The gels were then stained with 0.5% Coomassie brilliant blue in 40% methanol/10% acetic acid for 1 h and destained with 40% methanol/10% acetic acid. Protease activity was detected as a clear zone against a stained background.

Analytical procedures. Protein was determined by the method of Bradford (1976), while glucose was assayed enzymically by the method of Kingsley & Getchell (1960).

RESULTS

Stationary phase and acid tolerance

The acid tolerance of the test strains was assessed during the various phases of batch growth in complex medium (TYEG) at pH 7.5 and 5.5 by determining the numbers of cells capable of surviving an acid challenge at pH 3.5 for 3 h. As expected from previous results (Svensäter *et al.*, 1997), no survivors were observed with unadapted exponential-phase cells of the laboratory strain *S. mutans* LT11 (Fig. 1a) and the fresh isolate *S. mutans* H7 (Fig. 2a). However, a small number of survivors were observed during the transition between the exponential and stationary phases when the carbon source was depleted, but this increase was short-lived and did not extend to stationary-phase cells. Conversely, growth of the organisms at pH 5.5 (Figs 1b and 2b) resulted in the expected increase in survivors due to the induction of the exponential-phase ATR with *S. mutans* H7 generating significantly more survivors than strain LT11. Entry into the stationary phase, however, resulted in a major difference between the strains: the number of survivors of LT11 decreased to zero immediately the cells entered the stationary phase (Fig. 1b), while the numbers of

Fig. 1. Batch growth of *S. mutans* LT11 in TYEG at pH 7.5 (a) and pH 5.5 (b). Δ , Growth measured at OD₆₆₀; $\bullet$, numbers of survivors in culture samples following a 3 h exposure at pH 3.5 in the same medium; $\square$, glucose concentration in the medium (mM).

Fig. 2. Batch growth of *S. mutans* H7 in TYEG at pH 7.5 (a) and pH 5.5 (b). Δ , Growth measured at OD₆₆₀; $\bullet$, numbers of survivors in culture samples following a 3 h exposure at pH 3.5 in the same medium; $\square$, glucose concentration in the medium (mM).

strain H7 increased in cell samples removed during the transition and early-stationary phase of growth (Fig. 2b). This result indicates the presence of a pH-dependent stationary-phase ATR in the latter organism that is absent in LT11. Similar experiments were carried out with the laboratory strain *S. mutans* Ingbritt and the fresh isolate *S. mutans* BM71, and these confirmed the differences between the laboratory and fresh strains. In summary, the results indicate that while all four strains possessed the pH-dependent exponential-phase ATR, only the fresh strains, H7 and BM71, possessed the pH-dependent stationary-phase ATR.

Influence of the carbon source

The abrupt changes in survivors seen in Figs 1 and 2 upon depletion of the glucose suggested a possible link between carbon starvation and the ATR. To examine further the influence of glucose on survival, exponential-phase cells, grown at pH 7.5 in complex TYEG medium, were rapidly washed and incubated in the same medium at pH 7.5 and 5.5 without glucose (TYE) for 2 h to simulate entry into 'carbon-starved' stationary phase

Fig. 3. Effect of glucose starvation on the rate of acid killing of adapted and unadapted cells of *S. mutans* H7 (a) and *S. mutans* Ingbritt (b) at pH 3.5. $\square$, $\blacksquare$, Unadapted cells (pH 7.5) with and without glucose, respectively; $\circ$, $\bullet$, adapted cells (pH 5.5) with and without glucose, respectively. Cells were incubated for 2 h in TYE at either pH 7.5 or 5.5 with and without glucose before being concentrated, washed and incubated in the same medium at pH 3.5, with survivors assessed each hour for 3 h.

before being subjected to the acid challenge at pH 3.5. Control cells were incubated under the same conditions in medium with glucose (TYEG). As seen with *S. mutans* H7 (Fig. 3a), incubation at pH 5.5 in TYEG resulted in 2- to 135-fold higher number of survivors over the 3 h acid challenge when compared to TYEG cells at pH 7.5 demonstrating induction of the ATR. Incubation at pH 5.5 without glucose, however, generated 6- to 25-fold higher numbers of survivors than the pH 5.5 cells in TYE with glucose. In addition, the incubation of pH 7.5-unadapted cells in glucose-free TYE resulted in 2- to 50-fold higher numbers of survivors than the same cells incubated with glucose. Thus glucose starvation of *S. mutans* H7 enhanced acid resistance in both adapted and unadapted cells, implicating glucose starvation in both pH-dependent and pH-independent stationary-phase acid tolerance. Similar results were obtained with *S. mutans* BM71 (data not shown). When these experiments were repeated with the laboratory strain *S. mutans* Ingbritt (Fig. 3b), the results indicated that the organism was more acid sensitive than strain H7 with no

Fig. 4. Ratio of survivors of *S. mutans* LT11, *S. mutans* BM71 and *S. mutans* H7 in TYE versus MADM medium at pH 3.5 in the presence and absence of glucose. A, Unadapted cells with glucose; B, unadapted cells without glucose; C, adapted cells with glucose; D, adapted cells without glucose. Cells were incubated for 2 h in TYE with and without glucose before being concentrated, washed and incubated in the same medium at pH 3.5 for 2 h.

sustained effect of glucose starvation on acid tolerance, a result also seen with *S. mutans* LT11 (data not shown).

Influence of the amino acids/proteins

When the experiment in Fig. 3(a) was repeated with *S. mutans* H7 grown in a defined medium (MADM) supplemented with a low level of amino acids (0.02%), acid resistance was significantly diminished with little effect of the presence or absence of glucose. That a component in, or derived from, the complex medium enhances acid resistance can be seen by comparing the numbers of survivors in TYE to those in MADM (Fig. 4). In this comparison, the numbers of H7 survivors in TYE were two to four orders of magnitude greater than those obtained with cells in MADM, with similar results (e.g. two to three orders of magnitude) obtained with *S. mutans* BM71. A similar comparison with the laboratory strain *S. mutans* LT11 indicated that this organism was only marginally influenced by the nature of the growth medium with increases ranging from only two- to ninefold, a finding similar to that seen with *S. mutans* Ingbritt (data not shown).

Protein degradation

The increased acid resistance by cells of *S. mutans* H7 and BM71 in complex medium strongly suggested that the metabolism of the proteins and/or peptides present in the TYE medium, but not in the MADM medium, might have contributed to the increased acid tolerance of the fresh isolates by the generation of basic cellular

Fig. 5. Gelatin zymograms of cell-free culture supernatant fractions isolated during the growth of *S. mutans* H7 (a) and *S. mutans* LT11 (b) in TYEG. L, Mid-exponential phase; T, transition between exponential and stationary phases; S, stationary phase.

metabolic end products. This possibility was enhanced by the observation that cells of strain H7 induced protease activity in the transition phase prior to entry into the stationary phase, activity that was not observed in strain LT11 (Fig. 5). The H7 protease activity, which was transitory, being absent in exponential- or stationary-phase cells, was linked to at least two proteolytic zones with estimated molecular masses of 55 kDa and 25–32 kDa. Proteolytic activity was observed with gelatin, but not with casein, as the substrate.

This observation led to an examination of the proteins and peptides present in the medium during growth of *S. mutans* H7 and strain LT11 in TYE medium with 20 mM glucose. For this experiment, the cells were removed by centrifugation and the cell-free culture supernatant was sterilized by filtration, concentrated 10-fold by centrifugal filtration, and then subjected to 2D electrophoresis. The 2D gel analysis revealed that strain H7 generated 56 extracellular proteins in mid-exponential phase compared to 19 proteins by strain LT11 with proteins ranging in size from 5 to 200 kDa. Comparative analysis of the proteins in a selected gel

Fig. 6. Silver-stained 2D gels of cell-free culture supernatant fractions isolated during the growth of *S. mutans* H7 (a–d) and *S. mutans* LT11 (a, c*, d*) in TYEG. The gel in (a) is the original uninoculated medium, while (b)–(d) conform to the samples indicated on the growth curve.

Table 1. Glycogen present in cells of *Streptococcus mutans* strains H7 and LT11 during growth on tryptone-yeast extract medium at pH 7.5 with 10 mM glucose as the carbon source

Growth phase	<i>S. mutans</i> H7*	<i>S. mutans</i> LT11*	Ratio H7/LT11
Late exponential	41.2 ± 14.4	10.3 ± 10.8	4.0
Transition	43.8 ± 22.2	21.3 ± 1.9	2.1
Early stationary	29.1 ± 14.9	11.0 ± 0.6	2.7

* Values represent mg glycogen (g dry wt cells)⁻¹ ± SE.

area (pI range 4.5–6) showed that mid-exponential-phase cells of strain H7 (Fig. 6b) generated at least nine proteins that were either completely (spots 1, 2, 3, 6, 7

and 8) or partially (spots 4, 5 and 9) degraded by the time the cells had reached the stationary phase (Fig. 6d). *S. mutans* LT11, on the other hand, generated only one

protein (spot 1) that was degraded on entry to the stationary phase (Fig. 6c* vs d*).

Energy status of early-stationary-phase cells

Enzymic analysis of the cell-free supernatant samples in the experiment depicted in Fig. 6 indicated that the glucose was completely depleted upon entry into the transition period. This raised the question as to the source of energy available to cells for the maintenance of pH homeostasis, as well as for the reactions associated with protein degradation and amino acid/peptide transport in the transition and early-stationary phases of growth. With glucose as the exogenous energy source, oral streptococci have a variable capacity to synthesize intracellular glycogen and this was demonstrated in experiments comparing the glycogen synthetic activity of *S. mutans* H7 and strain LT11 during growth in TYE with 10 mM glucose. As seen in Table 1, strain H7 possessed two- to fourfold more glycogen than LT11 upon entry into the transition and stationary phases.

DISCUSSION

The results of the current research indicate that fresh isolates possess additional properties for protection against acid stress not observed with the strains transferred in laboratory medium for prolonged periods of time. None of the *S. mutans* strains in this study exhibited a sustained ATR during batch growth at pH 7.5 in either the exponential or stationary phases, although a small increase in survivors was seen in the transition between these two phases that was not sustained in the stationary phase (Figs 1 and 2). Growth at pH 5.5 for 2 h did, however, induce an ATR in exponential-phase cells of all strains, confirming earlier results (Svensäter *et al.*, 1997), but only the fresh isolates, strains H7 and BM71, were able to sustain a pH-dependent stationary-phase ATR. While not often acknowledged, fresh isolates of oral bacteria possess different properties to those transferred for long periods of time in complex laboratory medium. For example, *S. mutans* and other oral streptococci have been shown to undergo significant alterations in enzyme composition and activity in as little as 225 daily transfers in the laboratory (Cvitkovitch & Hamilton, 1994), not an unreasonable observation when one compares the nutrients available in dental plaque with that of laboratory medium comprised of preformed essential nutrients.

One feature not readily assessed in the growth experiments was the nature of the short transitory increase in survivors as the cells entered the stationary phase (Figs 1 and 2) at a point that coincided with the depletion of glucose. That the effect was due to the depletion of glucose was seen in the 'artificial stationary-phase' experiment which showed with strain H7 (Fig. 3a) that, while adapted cells were inherently more acid resistant than unadapted cells, the acid tolerance of unadapted and adapted cells was enhanced by the absence of

glucose during the acid challenge at pH 3.5 over the 3 h period. No such differential effect was seen with the laboratory strain *S. mutans* Ingbritt (Fig. 3b) and strain LT11. Earlier results with *S. mutans* H7 (Svensäter *et al.*, 2000) have demonstrated cross-protection of cells to acid killing by prior exposure of cells to starvation conditions. Starvation, induced by exposure of cells to fivefold diluted basal medium, was most protective when the glucose concentration was diluted from 20 to 4 mM, although an enhanced effect over cells adapted in full-strength medium was seen when the diluted medium was devoid of glucose.

Carbohydrate-starved cells of *Lactobacillus lactis* IL1403 exhibit enhanced resistance to acid, heat, ethanol, osmotic and oxidative stress with this cross-protection occurring progressively with the onset of stationary phase (Hartke *et al.*, 1994). Unlike the development of the ATR in exponential-phase cells of *L. lactis* (Rallu *et al.*, 1996), the stationary-phase response was independent of protein synthesis since it was not abolished, but enhanced, by chloramphenicol or rifamycin. The use of transposon mutagenesis with *L. lactis* MG1363 has suggested a link between acid tolerance and the stringent response since a number of acid-resistant mutants had defects in the biosynthetic pathway for the stringent response factor (p)ppGpp (Rallu *et al.*, 1996). Since (p)ppGpp is a key pleiotropic regulator of gene expression and survival in stationary phase (Nyström, 1993), it is conceivable that the stringent response may also be a factor in the regulation of stress in *S. mutans* and other oral streptococci.

The enhanced acid resistance of *S. mutans* H7 and BM71 in complex medium (TYE) compared to the low amino acid defined medium (MADM) clearly differentiates these strains from *S. mutans* LT11 (Fig. 4). One assumes that differences in the metabolism of proteins, peptides and amino acids by the former organisms are central to this enhanced resistance. The appearance of proteolytic activity with cells of strain H7 during the transition from exponential to stationary phase (Fig. 5), and the evidence of protein/peptide generation in the culture medium during the exponential phase with subsequent utilization during early-stationary phase (Fig. 6), support this contention. The appearance of a 55 kDa protease in *S. mutans* H7, using gelatin as a substrate, confirms an earlier report of such activity by Harrington & Russell (1994). As to the extracellular proteins/peptides, preliminary experiments indicate that a majority of the proteins in the culture medium seen in Fig. 6 are secreted by *S. mutans* H7 into the medium mainly during the mid-exponential phase (O. Björnsson & G. Svensäter, unpublished results). On-going mass spectrometric analysis, using peptide mass fingerprints for protein identification, indicates that the 60 kDa chaperonin DnaK and the glycolytic enzyme enolase are secreted in a manner similar to that recently reported for *Streptococcus pyogenes* (Chaussee *et al.*, 2001). Extracellular proteins are known to be important virulence factors and while information is emerging as to the regulation of their expression, less is known about their fate and

whether such proteins can be utilized to enhance acid tolerance.

Work with *E. coli* and *Shigella flexneri* has identified 'acid-resistance' systems protecting cells to pH 2.5 and requiring glutamate or arginine during the low pH challenge with the arginine-acid survival system in *E. coli* involving arginine decarboxylase (Lin *et al.*, 1995). More recently, a glutamate decarboxylase alkalization cycle was identified in *E. coli* to protect cells from cytoplasmic acidification (Hersh *et al.*, 1996), refining the early observations of Gale & Epps (1942). While there is relatively little specific information on the role of amino acids and peptides in acid resistance of *S. mutans*, oral bacteria are known to utilize salivary proteins for growth (Cowman *et al.*, 1979; De Jong *et al.*, 1984) and the uptake of arginine-containing peptides by mixed oral bacteria utilizing glucose has been shown to stimulate pH increases over that observed with glucose alone (Kleinberg *et al.*, 1976). As the cells enter the stationary phase and the exogenous glucose becomes depleted, an energy source is important for transport processes, consequently the utilization of endogenous carbon reserves, such as glycogen, becomes crucial to cell physiology. This energy source, the principal endogenous energy source for *S. mutans* (Hamilton, 1976), is also essential for the maintenance of pH homeostasis by the extrusion of proton via the H⁺/ATPase (Hamilton & Buckley, 1991). Thus the increased accumulation of glycogen by *S. mutans* H7 compared to strain LT11 (Table 1) would give the former organism a selective energy advantage as the cells entered the stationary phase of growth.

Clearly the current results, coupled with those on the multiple stress response of *S. mutans* H7 (Svensäter *et al.*, 2000), indicate a strong regulatory link between the acid stress and carbon/nitrogen starvation responses in the organism. In comparing the multiple stress response in *S. mutans* H7, it could be shown that the fivefold dilution of a defined medium resulted in the up-regulation of 58 proteins, 11 of which were specific to starvation; 20 additional proteins exhibited diminished synthesis. Acid shock from pH 7.5 to 5.5, on the other hand, resulted in the up-regulation of 64 proteins and the down-regulation of 49 proteins with 25 specific to the acid response. Of particular interest was the fact that 25 of those proteins that showed enhanced synthesis were common between the acid and starvation responses, and a number of these were associated with enzymes of the glycolytic pathway (unpublished results). Starvation-induced stress resistance is a common feature of both Gram-positive and Gram-negative bacteria with significantly more known about the response in enteric bacteria (Matin, 1991). The pH-independent general stress resistance in Gram-negative bacteria, such as *Sal. typhimurium* and *E. coli*, requires the growth-phase-dependent transcriptional factor σ^S , the product of the *rpoS* gene (Hersh *et al.*, 1996; Lin *et al.*, 1995). While σ^S has not been found in Gram-positive bacteria, *Bacillus subtilis* is known to possess a regulon controlled by the alternative sigma factor σ^B , regulating 60 general stress

proteins activated by various stresses and on entry into the stationary phase (Hecker *et al.*, 1996; Bernhardt *et al.*, 1997).

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