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Stress-responsive proteins are upregulated in *Streptococcus mutans* during acid tolerance

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Streptococcus mutans is an important pathogen in the initiation of dental caries as the bacterium remains metabolically active when the environment becomes acidic. The mechanisms underlying this ability to survive and proliferate at low pH remain an area of intense investigation. Differential two-dimensional electrophoretic proteome analysis of *S. mutans* grown at steady state in continuous culture at pH 7·0 or pH 5·0 enabled the resolution of 199 cellular and extracellular protein spots with altered levels of expression. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry identified 167 of these protein spots. Sixty-one were associated with stress-responsive pathways involved in DNA replication, transcription, translation, protein folding and proteolysis. The 61 protein spots represented isoforms or cleavage products of 30 different proteins, of which 25 were either upregulated or uniquely expressed during acid-tolerant growth at pH 5·0. Among the unique and upregulated proteins were five that have not been previously identified as being associated with acid tolerance in *S. mutans* and/or which have not been studied in any detail in oral streptococci. These were the single-stranded DNA-binding protein, Ssb, the transcription elongation factor, GreA, the RNA exonuclease, polyribonucleotide nucleotidyltransferase (PnpA), and two proteinases, the ATP-binding subunit, ClpL, of the Clp family of proteinases and a proteinase encoded by the *pep* gene family with properties similar to the dipeptidase, PepD, of *Lactobacillus helveticus*. The identification of these and other differentially expressed proteins associated with an acid-tolerant-growth phenotype provides new information on targets for mutagenic studies that will allow the future assessment of their physiological significance in the survival and proliferation of *S. mutans* in low pH environments.

INTRODUCTION

Streptococcus mutans is now well recognized as being associated with the initiation of dental caries, since its acid fermentation by-products can result in the demineralization of tooth enamel (Hamada & Slade, 1980; Harper & Loesche, 1984; Loesche, 1986; van Houte, 1994; van Ruyven *et al.*, 2000). A key to the survival of *S. mutans* at low pH is its ability to maintain a transmembrane pH gradient (ΔpH), with the interior of the cell more alkaline. This is achieved by upregulation of a proton-translocating F_1F_0-ATPase that extrudes H^+ as the external environment becomes more acidic. This results in an increased use of ATP for H^+extrusion and a consequent reduction in cell yield (Belli & Marquis, 1991; Hamilton & Buckley, 1991; Dashper & Reynolds, 1992; Quivey *et al.*, 2001). A series of recent physiological, mutagenic and proteome studies (Quivey *et al.*, 1995; Gutierrez *et al.*, 1996, 1999; Jayaraman *et al.*, 1997; Hamilton & Svensäter, 1998; Hahn *et al.*, 1999; Hanna *et al.*, 2001; Kremer *et al.*, 2001; Lemos *et al.*, 2001; Li *et al.*, 2002; Wilkins *et al.*, 2002; Len *et al.*, 2004), however, indicates that *S. mutans* regulates its phenotype in a far more complex fashion than simply increasing its ability to extrude H^+ in response to acid stress. For instance, our recent proteome analysis detected changes in metabolic pathways following acid-tolerant growth. Analysis of the data gave rise to the hypothesis that *S. mutans* redirects carbon from acidic fermentation by-products to more alkaline catabolites. These changes appear to occur in order to minimize the detrimental effects that result from the uncoupling of carbon flux from catabolism, as a consequence of the use of ATP for H^+extrusion (Len *et al.*, 2004). What remains to be elucidated is the breadth of the stress response in *S. mutans* that allows it to survive and proliferate at low pH.

Here we report the phenotypic changes, previously associated with the maintenance of bacterial viability

Abbreviations: 2-DGE, two-dimensional gel electrophoresis; ASB-14, amidosulfobetaine-14; *D*, dilution rate; DE, differential expression (values); IPG, immobilized pH gradient; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PMM, peptide mass mapping.

under a variety of imposed environmental stresses, that were observed when *S. mutans* was grown at steady state in continuous culture at low pH. Proteins required for the maintenance of DNA integrity, transcriptional fidelity, translational efficiency, and protein folding were uniquely identified during acid-tolerant growth at pH 5·0, or were present at higher levels than those in *S. mutans* grown at pH 7·0. The mode of action of these proteins is discussed in relation to current knowledge of their roles in responding to stress, particularly in Gram-positive bacteria associated with acidic environments.

METHODS

Growth conditions. Triplicate continuous cultures of *Streptococcus mutans* LT11 (Tao *et al.*, 1993) were grown under anaerobic conditions at a dilution rate (D) of $0{\cdot}100 \pm 0{\cdot}001\ h^{-1}$, at either pH $7{\cdot}0 \pm 0{\cdot}1$ or pH $5{\cdot}0 \pm 0{\cdot}1$, with glucose limitation as previously described (Jacques *et al.*, 1979). DMM medium, devoid of mucin, was used, but modified to include adenine, guanine and uracil at 20 μg ml^{-1}, and both KH_2PO_4 and K_2HPO_4 at 15 mM (Sissons *et al.*, 1991).

Preparation of cellular and extracellular proteins. When steady state had been achieved, the bacterial contents of the culture vessel were harvested, washed and lyophilized before aliquots of 10 mg dry wt of cells were treated with mutanolysin (Len *et al.*, 2003). Proteins that were to be separated on acidic immobilized pH gradient (IPG) strips (pH 4·0–6·7) were extracted as previously described (Len *et al.*, 2003), except that 1 % (w/v) amidosulfobetaine-14 (ASB-14) and 65 mM DTT were added to produce a modified solubilizing solution for two-dimensional electrophoresis (2-DGE). While the addition of these reagents increased the total number of protein spots that could be readily discerned on 2-DGE gels, their inclusion selectively inhibited the extraction or subsequent separation of a small number of weakly expressed proteins that had been previously visualized and/or identified on 2-DGE gels (Len *et al.*, 2003).

Proteins that were to be separated on basic IPG strips (pH 6–11) were obtained from the mutanolysin-treated cells by a two-fraction solubilization procedure. Following centrifugation of the cell lysate (12 000 ***g***, 4 °C, 10 min), the cell pellet was stored at −20 °C, and the proteins in the supernatant precipitated overnight at −20 °C with 15 % (w/v) trichloroacetic acid. After centrifugation and two washes in methanol (12 000 ***g***, 4 °C, 10 min), the precipitated proteins were solubilized in 300 μl of a 1 : 1 mixture of modified solubilization solution (without ASB-14) and Cellular and Organelle Membrane Solubilizing Reagent (Sigma-Aldrich) containing 1 % (v/v) Triton X-100 and 2 mM tributylphosphine. The frozen cell pellet was then thawed and resuspended by sonication (Branson Ultrasonics; 50 W, 10 × 10 s, 20–22 °C, with cooling on ice between each burst) in 700 μl of the same solubilization solution, before 150 U of exonuclease III was added and the suspension incubated at room temperature (20–22 °C) for 15 min to degrade any DNA. The two cellular fractions were then combined and centrifuged at room temperature (12 000 ***g***, 20–22 °C, 10 min). Prior to IEF, 100 μl 500 mM iodoacetamide was added, and the mixture incubated at room temperature (20–22 °C) for 2 h.

2-DGE and mass spectroscopic analyses of proteins. Both 2-DGE and mass spectroscopic analyses, using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, were performed using a PerSeptive Biosystems Voyager DE-STR mass spectrometer, with trypsin autolysis peptide masses of 842·5 and 2211·1 Da as internal standards, as previously described (Len *et al.*, 2003, 2004). All mass spectra were obtained in reflectron-delayed extraction mode. The density/volume, or differential expression (DE) value (arbitrary units), of SyproRuby-stained protein spots of 2-DGE gels was determined using the software package z3 (Compugen). The main source of error associated with this form of quantification is the reproducibility of the 2-DGE displays themselves, as biological variation is minimized when a chemostat is used to culture bacteria. That the 2-DGE displays were the main source of error was confirmed by comparing the DE values of 25 randomly chosen protein spots selected from 2-DGE displays separated on broad-range IPG strips over the pI range 4·0–7·0. Equivalent protein spots from triplicate samples from each of three repeat continuous cultures were analysed. The data confirmed that the main source of error in determining DE values was 2-DGE. As a consequence, triplicate experimental samples from cells grown at each pH were used for all 2-DGE analyses, and the increase in the level of expression of a protein spot was based on the difference in the mean DE values.

Protein identification. Peptide mass mapping (PMM) analyses of proteins were undertaken as previously described, making use of the six contigs of the *S. mutans* UA159 genome downloaded on October 6, 2001 that were translated in all six reading frames (Len *et al.*, 2003). All translated ORFs that matched PMM data were then used to query the annotated *S. mutans* genome at the Oral Pathogen Sequence Databases (http://www.stdgen.lanl.gov/oragen; Ajdić *et al.*, 2002), using the local BLAST search facility to determine the gene identification number. The original six contigs were used in this manner as some genes identified in these contigs were not present in the final annotated version.

Mass spectroscopic parameters for protein identification included a mass tolerance of 150 p.p.m. and a maximum of one missed cleavage per peptide while taking into consideration methionine sulfoxide and cysteine acrylamide modifications. Matches were defined on the basis of the number of matching peptide masses and the total percentage sequence covered by the peptides. As a general rule, a minimum total sequence coverage of 25 % was taken to match a given translated ORF of a high-M_r protein with confidence, though coverage as high as 80 % was observed with many low-M_r proteins. All translated ORFs that matched PMM data were then used to query the annotated *S. mutans* genome at the Oral Pathogen Sequence Databases (http://www.stdgen.lanl.gov/oragen), using the local BLAST search facility to determine the gene identification number. All gene names used are those associated with the *S. mutans* genome at the Oral Pathogen Sequence Database site. Theoretical M_r and pI were determined using MassLynx software version 3.4 (Micromass).

Protein isoforms. The term 'isoform' is used in the text to describe a protein that exists in multiple charged forms on 2-DGE gels, where the mean observed M_r for each form calculated from the second (SDS-PAGE) dimension deviates by up to 5 % and where there is no evidence from peptide mass mapping of any form of truncation or degradation (Len *et al.*, 2004).

RESULTS AND DISCUSSION

The increased expression of the proton-translocating F_1F_0-ATPase that extrudes H^+ from *Streptococcus mutans* (Belli & Marquis, 1991; Hamilton & Buckley, 1991; Dashper & Reynolds, 1992; Quivey *et al.*, 2001) is a key to the survival of the bacterium in an acidic environment. This was reflected in the previously reported increase in the α- (AtpA) and γ- (AtpC) subunits of the F_1 component of the ATPase when the bacterium was grown at pH 5·0 (Fig. 1, Table 1; Quivey *et al.*, 2001; Len *et al.*, 2004). A combination of steady-state continuous-culture technology and narrow-range IPG strips, however, resulted in the resolution of an additional 197

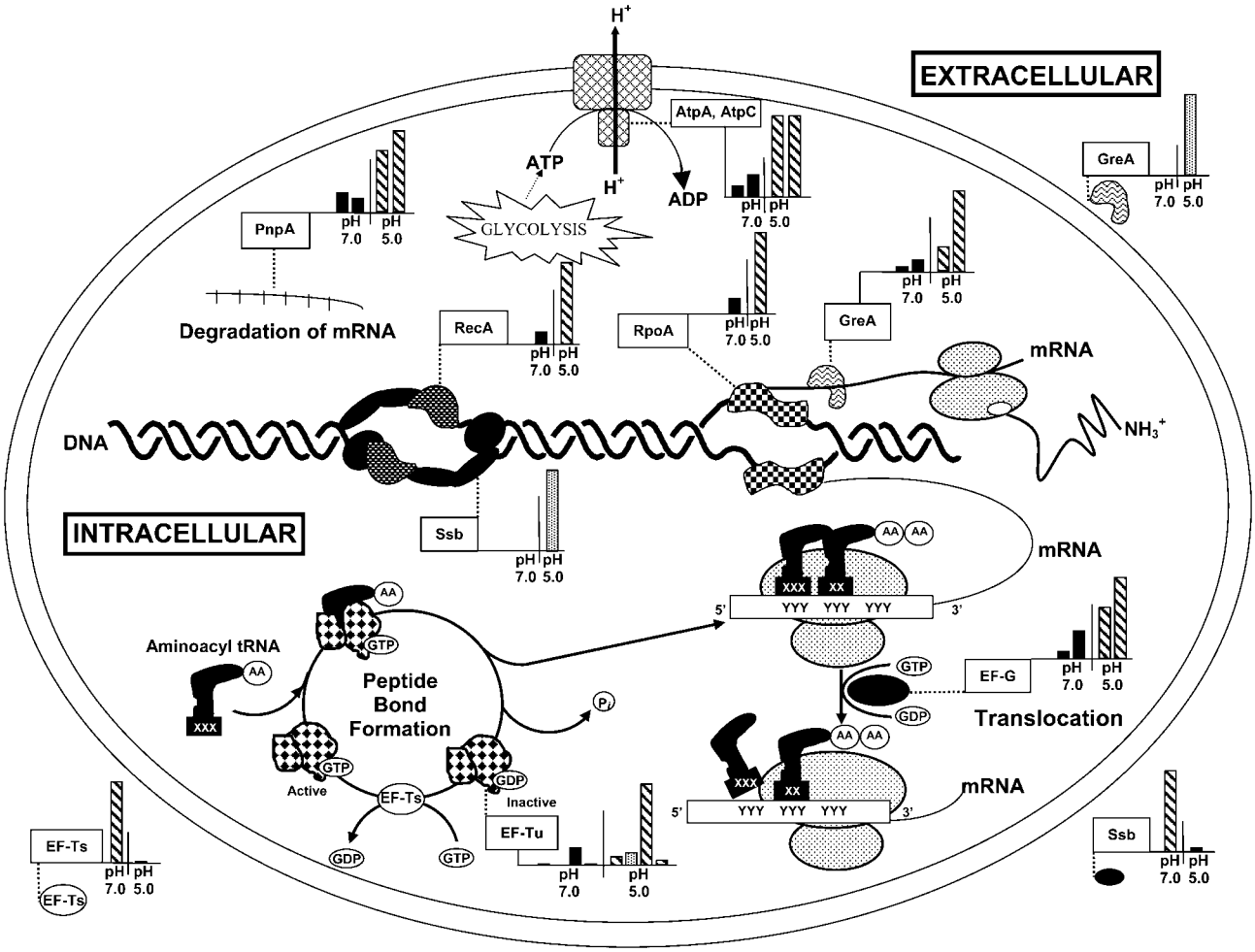

Fig. 1. Differentially expressed *S. mutans* proteins involved in replication, transcription and translation from cells grown at pH 7·0 or at pH 5·0. The columns represent the percentage mean DE values for each charged isoform identified on 2-DGE gels, relative to the most highly expressed isoform. Protein spots were either upregulated (cross-hatching), down-regulated (black), or uniquely expressed (dots), relative to the alternative pH. Truncated forms of proteins are not shown, except for those observed in the extracellular milieu.

differentially expressed protein spots on SyproRuby-stained 2-DGE gels, following acid-tolerant growth of *S. mutans* at pH 5·0. Of these, 167 (including all 44 extracellular protein spots) were identified by MALDI-TOF analysis, and 106 found to be associated with metabolism: glycolysis, alternative acid production and branched-chain amino acid synthesis, in particular (Len *et al.*, 2004). The remaining 61 protein spots were associated with regulatory and/or stress-responsive pathways. These proteins included those involved in DNA replication, transcription, translation, protein folding and proteolysis and are discussed below in light of current knowledge of the possible roles they play in Gram-positive bacteria, particularly oral streptococci.

DNA replication and chromosome integrity

One of the consequences of intracellular acidification is the loss of purines and pyrimidines from DNA, since deoxyribonucleotides are acid labile (Lindahl & Nyberg, 1972). Unrepaired non-instructive DNA damage blocks DNA replication and can be lethal. Aborted replication, however, exposes single-stranded DNA at replication forks and results in the binding of the recombinase RecA. The DNA-bound RecA protein undergoes a conformational transition to its active form, RecA*. In both *Escherichia coli* and *Streptococcus pneumoniae*, RecA* induces an SOS response that can lead to mutation of the DNA or acquisition of pre-evolved functions by horizontal gene transfer (Taddei *et al.*, 1997; Horst *et al.*, 1999; Steffen & Bryant, 2000; Volkert & Landini, 2001; Bjedov *et al.*, 2003; Katz & Bryant, 2003).

In *S. mutans*, RecA was found to be upregulated 6·8-fold when grown at pH 5·0 (Fig. 1, Table 1). The physiological effects of low pH on a RecA-deficient strain of *S. mutans* have previously indicated that RecA is required for survival

Table 1. Differentially expressed cellular stress-related proteins from *S. mutans* grown at pH 7·0 or pH 5·0

Numbers in the Gene ID column are associated with the *S. mutans* genome at the Oral Pathogen Sequence Database site (http://www.stdgen.lanl.gov/oragen).

Gene ID	Protein, EC no. (where applicable) and gene	2-DGE coordinates (pI/M_r)		DE value‡	
		Theoretical	Observed‡	pH 7·0	pH 5·0
Energy metabolism					
ATPase/proton motive force					
SMu1391	ATP synthase, alpha chain (EC 3.6.3.14) (*atpA*)	5·0/49 350	4·87 ± 0·02/53 310 ± 180	1 076 ± 417	8 105 ± 229
SMu1388	ATP synthase, epsilon chain (EC 3.6.3.14) (*atpC*)	6·50/15 882	6·50/15 880§	1 817 ± 567	6 468 ± 549
DNA replication					
DNA replication, restriction, modification, recombination and repair					
SMu1892	Recombinase A (*recA*)	5·0/36 987	5·40 ± 0·00/48 280 ± 480	300 ± 157	2 040 ± 661
SMu0268	DNA-directed DNA polymerase I (EC 2.7.7.7) (*polA*)*	5·3/99 455	4·70 ± 0·00/43 110 ± 370	764 ± 334	0·0
SMu1693	Single-stranded DNA-binding protein (*ssb*)	5·0/18 332	4·93 ± 0·03/17 930 ± 40	0·0	3 773 ± 183
Transcription					
RNA synthesis and modification					
SMu1817	DNA-directed RNA polymerase, alpha subunit (EC 2.7.7.6) (*rpoA*)	3·8/34 571	4·80 ± 0·00/41 340 ± 130	1 753 ± 472	9 428 ± 549
SMu1574	Transcription elongation factor (*greA*)	4·8/17 586	4·80 ± 0·00/15 870 ± 30	2 631 ± 495	19 600 ± 692
SMu1574	Transcription elongation factor (*greA*)	4·8/17 586	4·80 ± 0·00/17 590 ± 0·0	1 137 ± 131	5 775 ± 395
Degradation of RNA					
SMu0138	Polyribonucleotide nucleotidyltransferase, alpha chain (EC 2.7.7.8) (*pnpA*)	5·2/80 055	5·20 ± 0·00/77 000 ± 400	371 ± 57	1 157 ± 215
SMu0138	Polyribonucleotide nucleotidyltransferase, alpha chain (EC 2.7.7.8) (*pnpA*)	5·2/80 055	5·20 ± 0·00/77 200 ± 480	261 ± 132	1 535 ± 64
Translation					
Aminoacyl tRNA synthases					
SMu1441	Threonyl-tRNA synthase 1 (EC 6.1.1.3) (*thrS*)	5·4/74 758	5·33 ± 0·03/73 280 ± 790	41 ± 41	741 ± 81
SMu1441	Threonyl-tRNA synthase 1 (EC 6.1.1.3) (*thrS*)	5·4/74 758	5·40 ± 0·00/74 760 ± 0·0	101 ± 101	1 236 ± 384
SMu0592	Alanyl-tRNA synthase (EC 6.1.1.7) (*alaS*)	5·2/97 162	5·28 ± 0·02/88 230 ± 600	441 ± 147	1 283 ± 363
SMu1904	Arginyl-tRNA synthase (EC 6.1.1 NPT > 19), (*argS*)	6·0/63 574	6·10 ± 0·00/58 590 ± 580	1 715 ± 84	84 ± 84
SMu1374	Phenylalanyl-tRNA synthase, alpha subunit (EC 6.1.1.20) (*pheS*)	6·2/39 296	6·10 ± 0·00/40 200 ± 1 050	137 ± 137	6 936 ± 555
SMu1657	Glutamyl-tRNA (Gln) amidotransferase, B subunit (EC 2.6.−.−) (*gatB*)	5·1/53 501	5·08 ± 0·02/50 250 ± 280	604 ± 123	4 105 ± 127
SMu1658	Glutamyl-tRNA (Gln) amidotransferase, A subunit (*gatA*)	5·2/52 893	5·18 ± 0·03/52 960 ± 360	520 ± 41	2 233 ± 299

Table 1. cont.

Gene ID	Protein, EC no. (where applicable) and gene	2-DGE coordinates (pI/M_r)		DE value‡	
		Theoretical	Observed‡	pH 7·0	pH 5·0
Ribosomal proteins					
SMu1097	30S Ribosomal protein S1 (*rpsA*)	5·0/43 686	5·00 ± 0·00/50 170 ± 250	2 780 ± 72	13 569 ± 233
SMu1097	30S Ribosomal protein S1 (*rpsA*)	5·0/43 686	4·90 ± 0·00/50 250 ± 300	865 ± 381	7 463 ± 2 305
SMu1847	30S Ribosomal protein S2 (*rpsB*)	5·0/29 093	5·00 ± 0·00/33 150 ± 110	412 ± 75	2 240 ± 333
SMu1847	30S Ribosomal protein S2 (*rpsB*)	5·0/29 093	5·10 ± 0·00/33 050 ± 50	1 772 ± 88	8 036 ± 644
SMu1836	30S Ribosomal protein S3 (*rpsC*)	10·0/24 445	10·02 ± 0·20/27 300 ± 2 900	1 593 ± 68	6 411 ± 233
SMu1836	30S Ribosomal protein S3 (*rpsC*)	10·0/24 445	10·00 ± 0·36/27 610 ± 1 780	241 ± 121	3 990 ± 424
SMu1480	50S Ribosomal protein L1 (*rplA*)	9·4/26 674	9·35 ± 0·26/29 770 ± 2 070	1 415 ± 256	5 528 ± 591
SMu1480	50S Ribosomal protein L1 (*rplA*)	9·4/26 674	9·55 ± 0·15/28 190 ± 1850	2 188 ± 91	7 812 ± 833
SMu1480	50S Ribosomal protein L1 (*rplA*)	9·4/26 674	9·25 ± 0·03/30 050 ± 2 040	483 ± 173	2 003 ± 247
SMu0872	50S Ribosomal protein L7/L12 (*rplL*)	4·5/12 413	4·48 ± 0·02/9 580 ± 130	8 173 ± 5016	76 144 ± 7464
Translation elongation factors					
SMu0324	Elongation factor G (EF-G) (*fusA*)	4·8/76 664	4·80 ± 0·00/76 660 ± 0·0	1 141 ± 389	8 076 ± 1212
SMu0324	Elongation factor G (EF-G) (*fusA*)	4·8/76 664	4·80 ± 0·00/76 510 ± 0·0	4402 ± 1 045	12 935 ± 2204
SMu0324	Elongation factor G (EF-G) (*fusA*)*	4·8/76 664	5·00 ± 0·00/25 170 ± 40	925 ± 92	45 ± 17
SMu0651	Elongation factor Tu (EF-Tu) (*tuf*)	4·9/43 919	4·88 ± 0·04/56 820 ± 0·0	1 460 ± 204	10 156 ± 972
SMu0651	Elongation factor Tu (EF-Tu) (*tuf*)	4·9/43 919	4·88 ± 0·04/56 520 ± 0·0	0·0	11 115 ± 1751
SMu0651	Elongation factor Tu (EF-Tu) (*tuf*)	4·9/43 919	4·91 ± 0·04/57 000§	20729 ± 10 281	98 340 ± 4 379
SMu0651	Elongation factor Tu (EF-Tu) (*tuf*)	4·9/43 919	4·98 ± 0·04/57 410 ± 440	1 145 ± 707	5 303 ± 1 400
SMu0651	Elongation factor Tu (EF-Tu) (*tuf*)	4·9/43 919	4·98 ± 0·02/52 630 ± 320	5 189 ± 415	820 ± 169
SMu0651	Elongation factor Tu (EF-Tu) (*tuf*)*	4·9/43 919	4·80 ± 0·00/26 640 ± 200	3 827 ± 48	1 825 ± 161
SMu0651	Elongation factor Tu (EF-Tu) (*tuf*)*	4·9/43 919	5·00 ± 0·00/22 340 ± 40	3 017 ± 172	618 ± 73
SMu0651	Elongation factor Tu (EF-Tu) (*tuf*)*	4·9/43 919	5·10 ± 0·02/20 300 ± 70	2 280 ± 204	572 ± 76
SMu0651	Elongation factor Tu (EF-Tu) (*tuf*)*	4·9/43 919	5·36 ± 0·31/20 360 ± 234	2 625 ± 331	476 ± 55
SMu0651	Elongation factor Tu (EF-Tu) (*tuf*)†	4·9/43 919	4·45 ± 0·05/13 720 ± 170	4 353 ± 331	830 ± 52
SMu0651	Elongation factor Tu (EF-Tu) (*tuf*)†	4·9/43 919	4·54 ± 0·05/12 960 ± 130	1 531 ± 49	57 ± 67
SMu0651	Elongation factor Tu (EF-Tu) (*tuf*)†	4·9/43 919	5·00 ± 0·00/25 170 ± 330	3 868 ± 296	1 240 ± 177
SMu0651	Elongation factor Tu (EF-Tu) (*tuf*)†	4·9/43 919	5·30 ± 0·00/22 260 ± 190	3 247 ± 508	1 099 ± 76
Protein conformation					
Molecular chaperones					
SMu0074	Chaperone protein DnaK (*dnaK*)	4·6/65 286	4·60 ± 0·00/64 840 ± 620	1 204 ± 172	5 529 ± 1 165
SMu0074	Chaperone protein DnaK (*dnaK*)	4·6/65 286	4·60 ± 0·00/65 180 ± 90	576 ± 299	6 277 ± 1 936
SMu0074	Chaperone protein DnaK (*dnaK*)	4·6/65 286	4·60 ± 0·00/65 290 ± 0·0	5 673 ± 248	28 513 ± 3 337
SMu1774	60 kDa Chaperonin (*groEL*)	4·7/57 101	4·70 ± 0·00/58 090 ± 240	535 ± 168	10 692 ± 844
SMu1774	60 kDa Chaperonin (*groEL*)	4·7/57 101	4·70 ± 0·00/57 380 ± 220	6 405 ± 344	44 202 ± 4 753
SMu1774	60 kDa Chaperonin (*groEL*)	4·7/57 101	4·70 ± 0·00/57 100 ± 110	1 217 ± 251	8 576 ± 2 025

Table 1. cont.

Gene ID	Protein, EC no. (where applicable) and gene	2-DGE coordinates (pI/M_r)		DE value‡	
		Theoretical	Observed‡	pH 7·0	pH 5·0
SMu0082	Peptidyl-prolyl isomerase (trigger factor; *ropA*)	4·5/47 486	4·51 ± 0·02/54 560 ± 400	6 653 ± 623	16 183 ± 1 705
Degradation of proteins and peptides					
SMu0587	Oligoendopeptidase B (EC 3.4.24.15) (*pepB*)	5·0/69 724	4·80 ± 0·00/43 090 ± 0·0	5 517 ± 585	1 324 ± 113
SMu0868	ATP-dependent Clp protease, ATP-binding subunit (EC 3.4.21.–) (*ClpL*)	5·0/77 186	4·98 ± 0·05/71 010 ± 50	0·0	3 492 ± 379
SMu1190	Dipeptidase (EC 3.4.13.18) (*pepD*)	5·0/53 037	5·06 ± 0·03/52 120 ± 310	135 ± 9	1 665 ± 191

*C-terminus of protein.
†N-terminus of protein.
‡Mean ± SEM ($n = 3$).
§Part of the complement of random landmark proteins used to align gels and which were used for the calculation of the observed pI and M_r in accordance with the requirement of the *z*3 software.

in cells grown at neutral pH when subjected to a rapid drop in pH, but that cells grown at pH 5·0 can diminish the sensitizing effects of RecA deficiency (Quivey *et al.*, 1995). This physiological observation is a consequence of the induction, in *S. mutans* during growth at pH 5·0, of an abasic site-specific endonuclease activity which apparently acts independently of the RecA protein (Hahn *et al.*, 1999; Quivey *et al.*, 2001) in a similar manner to an error-prone form of DNA polymerase I (PolA) during the SOS response in *E. coli* (Lackey *et al.*, 1985; Wandt *et al.*, 1997). Even though a DNA polymerase I protein spot was identified in the 2-DGE proteome of *S. mutans*, it represented only the C-terminus of the protein, and was absent at pH 5·0 (Table 1).

Along with the enhanced expression of RecA, one of the two single-stranded DNA-binding proteins coded in the *S. mutans* genome, Ssb (Ajdić *et al.*, 2002), was uniquely expressed by growth of *S. mutans* at pH 5·0 (Fig. 1, Table 1). Both RecA and Ssb are essential for homologous genetic recombination as well as recombinational rescue and DNA repair of chromosomal replication. To the best of our knowledge, the only studies of this protein in streptococci relate to *S. pneumoniae* (Steffen *et al.*, 2002; Katz & Bryant, 2003), making this the first report of a role for Ssb in the acid tolerance of *S. mutans*. From the different roles for recombinational repair and the SOS response currently recognized in Gram-negative and Gram-positive bacteria, and the lack of information available on streptococci, it is clear that further research is needed to understand these processes in the acid-tolerant growth of *S. mutans*.

RNA synthesis and degradation

Under imposed stress conditions, it is self-evident that the DNA repair and protection responses needed for the survival of the cell require the transcription of appropriate genes. While the nature of these events has been well documented in *E. coli*, little is known of the mechanism(s) coordinating these events in streptococci, particularly oral streptococci (Volkert & Landini, 2001). Acid tolerance in *S. mutans*, however, led to upregulation of the transcription proteins, DNA-directed RNA polymerase α subunit, RpoA, and two isoforms of the transcription elongation factor (cleavage stimulatory factor) GreA, by 5·4-, 7·5- and 5·1-fold, respectively (Fig. 1, Table 1). In addition, one isoform of GreA was found to be uniquely expressed in the extracellular milieu at pH 5·0 (Fig. 1, Table 2). RNA polymerase forms an elongation complex with its template DNA and the nascent RNA product. While this complex is completely processive, it is responsive both to extrinsic regulatory factors and to intrinsic signals associated with the DNA and RNA that can alter the rate of elongation and lead to a transient pause or arrest of the complex (Nakasone *et al.*, 1998; Erie, 2002). This may be as simple as a lack of nucleotide substrates: a situation that may readily occur in *S. mutans* under acidic conditions, due to the disturbance of anabolic functions (Len *et al.*, 2004). The main function of GreA is to reactivate RNA polymerase once such a halt has occurred. This is achieved by enhancing the intrinsic

Table 2. Differentially expressed extracellular stress-related proteins from *S. mutans* grown at pH 7·0 or pH 5·0

Gene ID	Identification (gene)	2-DGE coordinates (pI/M_r)		DE value*	
		Theoretical	Observed*	pH 7·0	pH 5·0
Replication					
DNA replication and repair					
SMu1693	Single-stranded DNA-binding protein (*ssb*)	5·0/18 332	5·00 ± 0·00/17 120 ± 5	401 ± 95	18 ± 4
Transcription					
Transcription factors					
SMu1574	Transcription elongation factor (*greA*)	4·8/17 586	4·95 ± 0·04/18 640 ± 1 680	0·0	196 ± 43
Translation					
Translation factors					
SMu1846	Elongation factor Ts (*eftS*)	4·9/37 719	4·92 ± 0·04/41 650 ± 530	1 594 ± 644	12 ± 10
Ribosomal proteins					
SMu1097	30S Ribosomal protein S1 (*rpsA*)	5·0/43 686	4·88 ± 0·06/47 100 ± 500	1 162 ± 196	0·0
SMu0869	50S Ribosomal protein L10 (*rplJ*)	4·9/17 667	4·70 ± 0·00/14 960 ± 100	930 ± 1 030	11 ± 1
SMu0869	50S Ribosomal protein L10 (*rplJ*)	4·9/17 667	4·80 ± 0·00/10 300 ± 90	2 137 ± 273	81 ± 1
Cellular processes					
Molecular chaperones					
SMu0074	Chaperone protein DnaK (*dnaK*)	4·6/65 286	4·60 ± 0·00/65 290 ± 0	2 882 ± 739	85 ± 6
SMu0082	Peptidyl-prolyl isomerase (trigger factor; *ropA*)	4·5/47 486	4·47 ± 0·02/56 130 ± 340	1 787 ± 98	6 ± 6

*Mean ± SEM (n=3).

cleavage activity of RNA polymerase, thus releasing RNA from the elongation complex, and preventing backward translocation and hydrolysis of the RNA (Fish & Kane, 2002; Opalka *et al.*, 2003). Other than in the current study, elevated levels of GreA have been noted in the Gram-positive bacterium *Staphylococcus aureus*, when challenged with oxacillin (Singh *et al.*, 2001).

Two isoforms of the RNA-degrading enzyme polynucleotide phosphorylase [polyribonucleotide nucleotidyltransferase (PNPase; PnpA)] were also upregulated by at least 3·1-fold in *S. mutans* in acid-tolerant growth. (Fig. 1, Table 1). In *E. coli*, two 3′– 5′ exonucleases, RNase II and PNPase, are involved in mRNA decay, since the loss of both activities results in the cessation of growth (Donovan & Kushner, 1986). Recent analysis of RNA decay in *E. coli* (Mohanty & Kushner, 2003) has shown that even though RNase II constitutes about 90 % of the exonucleolytic activity in the cell (Deutscher & Reuven, 1991), PNPase plays a greater role in the degradation of mRNA, since it forms part of a multi-protein complex, the 'degradosome', that contains an RhlB RNA helicase capable of removing secondary structure that would impede PNPase activity (Mohanty & Kushner, 2003).

While the level of understanding of RNA decay in Gram-positive bacteria is not as advanced as that of Gram-negative bacteria, *in vitro* RNA decay does not appear to be as severely compromised by the absence of a PNPase activity (Wang & Bechhofer, 1996). In *Bacillus subtilis*, however, a *pnpA* deletion mutant shows pleiotrophic effects, including a cold-sensitive-growth phenotype, sensitivity to growth in the presence of tetracycline and multiseptate, filamentous growth (Wang & Bechhofer, 1996). It has been hypothesized that defective processing of specific RNAs in the *pnpA* mutant of *B. subtilis* results in these phenotypes, though there is no direct evidence in support of this contention (Wang & Bechhofer, 1996). To date, no studies of the role of PNPase in oral streptococci appear to have been undertaken, though a 1·9-fold down-regulation of the enzyme has been observed in *Streptococcus oralis* when inoculated and grown in batch culture under aerobic conditions at low pH (Wilkins *et al.*, 2001). Our observation of an increase in PNPase is clearly at odds with this observation. Whether the aerobic conditions used to cultivate *S. oralis* engender an additional oxidative stress that further influences PNPase expression requires investigation.

Translation

The incorporation of correctly encoded amino acids into proteins depends on the attachment of each amino acid to an appropriate tRNA by aminoacyl tRNA synthases (Cooper, 2000). Acid tolerance in *S. mutans* resulted in the upregulation of phenylalanyl- (PheS), alanyl- (AlaS) and two isoforms of threonyl- (ThsS) tRNA synthases, as well as subunits A (GatA) and B (GatB) of glutamyl-tRNA amidotransferase, which is required for the transamidation of misacylated Glu–$tRNA^{Gln}$ to form Gln–$tRNA^{Gln}$ in all Gram-positive bacteria (Curnow *et al.*, 1997; Harpel *et al.*, 2002; Table 1). In contrast, arginyl-tRNA synthase (ArgS)

was down-regulated 20-fold by growth at pH 5·0 (Table 1). The other four aminoacyl tRNA synthases previously identified (Len *et al.*, 2003) did not show any differential expression at pH 5·0 (data not shown).

In *S. mutans*, the 50S and 30S ribosomal subunits are composed of 51 proteins (Ajdić *et al.*, 2002). Of these, five were identified as being upregulated by growth at pH 5·0, four of which existed in more than one charged isogenic form and possessed an observed M_r higher than that predicted from their gene sequence (Table 1). While 16 ribosomal proteins have previously been mapped on 2-DGE gels (Len *et al.*, 2003), the inability to detect all 51 proteins is most likely due to their low M_r and/or very basic pI ($>10{\cdot}5$), which place them at the limit of the resolving power of current 2-DGE technology. Our data imply that the number of ribosomes may increase by a factor of four in a low-pH environment – a suggestion that will require independent confirmation. Protein S1 and the ribosomal protein L10 were also found in the culture medium, but at significant levels at pH 7·0 only (Table 2).

In translation, three elongation factors, EF-Tu, EF-Ts and EF-G, are responsible for escorting aminoacyl tRNAs to the ribosome and for translocation of the ribosome along the mRNA (Fig. 1; Cooper, 2000). A fourfold increase in the expression of the four charged isogenic forms of EF-Tu was observed by growing *S. mutans* at pH 5·0. In each case, the observed M_r of the protein was greater than that predicted from the gene sequence (Table 1). Two charged isogenic forms of EF-G were also upregulated at pH 5·0. Most notable, however, was the finding that the reduced amounts of multiple truncated forms of EF-Tu and a C-terminal fragment of EF-G in cells grown at pH 5·0 could be measured at all, as this meant that each protein spot had the same 2-DGE coordinates, irrespective of the growth pH (Table 1). This suggested that specific cleavage events had occurred, rather than random proteolysis. A similar observation has been made with *Salmonella enterica* serovar Typhimurium, where two specific proteinases are believed to be responsible for forming elongation factor artefacts (Adams *et al.*, 1999). Even if this were the case with *S. mutans*, the data in Table 1 indicate that the cumulative level of both EF-Tu and EF-G would be at least threefold higher during acid-tolerant growth at pH 5·0. Interestingly, no change in the third elongation factor, EF-Ts, was observed, except in the extracellular milieu, where its level was substantially upregulated at pH 7·0 (Fig. 1, Table 2).

The increase in EF-Tu and EF-G during acid-tolerant growth is also of interest from another standpoint, since it has recently been shown that both proteins behave like chaperones towards unfolded and denatured proteins in *E. coli* (Kudlicki *et al.*, 1997; Caldas *et al.*, 1998, 2000). EF-Tu, for example, recognizes the same hydrophobic binding motifs in proteins as the chaperone DnaK (see below; Malki *et al.*, 2002). Furthermore, EF-Ts has been shown to act as a folding template in a chaperone-like manner towards its substrate protein, EF-Tu (Krab *et al.*, 2001).

Molecular chaperones and degradation of misfolded proteins

Early pulse–chase experiments demonstrated an increase in DnaK levels during thermal stress in *S. mutans*, thus confirming the existence of a functional heat-shock response-system in this species (Jayaraman & Burne, 1995). The DnaK chaperone machinery prevents the misfolding and aggregation of ribosome-bound polypeptides (Szabo *et al.*, 1994; Rudiger *et al.*, 1997; Bukau & Horwich, 1998; Agashe & Hartl, 2000). In the current study, proteome analysis identified three isoforms of DnaK in *S. mutans*, which were upregulated 4·6-, 10·9- and 5·0-fold, respectively, at pH 5·0 (Fig. 2, Table 1). This confirmed a similar observation made by Jayaraman *et al.* (1997), who used the same values of D and pH to culture *S. mutans*.

It is now clear that the function of DnaK overlaps with that of another component, trigger factor, RopA (Deuerling *et al.*, 1999; Teter *et al.*, 1999). RopA is a major ATP-independent molecular chaperone with prolyl isomerase activity, which binds to the large ribosomal subunit proteins L23 and L29 near the polypeptide exit site and interacts with nascent polypeptide chains prior to DnaK (Hesterkamp *et al.*, 1996; Kramer *et al.*, 2002). A 2·4-fold increase in RopA during steady-state growth at pH 5·0 was observed in *S. mutans* (Fig. 2, Table 1). Other molecular chaperones, such as the bacterial group I chaperonin, GroEL, are indispensable for cell viability (Fayet *et al.*, 1989; Kubota *et al.*, 1995). GroEL is able to capture and refold non-native substrate proteins up to 50–60 kDa, while protecting them from aggregation with other non-native proteins (Braig *et al.*, 1994; Mayhew *et al.*, 1996; Weissman *et al.*, 1996; Xu *et al.*, 1997). Proteome analysis showed the existence of three isoforms of GroEL in *S. mutans*, which were enhanced 20·0-, 6·9- and 7·0-fold, respectively, by growth in an acidic environment (Fig. 2, Table 1). While similar results have been reported for a 60 kDa chaperonin (most probably GroEL) in aerobic batch cultures of *S. mutans* and *S. oralis* (Wilkins *et al.*, 2001, 2002), the current results differed from those previously reported in continuous culture (Lemos *et al.*, 2001). In the previous study, *S. mutans groEL* mRNA was induced 2·5-fold by an acid shock from pH 7·0 to pH 5·0, but no significant differences in the levels of *groEL* mRNA or GroEL (determined by Western blot analysis) were observed, once steady state was achieved at $D=0{\cdot}1\ h^{-1}$ (Lemos *et al.*, 2001).

DnaK and RopA were also identified in the *S. mutans* culture fluid. Both proteins were down-regulated, by factors of 34 and 313, respectively, at pH 5·0 (Fig. 2, Table 2). These levels of DnaK and RopA were equivalent to 39 % and 36 %, respectively, of the steady-state cellular levels at pH 7·0, but were equivalent to less than 0·2 % of the steady-state cellular levels at pH 5·0, implying a loss (or secretion) rate of approximately $4{\cdot}0\ \%\ h^{-1}$ of the steady-state cellular levels at pH 7·0, but a negligible rate of loss at pH 5·0 (Jacques *et al.*, 1985). Although previous studies have not considered the extracellular milieu as a source of these proteins

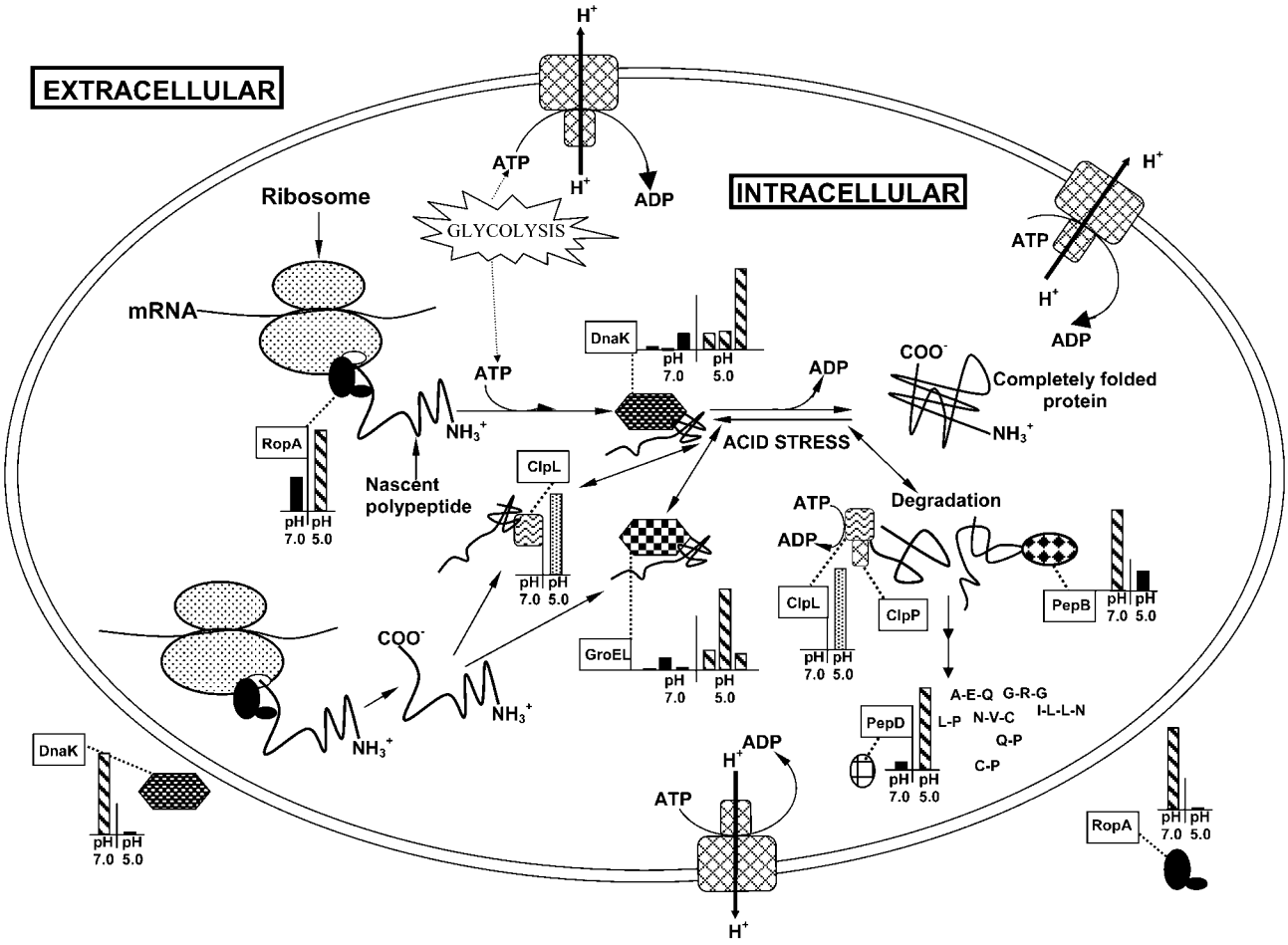

Fig. 2. Differentially expressed *S. mutans* molecular chaperones and proteinases from cells grown at pH 7·0 or at pH 5·0. The columns represent the percentage mean DE values for each charged isoform identified on 2-DGE gels, relative to the most highly expressed isoform in a given compartment. Protein spots were either upregulated (cross-hatching), downregulated (black), or uniquely expressed (dots), relative to the alternative pH. Truncated forms of proteins are not shown, except for those observed in the extracellular milieu.

(Lemos *et al.*, 2001; Wilkins *et al.*, 2001, 2002), DnaK has recently been identified on the surface of both *Streptococcus agalactiae* (Hughes *et al.*, 2002) and *Haemophilus influenzae* (Hartmann *et al.*, 2001), and is known to be highly immunogenic in *S. pneumoniae* (Hamel *et al.*, 1997). Whether *S. mutans* has surface-bound molecular chaperones is a matter of conjecture, as our technique of protein extraction did not discriminate between the various cellular compartments. Irrespective of this, the increase in the steady-state cellular levels of DnaK, RopA and GroEL in *S. mutans* at pH 5·0 is consistent with a need for an enhanced complement of molecular chaperones to counteract the denaturing properties of an acidic cytosol when cytosolic pH falls below 6·5 (Dashper & Reynolds, 1992).

Proteins which cannot be folded by molecular chaperones may be targeted for degradation, in order to recycle amino acids for *de novo* protein synthesis (Jenal & Hengge-Aronis, 2003). Among the proteins that can carry out such functions are the ATP-dependent proteases of the Clp family, which possess a dual chaperone/proteinase role. Proteolysis by Clp requires a serine type peptidase, the ClpP subunit, and a regulatory ATPase subunit consisting of several orthologues (Gottesman *et al.*, 1997; Porankiewicz *et al.*, 1999; Lemos & Burne, 2002). In *S. mutans*, only one ATP-binding subunit, ClpL, was expressed, and solely in growth at pH 5·0 (Fig. 2, Table 1). ClpL homologues appear to be specific to Gram-positive bacteria, as they have not been found in Gram-negative bacteria (Lemos & Burne, 2002; Kwon *et al.*, 2003). To date, information on the role of ClpL is limited to a single study of the heat-shock response in *S. pneumoniae*, in which mutations in the *clpL* and *clpP* genes were found to modulate virulence-gene expression, and purified recombinant ClpL was shown to possess molecular chaperone properties (Kwon *et al.*, 2003). Our data suggest that ClpL is upregulated and maintained under low pH conditions, with

the implication that it plays a vital role in pH tolerance. It remains to be seen whether ClpL operates solely as a molecular chaperone, or whether it interacts with ClpP to initiate proteolysis.

Two other differentially expressed proteinases were identified by MALDI-TOF, both belonging to the *pep* gene family of proteinases, for which 13 different genes exist in the *S. mutans* genome (Ajdić *et al.*, 2002). One of these proteinases, a truncated version of a putative PepB, was down-regulated by a factor of 4·2 in growth at pH 5·0 (Fig. 2, Table 1). In Group B streptococci, PepB has oligopeptidase activity and has been shown to degrade a variety of small bioactive peptides, as well as the synthetic collagen-like substrate *N*-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala *in vitro* (Lin *et al.*, 1996). The second proteinase was homologous to the dipeptidase PepD of *Lactobacillus helveticus* (Ajdić *et al.*, 2002). This enzyme does not appear to be similar to the cytoplasmic PepD isolated from *S. mutans* and *S. sanguis* which catalyses the hydrolysis of X-Pro dipeptides (Cowman & Baron, 1997), since the *Lactobacillus* enzyme is not able to hydrolyse di- and tripeptides containing proline (Vesanto *et al.*, 1996). PepD was upregulated 12·3-fold at pH 5·0 (Fig. 2, Table 1).

It is perhaps pertinent to note that proteolysis has another role additional to the recycling of amino acids for *de novo* protein synthesis, and that is to regulate cellular events by degrading regulatory proteins, thereby controlling the response of the cell to an imposed stress. A readjustment in the composition of the cellular proteolytic machinery would therefore most likely have pleiotropic consequences for the cell, as any change in the nature or complement of specific proteinases would be expected to affect proteins that are subject to regulation by proteolytic events (Jenal & Hengge-Aronis, 2003).

Concluding remarks

This study has made use of the steady-state conditions enabled by anaerobic continuous culture in a chemostat to study alterations in the stress-response proteome of *S. mutans*, following adaptation and tolerance to growth at low pH. The proteome literature relating to acid adaptation and tolerance in oral streptococci contains disparate findings. For instance, proteome analysis of *S. mutans* during acid adaptation, using an aerobic batch-culture model without pH control, identified eight of the 28 differentially expressed proteins found in the current study. The levels of six of these were down-regulated by an average of 2·6-fold, while DNA-directed RNA polymerase was down-regulated 33-fold. Only a 60-kDa chaperonin was upregulated to a similar extent to that observed with GroEL in the current study. It is difficult to assess whether the aerobic conditions, the use of a different culture medium and/or the dissimilar generation times of 1·0 h and 6·6 h, respectively, for the control and the experimental batch cultures influenced the outcome (Wilkins *et al.*, 2002). Since an arbitrary ratio of the levels of expression has been evaluated in both studies, it may be that, in batch culture, the control bacteria are adapting to a fall in the extracellular pH from a starting value of 7·0 to 6·2 at harvest (heading for a final of pH 5·3 at stationary phase) by initially overexpressing stress-related proteins. This would contrast with the steady-state levels measured in a chemostat, once *S. mutans* had adapted to the prevailing pH conditions. Such an overshoot in enzyme levels as an initial response to change has been noted previously, albeit in continuous culture (Carlsson & Elander, 1973; Koplove & Cooney, 1978), and if occurring in batch culture would explain the apparent reduction in the ratio between control and experimental protein values observed. Whatever the reason for the disparity, one must conclude that the two models are reflecting dissimilar events.

Although a number of stress-related proteins, in both Gram-positive and Gram-negative bacteria, are either well characterized or the subject of concerted ongoing study, others are not. This study has shown the involvement of at least three of these proteins in the acid-tolerant growth of *S. mutans*: the transcription elongation factor, GreA; the ATPase protease, ClpL; and the single-stranded DNA-binding protein, Ssb. The role of each of these proteins warrants further examination in light of the paucity of information regarding their mode of action. The subtle differences in homologous recombinational repair recently observed between *S. pneumoniae* and *E. coli*, along with the apparent differences in the complement of genes associated with the SOS-induced response in Gram-positive and Gram-negative bacteria (Steffen *et al.*, 2002; Katz & Bryant, 2003), emphasize this point, particularly as the regulons involved do not appear to have been studied in relation to acid tolerance or other stress responses in oral streptococci such as *S. mutans*.

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