Understanding the Starvation Adaptation of *Lactobacillus casei* through Proteomics

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ABSTRACT— ***Food microbes are exposed to several stress conditions in natural environments, which can have an effect on performances. Lactobacilli are highly adaptive group of food microbes which are able to survive in various environmental niches, including ones where preferred nutrients are deficient. This study was conducted to investigate the adaptation to lactose starvation in Lactobacillus casei using comparative proteomics. One-dimensional sodium dodecyl sulphate-polyacrylamide (1-D SDS PAGE) and two-dimensional electrophoresis (2-DE) were performed on L. casei cells cultivated in a semi-defined medium, with different initial levels of lactose, up to 8 days. Clear visual changes in the 1-D SDS PAGE profiles were seen for cells cultured in 0% lactose. The relative expression of xylulose-5-phosphate phosphoketolase, elongation factor G and DnaK increased in lactose starved cells during stationary phase when compared to the temporal expression of these proteins in cytosolic fraction of cells cultured in 0.2 or 1% lactose. Comparative spot analysis of 2-DE gels showed that 13 proteins were over expressed in lactose starved cells (0% lactose). Of these up-regulated proteins, nine were identified by MALDI-TOF/TOF mass spectrometry with functionalities in protein synthesis, general stress responses and carbohydrate metabolism, where enzymes involved in glycolysis, pyruvate metabolism and the pentose phosphate pathway were up-regulated. These results suggested that proteomic analysis can provide useful information on adaptation of lactobacilli. Identification of specific protein markers that involve in adaptation to a specific stress factor would be help in selecting strains with better performance in a given application of these beneficial food microbes.***

Keywords— Lactobacilli; Proteomics; Starvation; Adaptation; Metabolic enzymes

1. INTRODUCTION

Nutrient depleted environment is one of the conditions generally resulted by own activities of microbes. It is very common for food microbes to face starvation in their natural habitats. For example, the cheese ripening environment is likely to be challenging for microbial growth and survival due to poor availability of growth substrates, variable oxygenation, presence of growth-inhibitory concentrations of salt and pH plus temperatures that are well below optimum for growth [12]. It is well-documented fact that lactose is not the primary carbon and energy source for growth and metabolism during cheese ripening, given that lactose, glucose and galactose are rapidly removed by the starter and nonstarter lactic acid bacteria (NSLAB) soon after manufacture [39, 66]. Still NSLAB are able to survive and grow in number in the cheese matrix under nutrient deficient conditions during ripening.

Lactobacillus species have been found as dominant NSLAB population in cheeses made in different countries [1]. In Australian Cheddar, the dominant NSLAB species isolated were *Lactobacillus casei* or *L. paracasei* [8]. NSLAB therefore have developed, during their evolution in milk and other dairy products, a complex network of stress response pathways that promote their survival when facing such environmental challenges, allowing adaptation to the changing environment during cheese maturation. However, factors that influence the growth and dominance of strains within the cheese matrix are poorly understood.

Several investigations describing the physiological stress responses in NSLAB, particularly species in the genus *Lactobacillus*, have emerged. These reports include barotolerance in *L. sanfrancescensis* DSM [54], *L. plantarum* and *L. brevis* [38]; acid stress responses in *L. casei* [25, 72], *L. sanfrancescensis* CB1 [10], *L. acidophilus* CRL 639 [35, 36], *L.*

delbrueckii subsp. *bulgaricus* [13, 57]; heat shock responses in *L. plantarum* [11, 59]; bile tolerance in *L. casei*, [65], *L. plantarum* [6]; cold shock in *L. casei* [52]; oxidative stress in *L. plantarum* [55]; osmotic stress in *L. casei* [45]; and changes in expression of S-layer proteins in *L. acidophilus* in response to several stimuli related to growth phase and media composition [11, 14, 53] or aeration conditions in *L. brevis* [29]. Synthesis of 16 proteins in *L. acidophilus* induced as a result of starvation was reported [38]. Several of these proteins were induced by stationary phase or were attributed to low pH [12]. It is well documented in many species that stress induces the synthesis of generalized stress proteins, including molecular chaperones and proteases [17, 20], and that one form of stress can lead to cross-protection to other stress factors [12]. In contrast, relatively few reports address starvation stress responses in *L. casei* despite the continuing speculation on the nature and source of carbohydrates, or other nutrients, that sustain growth and survival during extended periods of cheese maturation [1].

The present study investigated the proteomics of transition into carbohydrate starvation in an Australian Cheddar cheese isolate, *L. casei* GCRL163. While not attempting to simulate a cheese matrix, this study is the first attempt to approach stationary phase adaptation of NSLAB experimentally using a chemically defined medium (called as S3 broth) where lactose concentration and medium pH were controlled [26] and focuses on the analysis of differentially expressed cytosolic proteins in lactose starved cells through comparison of two-dimensional electrophoresis (2-DE) profiles and identification of presumptive protein function using MALDI-TOF/TOF mass spectrometry.

2. EXPERIMENTAL DESIGN AND METHODS

2.1 Bacterial strain

The bacterial strain *L. casei* GCRL163 used in this study was previously isolated from Australian Cheddar cheese and identified by 16S rDNA sequencing [8] and maintained in 40% glycerol in MRS (Oxoid, Australia) broth. The stock cultures were maintained in cryogenic storage at −80 °C in glycerol storage broth and working stocks at –20 ^{o}C. The inoculum preparation and a semi-synthetic liquid medium (S3 broth) were described previously by [28]. When used, lactose was added at 0.2 or 1% (w/v) from a filter-sterilized 20% stock solution.

2.2 Starvation adaptation and harvesting of cells

Strain *L. casei* GCRL163 was grown in S3 broth containing different levels of lactose: S3-0%, no lactose; S3-0.2% lactose, which was growth-limiting; and S3-1% lactose. Cells were initially cultured in MRS broth overnight at 30g^{o}C under anaerobic conditions (Oxoid anaerobic jars system) then cells were harvested by centrifugation, washed twice in S3-0% broth before suspending in the same medium (S3-0%) to give an OD_{600} of ~0.5. After incubation for 24 h at 30 ^{o}C, cells were harvested by centrifugation, washed twice in S3-0% broth and sub-cultured in the different S3 broths to give a starting OD_{600} of 0.01 (between 10^6 and 10^7 colony forming units, cfu ml^{-1}).

Cultures were set up in multiple replicates of 1,800 ml in 2 l Schott bottles to provide sufficient volume of cells for later analysis by 2DE. Anaerobic conditions were maintained initially and throughout incubation at 30 ^{o}C for up to 8 days by sparging the headspace with filtered (0.22 µm Millipore filters used in-line) oxygen-free nitrogen (Linde, Melbourne, Australia). Samples (50 to 1,500 ml, depending on the measured OD_{600}) were collected after 1, 2, 4 and 8 days. Cells were collected by centrifugation (12,000 x *g* for 10 min at 4 °C), washed (10 ml of sterile 40 mM Tris-buffer, pH 7.0), and resuspended in 40 mM Tris-buffer to achieve a final OD_{600} of 20, then stored at -80 ^{o}C until protein extraction. Each growth condition was tested in duplicate within each experiment and each set of experimental conditions was tested at least twice.

2.3 Viable cell count and growth

Cell viability was measured by determining cfu ml^{-1} and viable counts were performed in triplicate by plating 0.1 ml of serial dilutions in MRS broth onto MRS plates. Colonies were enumerated after 72 h at 30^{o}C; replicates were typically within 10% variance. OD_{600} was measured to monitor the growth of cells.

2.4 1-D PAGE analysis

1-D SDS-PAGE was performed as described by Laemmli [33]. To determine the protein profile of whole cells, which would include cell surface, membrane and cytosolic proteins, 200 µl of cell suspension was added to 0.5 g of beads (0.1 mm diameter) in a 2 ml-capacity screw-top plastic tube and a Mini Bead Beater-8 (Biospecs Products, Bartlesville, OK, USA) was used to lyse the cells using three bursts of 90, 60 and 60 s. Samples were cooled on ice for 5 min between each

burst. After bead beating, 200 µl of double strength loading buffer was added and mixed well by vortexing for 30 s. The suspension was boiled for 10 min, cooled on ice and centrifuged (4,000 rpm, 10 min, 4°C) to settle glass beads and unbroken cells and 30 µl of each sample loaded onto a SDS-PAGE gel (12%). Electrophoresis was performed at 150 V (constant voltage). After electrophoresis, proteins were visualized using Coomassie blue (Brilliant blue R 250, Sigma) stain [42]. The BenchMark™ Protein Ladder (Invitrogen), 10 to 220 kDa, molecular weight range was used as marker. Cytosolic proteins preparation and 1D SDS-PAGE method was described elsewhere [27].

2.5 2-DE, spot analysis and MALDI-TOF/TOF analysis

2-DE coupled with MALDI-TOF/TOF mass spectrometry analysis was used to identify deferentially expressed proteins in cells exposed to various starvation conditions. Briefly, isoelectric focusing (150 µg of protein in sample, pH 4-7, 24 cm IPG dry strips) was performed using the Ettan IPGphor electrophoresis system (GE Healthcare) and 12% SDS-PAGE large format gels were used to separate the proteins. Gels were stained in 1 µM ruthenium II tris bathophenanthroline disulphonate (RuBPS) according to protocols described previously [47] and imaged using a cooled scanning CCD camera (ProXPRESS, Perkin Elmer Life Sciences).

Protein spot analysis was performed on a data set that contained nine large-format 2DE gels (3 growth conditions with varying amounts of lactose x 3 replicates) using Progenesis PG 240 v2006 software (Non-linear Dynamics, UK). Data reported here are protein spots that are differentially expressed in cytosolic fractions of cells cultured in 0%, 0.2% and 1.0% lactose for 8 days. A Pro-Pick Investigator Robot (Genomic Solutions, USA) was used to excise the target bands. MALDI-TOF/TOF mass spectrometry was used for protein identification [56]. An identity was assigned on the basis of significant match of peptide sequence query from Mascot database search [43]. Protein identity was only reported for samples that gave a significant ($P<0.05$) MOWSE score.

Statistical analysis was performed on triplicate set of 2DE gels using the ANOVA function of Progenesis PG 240 v2006 software and differences were considered significant at *P value* < 0.05. In the ANOVA test lactose level was treated as the independent variable.

3. RESULTS AND DISCUSSIONS

3.1 Viable cell counts in S3 broth with different levels of lactose

Following initial culture in MRS broth and pre-incubation in S3-0% lactose, *L. casei* GCRL163 was sub-cultured into S3 broth containing 0, 0.2 or 1% lactose with initial cell densities of ~ 5 x 10^6 cfu ml^{-1} and growth monitored into stationary phase by viable count measurement (Figure 1). These conditions were selected to eliminate carry-over of

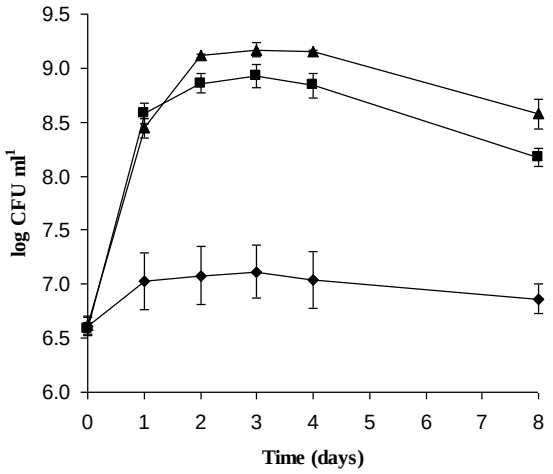

Figure 1: Growth of *Lb. casei* GCRL163 in S3 broth. This figure shows viable count (cfu ml^{-1}) in S3 broth supplemented with 0% (♦), 0.2% (■) or 1% (▲) lactose over 8 days incubation anaerobically at 30 °C. The data represents the mean of triplicate counts.

nutrients from MRS starter cultures and would enable observation of proteins expressed when cells either continued in a starvation environment or readapted to growth in a carbohydrate-limited (0.2% lactose) or non-limited (1% lactose) environment. For all three conditions tested, cells entered stationary phase within 24 to 48 h of inoculation depending on the broth used and the maximum viable count was dependent on the amount of lactose supplied [28]. The pH of the medium remained at 5-5.5 throughout the period of incubation in all media.

3.2 Changes in 1-D SDS-PAGE profiles

The protein profiles of *L. casei* GCRL163 grown in the presence of different concentrations of lactose were analyzed by 1-D SDS-PAGE for both whole cell proteins and the soluble proteins fraction obtained after lysis by bead beating (Figure 2). Analysis of both types of samples provided some indication about the sub-cellular location of differentially expressed proteins within the cytosol or at the cell surface. The protein banding patterns observed for whole cells (Figure 2A) and soluble fractions (Figure 2B) were generally similar. However, there were obvious differences in the relative level of expression of specific proteins seen in both whole cells and the soluble fraction, particularly in the molecular weight ranges between 50 and 90 kDa, and in the 15-20 and 30-40 kDa regions for the soluble fractions for cells cultured in S3-0% lactose.

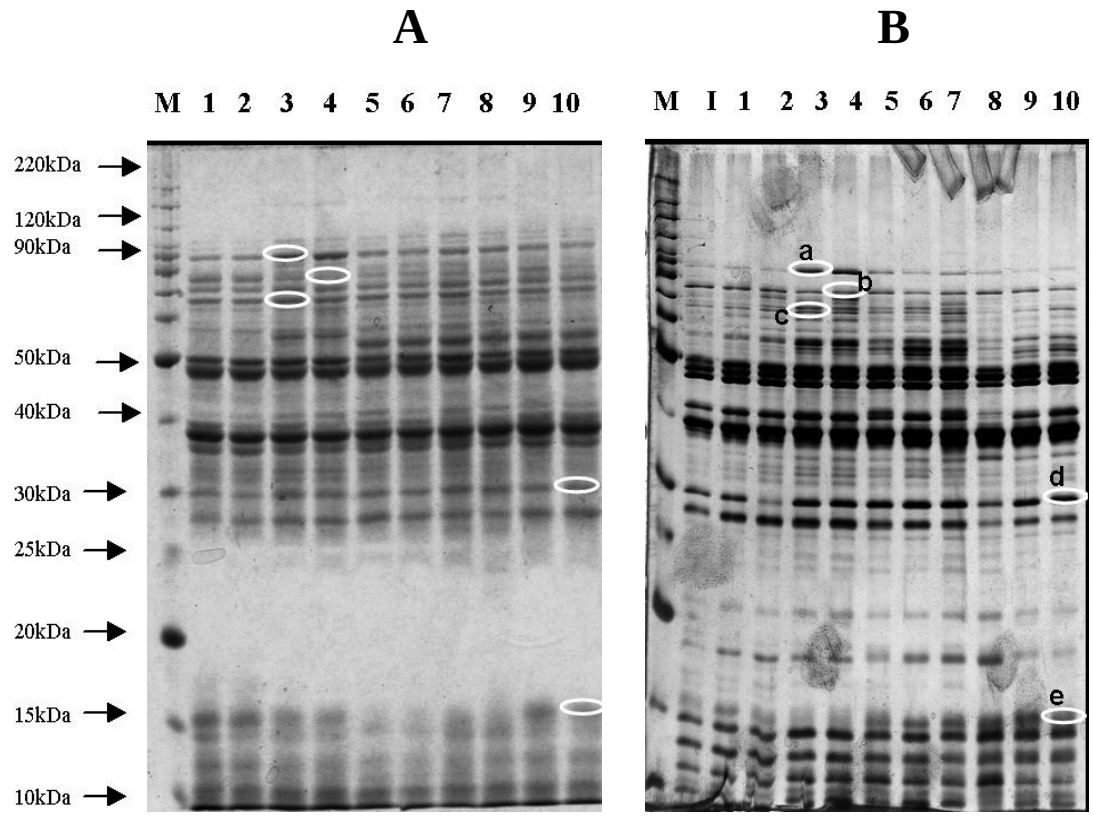

Figure 2: 1D SDS-PAGE (12% acrylamide) protein profiles of whole cells (A) and the soluble fraction obtained following cell lysis by bead-beating and centrifugation (B) of *L. casei* GCRL163. The inoculum (lane I) was grown in MRS broth then sub-cultured into S3 broth buffered at pH 6.4 with 0.35M phosphate buffer for 24 hours at 30 ^{o}C (lane 1) before further sub-culturing in buffered S3 broth with different concentrations of lactose (see Fig. 1). Cells were collected after 2, 4 and 8 days for each growth condition: 0% lactose (lanes 2-4), 0.2% lactose (lanes 5-7)) and 1% lactose (lanes 8-10). Marked bands were excised for analysis by MALDI-TOF/TOF mass spectrometry. Lane M contained the molecular weight markers.

MALDI-TOF/TOF analysis was carried out for the five bands that showed clear visual differential expression between culture conditions and where the bands were sufficiently resolved to allow analysis, an approach that has been used by other authors for bands on 1-D SDS-PAGE gels [6]. The proteins identified were: (a) xylulose-5-phosphate phosphoketolase (88,645 Da), which was strongly and increasingly up-regulated in stationary phase only in S3-0% lactose, although expression of this protein was detected in both S3-0.2% and -1% lactose; (b) elongation factor G (76,495 Da), which was differentially over-expressed in cells grown in S3-0% lactose and clearly detected in the day 2 soluble fraction sample; and (c) molecular chaperone (DnaK homologue) (67,523 Da), which was up-regulated progressively in stationary phase in 0% and 0.2% lactose broths but not in S3-1%. Fructose/tagatose biphosphate aldolase (d) (31,640 Da) was more strongly expressed in S3-0.2 and 1% lactose in early stationary phase relative to S3-0% and ribose-5-phosphate isomerase RpiB (e) (15,686 Da) was more strongly expressed in cultures containing 0.2 and 1 % lactose in younger cultures (Table 1 and Figure 2). The banding pattern for the inoculum grown in MRS and for cells after 24 h in S3-0% lactose were similar, indicating that the pre-conditioning step did not visually alter the protein banding patterns were seen. However, it was clear that several proteins present in the day 2 samples for cell grown in

S3-1% lactose (molecular weight ~18 kDa and 21 kDa, possibly corresponding to DNA polymerase III and hypothetical protein LcaA01002425 respectively) declined in relative proportions as cells progressed into stationary phase. Definite identity could not be assigned to these proteins due to low identity scores. Further analysis of differentially expressed proteins was pursued by 2-DE.

Table 1: Identification of proteins by MALDI-TOF/TOF mass spectrometry for bands excised from 1D SDS-PAGE gels.

Spot ID	Accession no. (gi)[a]	Name	Mr[b] (Da)	pI[b]	Source	MOWSE score[c]	Function
a	81427904	Xylulose-5-phosphate phosphoketolase	88645	5.14	*Lactobacillus sakei* subsp. *sakei* 23K	61	Carbohydrate metabolism
b	52401817	Elongation factor G	76495	4.83	*Bacillus licheniformis* ATCC14580	88	Protein synthesis
c	62514176	Molecular chaperone DnaK	67523	4.77	*Lactobacillus casei* ATCC 334	51	Protein folding/ repair
d	62512624	Fructose/tagatose biphosphate aldolase	31640	5.08	*Lactobacillus casei* ATCC 334	127	Glycolysis, gluconeogenesis
e	62511409	Ribose 5-phosphate isomerase RpiB	15686	5.32	*Lactobacillus casei* ATCC 334	69	Carbohydrate metabolism

[a]NCBInr accession number. MS/MS ion search data were used to query the *Firmicutes* (gram-positive bacteria) by accessing the NCBInr database through Mascot.

[b]Database molecular weight and isoelectric point values.

[c]Protein MOWSE scores greater than 67 are significant ($p<0.05$). Proteins with score less than 67 were tentatively identified.

3.3 Differential expression of protein on 2-DE gels

The analysis of the cytosolic proteins in a pI range of 4 to 7 expressed under lactose starvation was carried out. 2-DE protein profiles of the soluble fraction obtained from *L. casei* GCRL163 cultures harvested after 8 days of incubation are shown in Figure 3 and these are representative of the gels used to compare differential expression by spot analysis, followed by picking of marked spots. Differential comparison using computer software (Progenesis PG240, Non-Linear Dynamics, UK) facilitated the marking of protein spots on 2DE gels and assigning a unique identification number in each case which provided the basis to align and match the gels. A total of 125 spots on 2-DE gels from each culture condition were selected for analysis on the basis of protein intensity and clear separation. However, due to streaking in some parts of the gels, marking and analysis of spots present in those regions were not possible. This streaking was most probably caused by the high amounts of proteins in the samples in the molecular weight range of 40-50 kDa. The patterns of protein synthesized under starvation adaptation and lactose growth-limiting conditions were compared to each other than these compared with the proteome pattern for cells grown in S3-1% lactose as the control condition.

The comparisons revealed that 49 and 59 spots respectively from cells grown in S3-0% and S3-0.2% lactose cultures were matched with spots detected in cells from S3-1% lactose culture. Spot analysis showed that 42 spots were expressed differentially ($p< 0.05$) between the three culture conditions (data not shown). Expression of 13 and 17 proteins out of 49 was up- or down-regulated respectively in starved cultures (S3-0%) in comparison to the control condition (S3-1%) and the remainder showed no significant difference in standardized spot density. In the case of cultures grown in growth-limiting (S3-0.2%) conditions, 25 proteins showed the same relative expression as S3-1% lactose while 10 proteins were over-expressed and 24 spots were down-regulated. Up-regulation of four spots (707, 735, 917 and 936) was found common in both S3-0% and S3-0.2% cultures relative to S3-1% cultures. The expression of 14 of these protein spots (only those which were identified) from S3-0% and S3-0.2% lactose growth, relative to cells grown in S3-1% lactose, is shown in Table 2. These data indicated that some proteins were over-expressed only in S3-0% lactose (e.g. spots 593 relative expression was 4-fold above S3-1% lactose), while others were either over-expressed in both 0 and 0.2% lactose (e.g. 917, 707) or down-regulated (e.g. 513, 1473) relative to cells grown in 1% lactose.

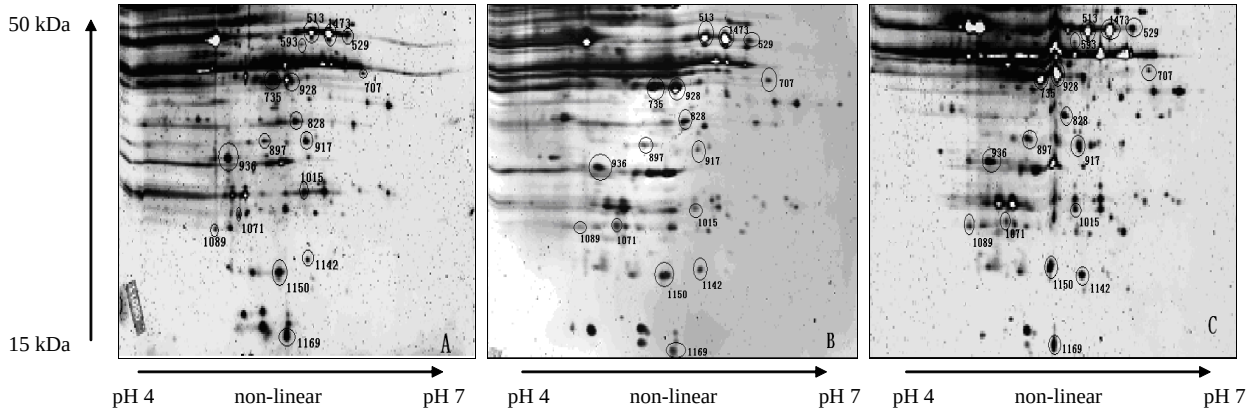

Figure 3: 2-DE gels of cytosolic cell protein from *L. casei* GCRL163 cultured in S3 broths with different levels of lactose. This figure shows representative 2-DE gel images (24 cm IPG strip) of cytosolic protein fractions obtained from *L. casei* GCRL163 grown in S3-0% (A), S3-0.2% (B) and S3-1% lactose (C) harvested after 8 days, separated between pH 4–7 in the first dimension and resolved by molecular weight in the second dimension by SDS-PAGE using a 12% acrylamide gels. These gels were used to compare the proteins profiles using spot analysis software. Differentially expressed spots were excised for MALDI-TOF/TOF mass spectrometry analysis.

Table 2: Comparative data of spots identified on two-dimensional gels for cytosolic proteins extracted from *Lactobacillus casei* GCRL163 cells grown in S3-0%, S3-0.2% and S3-1% lactose broths and harvested after 8 days of incubation at 30 ^{o}C. Progenesis PG 240 software was used to analyze the data.

Ref. Spot	S3-0%	S3-0.2%	S3-1%	ANOVA (p)	Regulation[b]	
	Norm. Vol. (SEM[a])	Norm. Vol. (SEM)	Norm. Vol. (SEM)		S3-0%	S3-0.2%
936	2.549 (0.217)	1.607 (0.047)	1.411 (0.048)	0.001	+ 1.81	+ 1.14
513	0.675 (0.089)	3.684 (0.385)	5.729 (0.848)	0.008	- 8.49	- 1.55
1473	0.861 (0.069)	3.640 (0.531)	6.862 (1.404)	0.018	- 7.97	- 1.89
529	0.943 (0.136)	0.355 (0.056)	0.486 (0.091)	0.017	+ 1.94	- 1.37
735	4.258 (0.386)	7.082 (0.465)	2.960 (0.253)	0.01	+ 1.44	+ 2.39
917	1.172 (0.210)	1.294 (0.105)	0.388 (0.019)	0.003	+ 3.02	+ 3.33
1142	0.304 (0.050)	0.866 (0.089)	0.415 (0.037)	0.004	- 1.37	+2.09
1150	2.198 (0.354)	1.186 (0.148)	1.289 (0.052)	0.023	+ 1.71	- 1.09
1015	1.134 (0.051)	0.580 (0.081)	1.003 (0.100)	0.016	+ 1.13	- 1.73
707	0.218 (0.016)	0.307 (0.019)	0.102 (0.015)	0.015	+ 2.14	+ 3.01
1173	3.050 (0.582)	1.103 (0.016)	1.918 (0.176)	0.008	+ 1.59	- 1.73
897	0.453 (0.044)	0.495 (0.052)	0.661 (0.034)	0.047	- 1.46	- 1.33
1169	2.503 (0.363)	0.142 (0.021)	0.331 (0.049)	0.004	+ 1.56	- 2.33
593	1.366 (0.171)	-	0.326 (0.009)	0.004	+ 4.19	-

[a] Standard error mean
[b]The volume value for each spot was normalized against the volume value detected for the corresponding spot for proteins extracted from cells grown in S3-1% lactose cultures, to obtain a measure of change in regulation of protein expression.

3.4 Identification of differentially expressed proteins

Proteins that may have a role in the ability of *L. casei* to adapt to carbohydrate depleted conditions were identified. From all the three gels 34 differentially expressed dense spots (out of 42) were picked and analyzed by MALDI-TOF/TOF mass spectrometry and identified by comparison on Mascot database search [43]. Out of the 34 picked, 9 proteins were unambiguously identified by peptide mass fingerprinting and a further 4 protein spots were tentatively identified (Table 3). Cysteine synthase, L(+)–lactate dehydrogenase, phosphoglycerate mutase 1, hypothetical protein LcasA01002425, three isoforms of phosphoglycerate kinase and two isoforms of dTDP-4-dehydrorhamnose 3, 5-epimerase and related enzymes were identified by Mascot database search with MOWSE score higher than 67 (MOWSE scores greater than 67 are significant $p< 0.05$). This identification was confirmed from the isoelectric point, molecular weight and known occurrence in *L. casei* (Table 3). Among the proteins identified, 10 revealed homology with *L. casei* ATCC 334, two with *Bacillus subtilis* subsp. *subtilis* str. 168 and one with each of *Bacillus cereus* ATCC 10987, *Streptococcus salivarius* and *Pediococcus pentosaceus* ATCC 25745 proteins.

Table 3: Differentially-expressed proteins detected in two-dimensional electrophoresis (Progenesis PG 240) identified by MALDI-TOF/TOF.

Ref Spot	Accession no. (gi)[a]	Name	Mr[b] (Da)	pI[b]	Source	MOWSE score[c]	Function
513	62513219	3-phosphoglycerate kinase (⇓) (↓)[#]	42211	5.64	*Lactobacillus casei* ATCC 334	297	Glycolysis
1473	62513219	3-phosphoglycerate kinase (⇓)(↓)	42211	5.64	*Lactobacillus casei* ATCC 334	489	Glycolysis
529	62513219	3-phosphoglycerate kinase (↑) (↓)	42211	5.64	*Lactobacillus casei* ATCC 334	299	Glycolysis
735	7246033	L(+) – lactate dehydrogenase (↑)	35508	5.24	*Lactobacillus casei*	377	Lactate metabolism
917	62513242	Cysteine synthase (⇑)	33290	5.26	*Lactobacillus casei* ATCC 334	170	Protein synthesis
1142	62513563	dTDP-4-dehydrorhamnose 3, 5-epimerase and related enzymes (↓) (↑)	21090	5.03	*Lactobacillus casei* ATCC 334	120	Cell envelope, Surface polysaccharides
1150	62513563	dTDP-4-dehydrorhamnose 3, 5-epimerase and related enzymes (↑) (↓)	21090	5.03	*Lactobacillus casei* ATCC 334	100	Cell envelope, Surface polysaccharides
1015	48869719	Phosphoglycerate mutase 1 (↑) (↓)	25882	4.96	*Pediococcus pentosaceus* ATCC 25745	72	Glycolysis
936	62512535	Hypothetical protein LcasA01002425 (↑)	19970	4.81	*Lactobacillus casei* ATCC 334	71	-
593	62512667	Pyruvate/2-oxoglutarate dehydrogenase complex (⇑)	31210	5.57	*Lactobacillus casei*	53	Putative glutathione reductase
707	62513441	Phosphomannose isomerase (↑)	37933	5.57	*Lactobacillus casei* ATCC 334	52	Glycolysis
1173	92111610	DNA polymerase III (↑) (↓)	18381	4.63	*Streptococcus salivarius*	42	DNA repair
897	2633920	Transcriptional attenuator and uracil phosphoribosyltransferase activity (minor) (↓)	20308	5.21	*Bacillus subtilis* subsp. *subtilis* str. 168	41	-
1169	2633865	ylbA (↑) (↓)	13485	5.15	*Bacillus subtilis* subsp. *subtilis* str. 168	40	-

[a] NCBInr accession number. MS/MS ion search data were used to query the *Firmicutes* (Gram-positive bacteria) by accessing the NCBInr database through Mascot.

[b] Database molecular weight and isoelectric point values. These values were compared with apparent pI and Mr values estimated from 2DE gels.

[c] Proetien MOWSE scores greater than 67 are significant ($p<0.05$). Proteins with score less than 67 were tentatively identified.

[#] Symbols in parentheses indicate up- or down-regulation relative to the same protein spot for S3-1% lactose (see Table 1): ↓ or ↑ means moderate relative decrease or increase, ⇓ or ⇑ means major decrease or increase (>3-fold change). When a second symbol occurs, this indicates a difference seen for S3-0.2% lactose.

In this study we used proteomic approach to identify proteins expressed in response to lactose starvation in a NSLAB isolate. A limited number of reports have addressed the responses to starvation in lactobacilli [9, 22, 35, 50], or in LAB more generally [7, 31]. Most studies have been concerned with characterising oxidative, acid and heat or cold stress

responses in *Lactococcus lactis*, or tolerance to assaults likely to impact on probiotic activity (such as bile tolerance) [12, 49, 51]. However, numerous studies in other species have shown that bacteria generally adapt to nutritional stress by adopting a physiological state characterised by the down-regulation of nucleic acid and protein synthesis, and the simultaneous up-regulation of protein degradation and amino acid synthesis, typified by movement from growth into stationary phase [40, 41]. Our contention was that *L. casei* must adapt similarly to carbohydrate starvation during cheese ripening, given the observation that different biotypes with different capabilities for degrading protein, peptides and amino acids emerge in the cheese matrix as the nutritional environment changes [70] and that alternative carbohydrate sources would be in limited supply.

Physiological traits of *L. casei* GCRL163 showed the adaptation ability and metabolic capacity of the strain in S3 broth lacking lactose. *L. casei* produced end-products that can arise from amino acids degradation, including isopropanol, 3-methyl-1-butanol, benzylacetaldehyde and acetate [39] indicating that Tryptone was being utilized. This is consistent with our previous findings that showed peptide degradation in long-term cultures of GCRL163 in S3 broth [28, 50]. Viability in the S3-0% broths remained high relative to the maximum numbers seen for each growth condition, an observation reported for other strains of lactobacilli in carbohydrate-free chemically defined media [22, 26]. We have previously shown that during extended culture, this strain continued to lose viability in lactose-supplemented broths relative to S3-0%L although metabolite production continued. These data suggested that cells remained viable and metabolically active but not recoverable on MRS plates [28]. This phenomenon is also reported in other species [16, 63].

Survival and adaptation to growth in media containing low or no lactose was expected to involve expression of stress proteins during adaptation to environmental stress conditions as suggested by a number of authors [5, 30, 62, 73]. Using proteomic approach, significant changes in the relative expression of proteins was observed when cells entered into stationary phase over the sampling period of 8 days. The most notable identified proteins in the soluble fraction of cells were known to be involved in general stress responses, protein synthesis and carbohydrate metabolism. Up-regulation of general stress response proteins, molecular chaperone DnaK, and elongation factor G, which is involved in protein synthesis, was seen in lactose starved cells, which commonly occurs in response to several stresses [5]. An important enzyme in sulfur assimilation, cysteine synthase, was up-regulated 3-fold in starved cells. Cysteine and other sulphur containing molecules participate in protection against oxidative and non-oxidative stresses. Several reports showed induction of cysteine synthase in *P. freudenreichii* CIP103027, *B. subtilis* and *S. aureus* in response to various stress conditions and survival mechanisms [2, 34]. These proteins are important for maintenance of cell integrity, physiology and adaptation under adverse growth conditions [2, 4, 18, 24, 37, 46, 60, 67].

The major group of proteins showing differential expression consisted of the enzymes involved in glycolysis and other related metabolic pathways involved in energy production or regeneration of cofactors. These enzymes were: xylulose-5-phosphate phosphoketolase (XpkA), phosphomannose isomerase, phosphoglycerate mutase 1, pyruvate/2-oxoglutarate dehydrogenase complex, 3-phosphoglycerate kinase, dTDP-4-dehydrorhamnose 3, 5-epimerase and related enzymes, and L(+)–lactate dehydrogenase. Strong up-regulation of protein identified as pyruvate/2-oxoglutarate dehydrogenase was also seen. This complex has putative glutathione reductase activity which may be linked to oxidative stress responses in other bacteria: given that the cells were cultured in anaerobic conditions, up-regulation of cysteine synthase and pyruvate/2-oxoglutarate dehydrogenase may be aligned with a generalized stress response in *L. casei* GCRL163. Enzymes like XpkA, phosphomannose isomerase, phosphoglycerate mutase 1 and L(+)–lactate dehydrogenase showed a relative higher expression in starved cells. These metabolic enzymes were reported to have an essential role in cell survival and adaptative response to numerous stresses [6, 19, 32, 68, 72]. A hypothetical protein LcasA01002425 was also up-regulated in starved cells.

Isoforms of some proteins were found on the gels: 3-phosphoglycerate kinase was present in three isoforms (spots number 513, 1473 and 529, Table 3) and interestingly two of the isoforms were expressed in higher level in 1% lactose cultures but one isoform (spot number 529) was up-regulated in cells grown without lactose. Similarly, isoforms of dTDP-4-dehydrorhamnose 3, 5-epimerase and related enzymes (spot number 1142 and 1150, Table 3) were identified where one form was up-regulated in starved cells and second in non-starved cells. The presence of isoforms proteins had been reported for *Lac. lactis* [69] and *Lactobacillus* spp. [44]. Their occurrence is likely to be the result of post-translational changes including phosphorylation, which has been described in eukaryotic and prokaryotic systems [15, 30]. Protein phosphorylation was not investigated in this study nor was the sub-cellular location of the isozymes upregulated, although this analysis may inform the role of these specific isozymes.

The expression levels of several proteins (some in the MW range 50-60 kDa and fructose/tagatose biphosphate aldolase, 31 kDa) increased over the 8 days but this was not linked to the presence, or absence, of lactose: presumably up-regulation of these proteins was a general stationary-phase response rather than being linked to lactose starvation specifically. Other proteins (RpiB, 16 kDa) were only detected in cells from 0.2 and 1% lactose broths, indicating that their presence or up-regulation in stationary phase was related to the prior history of growth in lactose. Some proteins (DNA polymerase III) were tentatively identified by searching the peptide sequence query matches from two databases,

MSDB and NCBInr, and using a minimum benchmark MOWSE score >40 to consider character compatibility. Identification of other differentially expressed protein spots was not successful in this study.

Induction of general stress response proteins, along with other proteins involved in sugar metabolism has been demonstrated in two LAB species and *B. subtilis* previously [21, 57, 71]. The overlap between starvation and other stress responses, particularly acid stress, is well known and considerable information is now available on the responses to various nutrients in *E. coli* [40, 64], *L. lactis* [23], *B. subtilis* [3] and *Sal. Typhimurium* [60]. Gagnaire et al. [15] demonstrated that LAB encountered numerous stresses during Emmental manufacture and, in turn, generated a severe stress response. Findings of these studies suggested that fingerprints of bacterial enzymes releases during *in vitro* [19] or *in situ* [15] conditions have significant applications at different key steps of cheese manufacture and ripening. We assume starved *L. casei* hunt around alternative energy sources i.e. amino acids and pentose sugars. Identification of protein revealed that enzymes associated with amino acid and phosphoketolase metabolic pathways were expressed in long-term starvation.

4. CONCLUSIONS

This work showed that stationary phase in *L. casei* GCRL163 is an active phase where metabolism continues after growth has ceased, for cells grown in a lactose-free or supplemented media. This study on starvation responses of *L. casei* using proteomic approach indicates the involvement of general stress response proteins and metabolic enzymes for survival of lactose starved cells. Identification of proteins associated with generalized stress responses following growth without lactose (DnaK, elongation factor G, cysteine synthase) was consistent with previous reports for NSLAB and many other species of bacteria. Induction of glycolytic flux and other metabolic enzymes particularly XpkA suggests that alternative metabolic regulation may be involved in starvation responses of *L. casei.* A recent report also concluded that starved *L. brevis* metabolized arginine, glycine, and histidine from dead cells as alternative nutrient sources [74]. Finally, this study provided information on expression of proteins involved in the survival and metabolic activity of a NSLAB isolate in lactose deprived conditions. Understanding how *L. casei* reacts and performs in stationary phase, and nutrient starvation, may inform strategies for strain improvement for several applications in the dairy industry.

5. ACKNOWLEDGEMENT

The research was funded by an International Postgraduate Research Scholarship (Australian Commonwealth Government) and a Melbourne International Research Scholarship to support MA Hussin's PhD studies. The authors thank Food Science Australia, CSIRO, for supplying the strain of *L. casei* and Biosciences Research Division, Primary Industries Research, Victoria, Australia for access to proteomic analysis facilities.

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