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The effect of starvation stress on *Lactobacillus brevis* L62 protein profile determined by *de novo* sequencing in positive and negative mass spectrometry ion mode

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RATIONALE: We describe a novel negative chemically activated fragmentation/positive chemically activated fragmentation (CAF−/CAF+) technique for protein identification. The technique was used to investigate *Lactobacillus brevis* adaptation to nutrient deprivation.
METHODS: The CAF−/CAF+ method enables *de novo* sequencing of derivate peptides with negative and positive ion mode matrix-assisted laser desorption/ionization (MALDI) tandem mass spectrometry (MS/MS). Peptide sequences obtained from MS/MS spectra were matched against the National Center for Biotechnology Information (NCBI) non-redundant (nr) database and confirmed by the mass spectrometry data of elucidated peptide mass sequences derived from the annotated genome. This improved protein identification method highlighted 36 differentially expressed proteins in the proteome of *L. brevis* after 75 days of starvation.
RESULTS: The results revealed the key differences in the metabolic pathways that are responsible for the survival of *L. brevis* in a hostile environment. Proteomics analysis demonstrated that numerous proteins engaged in glucose and amino-acid catabolizing pathways, glycerolipid metabolizing pathways, and stress–response mechanisms are differentially expressed after long-term starvation. Amino acid and proteomics analysis indicated that starved *L. brevis* metabolized arginine, glycine, and histidine from dead cells as alternative nutrient sources. The production of lactic acid also varied between the parent cells and the starved cells.
CONCLUSIONS: Differentially expressed proteins identified exclusively by peptide sequence reading provided promising results for CAF−/CAF+ implementation in a standard proteomics workflow (e.g., biomarker and mutation discovery and biotyping). The practical performance of a reliable *de novo* sequencing technique in routine proteomics analysis is emphasized in this article. Copyright © 2013 John Wiley & Sons, Ltd.

A novel method of negative chemically activated fragmentation/positive chemically activated fragmentation (CAF−/CAF+) based on N-terminal disulfonation and peptide *de novo* sequencing in two mass spectrometry (MS) modes (positive and negative ion mode) was upgraded with the BLASTp (Basic Local Alignment Search Tool) algorithm.[1] The usage of disulfonic acid reagent (4-formyl-1,3-benzenedisulfonic acid) for short sequence elucidation has been described by Han *et al.*[2] and Hassell *et al.*,[3] but the method was limited to a narrow repertoire of the gas-phase ion/ion interactions and the reagent used exhibited relatively low reactivity caused by steric interaction between the sulfonic group and reactive centre (aldehyde functional group). The method presented herein is based on in-solution peptide derivatization by 5-formyl-1,3-benzenedisulfonic acid and incorporates *de novo* sequencing proteomics data into a high-throughput *de novo* sequencing platform, which consists of determined tryptic peptide amino acid sequences obtained by negative and positive ion mode matrix-assisted laser desorption/ionization (MALDI) tandem mass spectrometry (MS/MS). The elucidated sequences were matched against the annotated genes of *Lactobacillus brevis* ATCC 367 (the National Center for Biotechnology Information (NCBI) BLAST/BLASTp suite). The method was used to determine *L. brevis* proteins by comparing the peptide readings of certain proteins against protein/DNA databases. With the use of unambiguously determined peptide sequences (6–20 amino acids in a row) combined with peptide precursor masses,[4,5] the proteins could be accurately identified and particular mutations could be easily determined (Fig. 1; and Supplementary Fig. S1 in leave Supporting Information).

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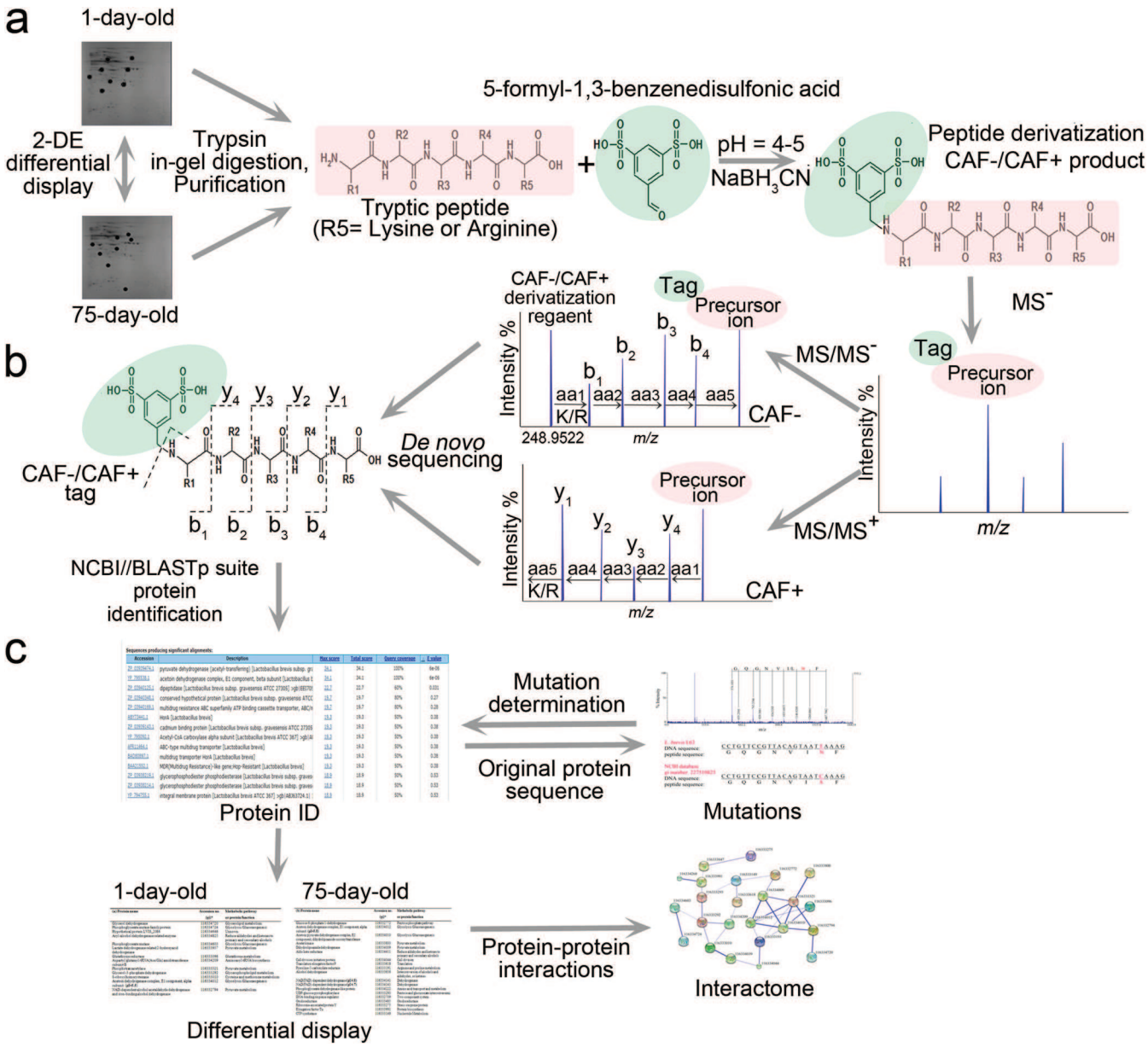

Figure 1. *Lactobacillus brevis* proteome differential display of starved and non-starved cells obtained through protein identification performed by CAF−/CAF+ mass spectrometry (MS). (a) In-solution chemical derivatization of tryptic peptides by 5-formyl-1,3-benzenedisulfonic acid (reductive aminations) under controlled pH and selective reduction conditions ($NaBH_3CN$). (b) *De novo* sequencing of derivatizated peptides by MS in positive and negative ion mode (b-series ions in negative MS/MS, reading direction from N- to C-terminus; y-series ions in positive MS/MS, reading direction from C- to N-terminus). (c) BLASTp data processing of peptide readings. Differential display of identified proteins (starved vs. non-starved proteome), nutrition starvation interactome analysis and mutation determination.

Lactic acid bacteria (LAB) have been used widely for centuries in the commercial production of fermented dairy products, meat, fish, bread, cereals, and vegetables. Many LAB metabolize polysaccharides in animal and human gastrointestinal tracts.[6] Variations in temperature, the presence of sodium chloride, oxidative shock, low pH, and nutrient limitation affect LAB growth in both industrial processes and the gastrointestinal tract.[7] In general, bacteria have developed different strategies and response mechanisms to adapt to and to survive such stressful and unfavorable environmental conditions.[8] The examination of metabolic adaptation in bacteria exposed to stress conditions provides insight into the regulatory processes and adaptive pathways on which bacteria rely when they are exposed to environmental stress.

Lactobacillus brevis is a Gram-positive, non-sporulating, facultative anaerobic bacterium that metabolizes hexoses by the 6-phosphogluconate pathway. The products of hexose fermentation are lactic acid, CO_2, and ethanol and/or acetic acid in equimolar amounts.[9] When non-sporulating bacteria are inoculated in rich medium in a batch system, they pass through five phases of growth: lag, exponential, stationary, death, and prolonged stationary phase. To survive under stressful conditions, during the prolonged stationary phase,

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bacteria activate stress response genes and alternative metabolic pathways. Adaptive metabolic response enables bacterial cells to metabolize detritus from dead cells as alternative nutrient sources: amino acids, carbohydrates, lipids, and DNA.[8,10] Previous studies described the differences in gene expression of several LAB species during starvation based on proteomics approaches.[7] For example, from the proteomics analysis of *Lactobacillus casei* that was lactose-starved for 72 h, numerous glycolytic enzymes were found to be differentially regulated.[11]

In the present study, we monitored *L. brevis* L62 growth in spent glucose medium for 75 days. Although the growth curve for *L. brevis* was based on experimental data without literature references (Fig. 2), the experimental results showed the expected similarity with the growth curves of other non-sporulating bacterial species during long-term survival in a stationary phase.[8,12] We recorded the differences in the protein profile of the cells, the availability of amino acids during growth as an alternative carbon source, the alternations in gene expression and the amounts of metabolic products after long-term starvation. The amino acid composition of *L. brevis* cells was determined by amino acid analysis to record the level of amino acids that were available to bacteria as an alternate source of carbon during long-term incubation. Information gained from amino acid analysis indicates that *L. brevis* metabolizes several amino acids during long-term incubation (arginine, glycine, and histidine). Changes in gene expression as a consequence of long-term incubation were determined in clones isolated from 75-day-old cultures. Two-dimensional electrophoresis (2-DE) in conjunction with MS was used as a proteomics tool for mining protein sequence databases. The 2-DE technique allowed protein differential display between 1-day-old and 75-day-old clones. The CAF−/CAF+ method enabled the determination of 36 differentially expressed proteins and is presented here for the first time as a promising *de novo* sequencing tool. This approach allowed us to link long-term starvation with differentially expressed proteins involved in the glucose catabolism pathways, glycerolipid metabolizing pathways, and stress–response mechanisms of *L. brevis*.

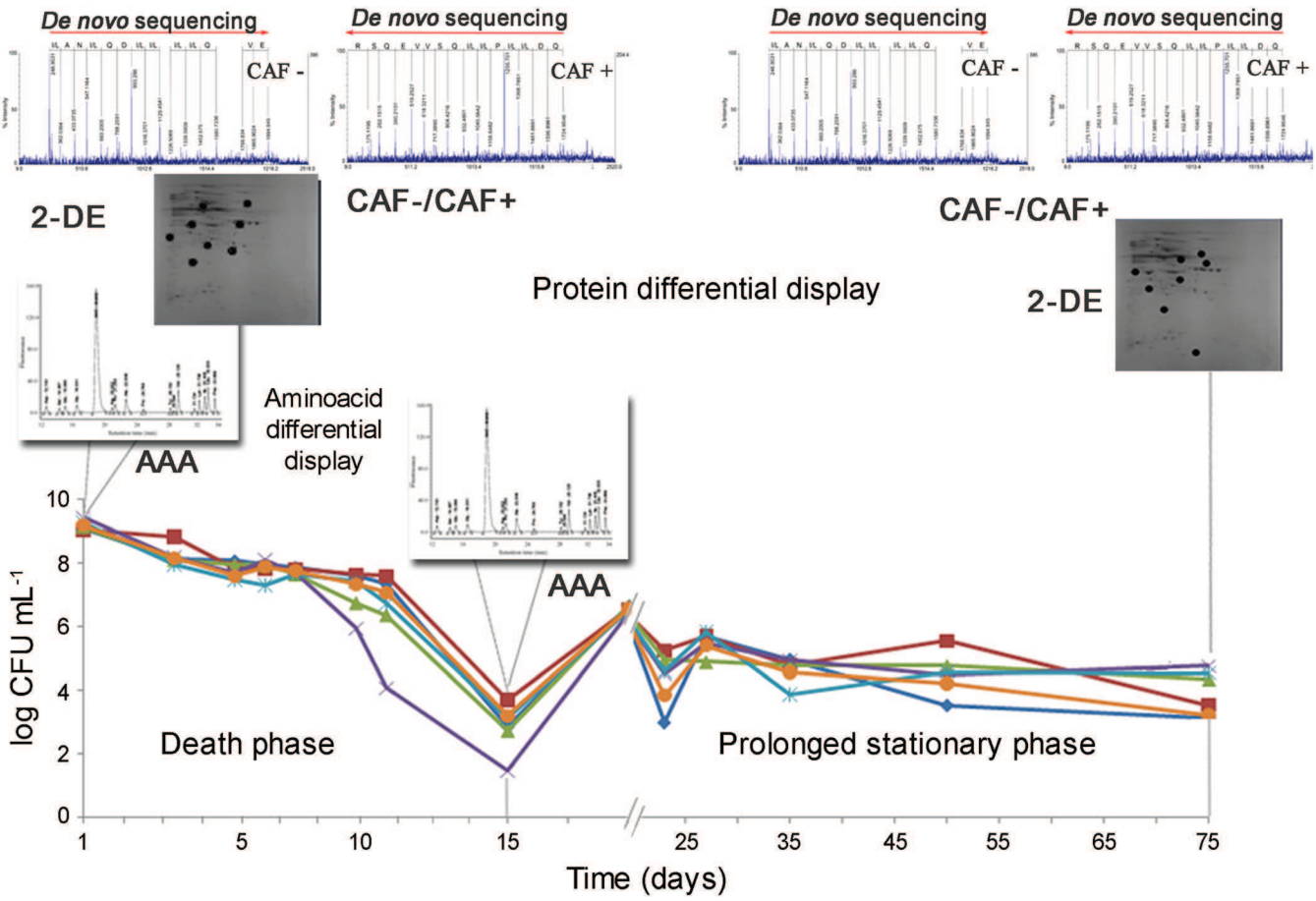

Figure 2. *Lactobacillus brevis* growth monitoring in long-term nutrition deprivation culture systems and experiments performed during different growth phases. The schematic growth curves of six parallel cultures during long-term incubation are presented with different colors. The bacterial growth curves are basically divided between death phase and prolonged stationary phase. Experiments that were performed during different growth phases, namely 2-DE, CAF−/CAF+ MS/MS and amino acid analysis (AAA), exhibited significant differences between compared cultures. Differential display was estimated between protein expression of parent cells (1-day-old cultures) and proteins isolated from 75-day-old clones. The amino acid composition of parent cells was compared with the amino acid composition of cells that were grown in spent medium for 15 days when the measured number of viable cells reached its lowest level.

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EXPERIMENTAL

Bacterial strain and long-term growth

The *L. brevis* L62 strain was used for the nutrient starvation experiments. The cells were inoculated in 10 mL of de Man Rogosa Sharpe (MRS) broth (Biolife, Banbury, UK) supplemented with 2% glucose and incubated at 37 °C for 75 days in a laboratory shaker (New Brunswick Scientific, NJ, USA). The colony-forming units (CFU) per milliliter were recorded periodically (Fig. 2) by the plating of serial dilutions of the bacterial samples (in phosphate buffer solution) onto MRS agar plates (Biolife).

Sample preparation

We inoculated 75-day-old clones (six clones isolated from the solid plates after 75 days of incubation) and 1-day-old cells (parent cells) in 10 mL of MRS medium and incubated them at 37 °C for 24 h. The cell suspensions were then transferred into 200 mL of MRS broth and grown until they reached an optical density (OD) of 1.2 at 600 nm. The cells were harvested at 7000 *g* (Beckman Coulter, High Wycombe, UK) for 7 min, washed twice in 20 mM Na_2HPO_4 and lysed with glass beads (0.10 to 0.11 mm in diameter).[12] The DNA was removed after treatment with phenol/chloroform/isoamylalcohol solution (25/24/1, v/v/v), and the reconstituted proteins were precipitated overnight at −20 °C with ice-cold acetone (20 min at 5000 *g*).[13] The proteins were resuspended in rehydration solution for isoelectric focusing (IEF). Protein concentration in solution was estimated by the Bradford protein assay. All chemicals and materials, unless stated otherwise, were purchased from Sigma Aldrich Co., Munich, Germany.

Amino acid analysis

One-day-old and 15-day-old cells were grown, harvested, washed, and lysed as described above. The soluble cell fractions were purified with spin-columns (Agilent, Santa Clara, CA). Amino acid analysis was performed according to the procedure described by Bosch *et al.*[14] and Eriksson and Jonasson.[15] Briefly, samples were hydrolyzed in HCl vapor under vacuum at 110 °C for 22 h. After protein hydrolysis, the amino acids were derivatized with the AccQ-Tag amino acid analysis kit. Analysis was performed using a Waters Alliance 2695 (Milford, MA) high-performance liquid chromatograph equipped with a 474 scanning fluorescence detector and Empower data station software. Separation was carried out with a 1.0 mL/min flow rate and an AccQ-Tag Nova-Pak C18 column. Eluent A consisted of AccQ-Tag Eluent A acquired from Waters Corporation, and eluent B consisted of a 60/40 ACN/water mixture (J.T. Baker, Deventer, The Netherlands). All chemicals and materials were purchased from Waters Corporation unless stated otherwise. An amino acid hydrolysate standard mixture (2.5 mM) was purchased from Pierce Chemical Co. (Rockford, IL, USA).

Two-dimensional electrophoresis

Immobilized pH gradient strips (IPG; 17 cm, linear pH 4–7) were rehydrated for 14 h with 350 µL of rehydration solution containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate hydrate (CHAPS) (w/v) and 2 mg/mL of total protein. The IEF was carried out with a Protean IEF cell (Bio-Rad, Hemel Hemstead, UK) with a low initial voltage and an applied voltage gradient up to 7000 V. The total $V \times t$ product applied was 90 000 Vh for each strip at 20 °C. The strips were equilibrated in equilibration buffer containing 20 mM dithiothreitol (DTT), 50 mM tris(hydroxymethyl)aminomethane (Tris) adjusted to pH 6.8, 6 M urea, 2% sodium dodecyl sulfate (SDS) (w/v), 30% glycerol (v/v) and 0.01% bromophenol blue (w/v) on a tilt table for 15 min. The solution was discarded and the same equilibration buffer solution without the addition of DTT and with the addition of 25 mM iodoacetamide was used for a 15 min protein alkylation reaction. The strips were placed on a 1 mm thick 12% polyacrylamide gel and sealed with 0.1% (w/v) agarose in SDS-electrophoresis buffer containing 0.01% (w/v) bromophenol blue. In the second dimension, the electrophoresis was run for 1 h at 15 mA per gel and then at 20 mA for 600 Vh. The electrophoresis was terminated after 30 mA per gel until the bromophenol blue reached the bottom of the gel. Tris-glycine running buffer containing 25 mM Tris, 190 mM glycine and 0.1% (w/v) SDS was used in the second dimension. All chemicals were of electrophoresis grade (Bio-Rad).

Differential display analysis

Differential display analysis of the gel data sets was undertaken by comparing images of two control gels (1-day-old cultures) with six 75-day-old clone gels of eight independent experiments (each sample was analyzed in two replicates). Densitometry analysis was performed with image analysis software (Discovery Series PDQuest 2-DE analysis software package version 7.4.0.) integrated with a VersaDoc 4000 Imaging System (Bio-Rad). Master gels were used to obtain the differences between protein profiles of 1-day-old and 75-day-old clones.

In-gel digestion

Differentially displayed protein spots were excised from 2-DE gels into small pieces and subjected to in-gel digestion with trypsin according to the procedure described by Shevchenko *et al.*[16] Briefly, after the Coomassie destaining procedure [acetic acid/methanol/water (1/4/5, v/v/v)], the gel pieces were washed with NH_4HCO_3 (Sigma Aldrich Co.) and CH_3CN (Merck KGaA, Darmstadt, Germany). Trypsin proteolysis (trypsin from porcine pancreas; Merck KGaA) was performed at 37 °C for 18 h. After digestion, the extracted peptides were purified with C4 ZipTip columns (Millipore Co., Billerica, MA, USA) and evaporated to dryness with a SpeedVac (Eppendorf, Germany).

Derivatization reaction

Each sample that contained the evaporated tryptic peptide mixture was reconstituted with a 15 µL volume of derivatization solution. The derivatization solution contained 0.8 µM 5-formyl-1,3-benzenedisulfonic acid, disodium salt hydrate (pro analysis synthetic product, "Ruder Bošković" Institute) and 16 µM of $NaBH_3CN$ (Merck KGaA) dissolved in 10 mM KH_2PO_4 and adjusted to pH 4.5 with H_3PO_4 (Merck KGaA). The derivatization mixture was maintained at 4–8 °C for at least 3 h, as described in Cindrić *et al.*[1] Commercially available 4-formyl-1,3-benzenedisulfonic acid

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(Sigma-Aldrich, St. Louis, MO, USA) can be used as derivatization reagent instead of 5-formyl-1,3-benzenedisulfonic acid under the same aforementioned conditions, except prolonged incubation time (at least 12 h). After derivatization, the mixture of derivate and non-derivate peptides was purified with C_4 ZipTip columns (Millipore Co.). Comparative derivatization analysis performed by Lys-Tag/SPITC derivatization was accomplished on a control set of 10 tryptic peptide mixtures.[17,18]

MS acquisition (CAF−/CAF+) was performed with a 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems, Carlsbad, CA, USA) equipped with a 200 Hz, 355 nm Nd:YAG laser. Ions were analyzed in reflectron mode using positive and negative polarity. Samples were mixed with α-cyano- 4-hydroxycinnamic acid 1:5, v/v (5 mg/mL; Waters Corporation) and spotted onto a metal plate. The instrument parameters were set using the 4000 Series Explorer software (version 3.5.3, Applied Biosystems). Mass spectra were obtained by averaging 1800 laser shots covering a mass range of *m/z* 1000 to 5000. Internal calibration of the mass range was performed with trypsin autolysis fragments. MS/MS of the 10 most intense precursor signals after derivatization (excluding trypsin autolysis fragments) from MS negative spectra was achieved by 1 keV collision energy in positive ion mode with air as a collision gas, CAF+. In the negative ion mode, the same 10 most intense fragile precursor signals (obtained after derivatization) were fragmented without using collisionally induced dissociation (CID), CAF−.

Protein identification

Protein identification and a protein mutation search were performed by *de novo* sequencing of the MS and MS/MS spectra from both positive and negative ion modes followed by a BLASTp search against the NCBI nr database. Exploration of the protein–protein interaction map was performed to ascertain the interconnectedness of the identified proteins. A STRING web resource[19] was used to construct an integrated map of protein–protein interactions for the identified differences in the protein expression profiles.[20]

Determination of lactic acid and acetic acid by HPLC

One-day-old cells and 75-day-old *L. brevis* clones were inoculated in 10 mL MRS medium and grown until the OD_{600} reached 1.2. The liquid cultures were centrifuged at 7000 *g* for 10 min (Rotina 35, Hettich, Germany). Carrez reagent (Kemika, Zagreb, Croatia) was added to the supernatant, and the precipitated proteins were removed by filtration. Lactic and acetic acid concentrations were quantitatively determined at 340 nm by a ProStar Varian 230 (Walnut Creek, CA, USA) analytical HPLC system with a Varian MetaCarb 67H column (300 × 6.5 mm) heated to 60 °C in the isocratic mode of elution with 0.005 M sulfuric acid at a constant flow rate of 0.6 mL/min.[21]

RESULTS AND DISCUSSION

The *L. brevis* cells were grown and maintained during long-term incubation in MRS medium, and the number of viable cells in the medium was determined as CFU per milliliter for 75 days (Fig. 2). According to the CFU value, the cells entered the death phase 24 h after inoculation. Because the number of viable cells decreased steadily, the lowest value of viable cells was reached after 15 days. In the following days, the survivors entered a prolonged stationary phase. During long-term growth, *L. brevis* cells experienced adverse conditions, such as an unfavorable pH (the pH value reached 4.3 after 24 h of incubation) and carbon source deprivation. The final impact of starvation on bacterial growth was observed through the differential display of quantitative amino acid profiles, metabolic end-products (lactic and acetic acid), and proteomics analysis.

During nutrition deprivation, *L. brevis* cells metabolized amino acids from dead cells as an alternative source of carbon. Quantitative comparison of the amino acid profile from 1-day-old cells and the 15-day-old cultures indicated decreased concentrations of three amino acids in the 15-day-old cultures: arginine, glycine and histidine (Fig. 3). This finding demonstrated that *L. brevis* consumed these specific amino acids during the 15 days of nutrition deprivation. Examples from the literature suggest that lactic acid bacteria

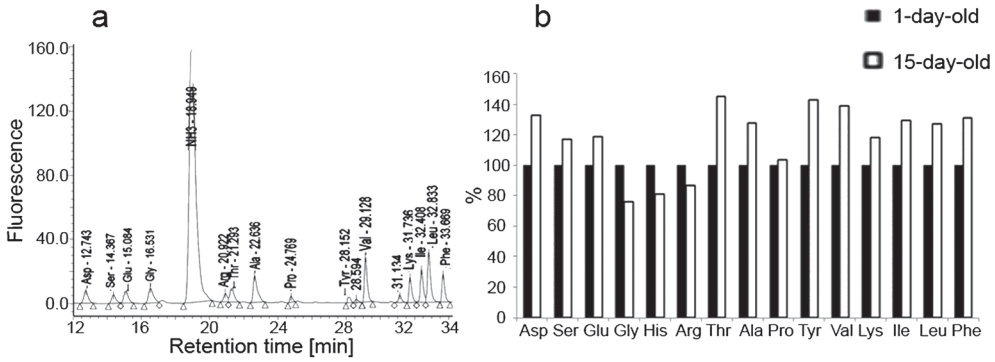

Figure 3. Amino acid composition analysis. (a) A representative chromatogram showing the amino acid composition analysis detected in 15-day-old cultures, and (b) comparison profile of amino acid composition of 1-day-old cells and 15-day-old cultures. Amino acid concentrations from 1-day-old cells are expressed as 100% (white bars) and compared with data generated from 15-day-old cultures (black bars).

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shift their metabolism from glycolysis to amino acid catabolism under glucose deprivation,[6] e.g., the catabolism of arginine by the arginine deiminase pathway, to access an alternative source of energy.[22] Because *L. brevis* cells produce enzymes that are involved in the arginine deiminase pathway,[23] the activation of this metabolic pathway turns arginine and proline into an alternative source of carbon. The decrease in arginine concentration likely stems from the induction of the deiminase pathway that mobilizes extracellular reserves of arginine, which is then used as a source of adenosine 5′-triphosphate. Because genes for glycine and histidine biosynthesis are not present in *L. brevis*,[23] the decreased concentration of these amino acids in the 15-day-old cells could be indicative of amino acid recycling from the dead cells.[24] The observed higher concentrations of 14 other measured amino acids in the 15-day-old samples, compared with the 1-day-old cells, stem from the amino acid accumulation of dead cells that cannot be used as an energy source.

The pH and concentrations of lactic acid and acetic acid were monitored in six clones isolated from the agar plates (75-day-old clones recultivated in rich medium). The recorded pH reached 4.3 in the 1-day-old cultures that served as a control, but the measured pH in cultures with 75-day-old clones was 5.3. The pH and acid concentrations were compared to evaluate the relationships between pH variability and the observed production of lactic and acetic acids. These results demonstrated that *L. brevis* produced less lactic acid after 75 days of growth (observed decrease in concentration from 5.9 mg/mL to 3.2 mg/mL) under starvation conditions, which led to the observed increase in pH.

To identify proteins that were responsible for the metabolic pathway alteration of 75-day-old clones and the lower production of lactic acid, the proteome of 1-day-old cells and 75-day-old clones was separated by 2-DE (Fig. 4) followed by a differential display analysis of expressed proteins by MALDI-TOFTOF (time-of-flight) mass spectrometry. The proteins were identified by *de novo* sequencing of the CAF−/CAF+ spectra (Figs. 5(a) and 5(b)) followed by a BLASTp search against the NCBI nr database. By comparison with the corresponding spectra of the Lys-Tag /SPITC derivatized spectrum (Fig. 5(c)) and the intact peptide spectrum (Fig. 5(d)), it was possible to interpret the complete sequence mostly after reading of overlapped sequences deduced from CAF−/CAF+ spectra. Intact peptide or Lys-Tag/SPITC methods of *de novo* sequencing were found difficult to accurately interpret *de novo* relative to CAF−/CAF+ *de novo* sequencing accomplished by reading from positive and negative MS/MS spectra.

The concept of CAF−/CAF+ peptide sequence reading was successfully used to find changes in the proteome occurring after starvation. Enzymes involved in various metabolic pathways were found to be differentially expressed in 75-day-old clones (Table 1). These enzymes included glucose catabolizing pathways (glycolysis/gluconeogenesis, pyruvate metabolism, and pentose phosphate), amino-acid catabolizing pathways (amino-acid transport, arginine and proline metabolism, cysteine and methionine metabolism), lipid and glycerolipid metabolizing pathways (fatty acid metabolism, glycerolipid and glycerophospholipid metabolism), and nucleotide metabolism. Proteins involved in stress–response (ribosome-associated protein Y), homologous recombination (recombinase A), translation (translation elongation factor P), and cell division (cell division initiation protein) were also detected as being differentially expressed. The differentially expressed proteins were integrated into a map of protein–protein interactions (Supplementary Fig. S2 in the Supporting Information). By adding proteins to the empirically determined protein set, the proteins involved mainly in primary metabolism were integrated into a single connected map. Taken together, these data showed that 22 pathways were altered after starvation. Bacterial evolution is a random process, and it is likely that every bacterial clone exhibited a slightly different protein profile. However, a strong correlation between the 75-day-old clones was found as a result of the same environmental selection pressures affecting all cultures (low pH and starvation).

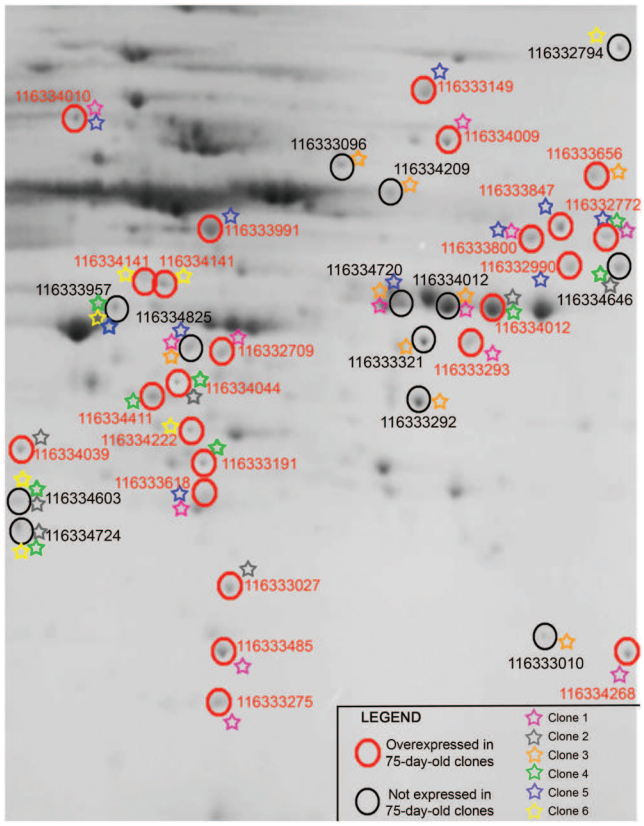

Figure 4. *Lactobacillus brevis* 2-DE proteome differential display of starved and non-starved cells. Superimposed 2-DE images of cytosolic protein fractions indicating differences in protein synthesis between 1-day-old cells (black circles) and 75-day-old clones (red circles). Superimposed images were generated by combining the digitalized images after Gaussian modeling analysis in PDQuest 2-DE analysis software. Six 75-day-old clone gels and two control gels (1-day-old culture) of eight independent experiments were fused and overlaid (each sample was analyzed in two replicates). Therefore, red and black circles represent proteins that are expressed or not expressed in 75-day-old cells, respectively. Proteins of 75-day-old cells that are differentially expressed in each clone are indicated by different colored stars(grey, yellow, pink, blue, green, and orange). Protein spots are identified after *de novo* sequencing (NCBI nr accession number) and results are summarized in Table 1.

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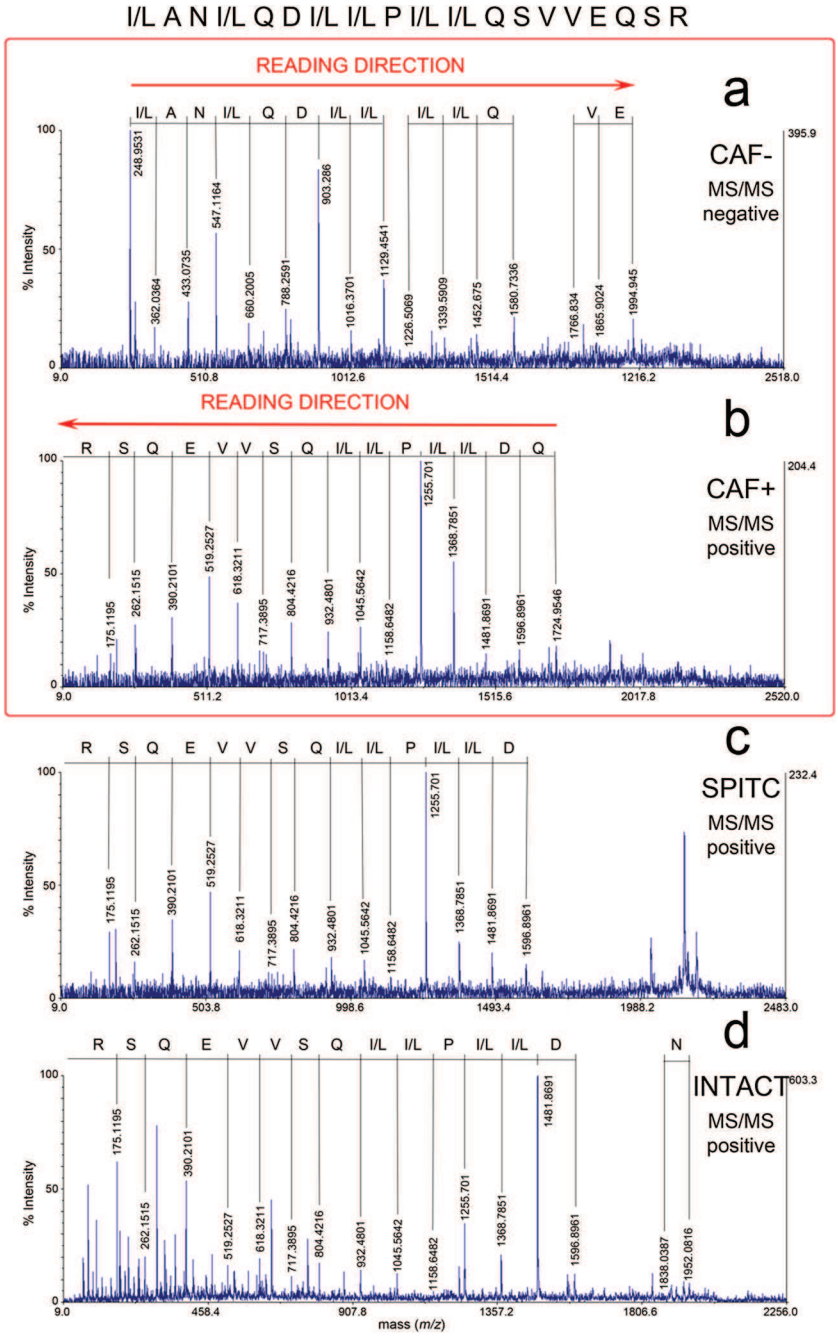

Figure 5. Comparison between CAF−/CAF+, SPITC, and intact peptide *de novo* sequencing. MALDI-MS/MS spectra of IANIQDILPLLQSVVEQSR peptide (*m/z* 2136.1674 positive MS of the intact peptide, *m/z* 2351.1792 positive MS after SPITC derivatization and *m/z* 2384.114 negative MS after CAF−/CAF+ derivatization) assigned as *L. brevis* molecular chaperone GroEL (NCBI nr accession number 116333267). Peptide was assigned according to the NCBI nr protein database from (a) the amino acid sequence reading from the negative ion MS/MS spectrum, CAF−, and (b) the amino acid sequence reading from the positive MS/MS spectrum, CAF+. The peptide was partially assigned from (c) the amino acid sequence reading from the positive ion MS/MS spectrum, SPITC, and (d) confirmed by database matching of the intact peptide. Red frame represents peptide data set derived from negative and positive MSMS spectra.

The lower production of lactic acid in 75-day-old clones was due to the differential expression of glucose-catabolizing enzymes (e.g., lactate dehydrogenase). Lactate dehydrogenase enzyme reduces pyruvate for the regeneration of NAD^+, and lactate is the end product of this reaction. According to the literature, inactivation of this enzyme affected the lactic acid production of another LAB, *Lactobacillus johnsonii*.[25] Additionally, the acetoin dehydrogenase complex, E1 component, alpha

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Table 1. Identification of differentially expressed proteins in 1-day-old cells and 75-day-old clones after 2-DE by CAF−/CAF+ analysis

Protein name	Accession no. (gi)[a]	Metabolic pathway or protein function	Protein expression	ID/6[b]
(a) Proteins that are not expressed in 75-day-old clones				
Glycerol dehydrogenase	116334720	Glycerolipid metabolism	–	3/6
Phosphoglycerate mutase family protein	116334724	Glycolysis/gluconeogenesis	–	3/6
Hypothetical protein LVIS_2086	116334646	Unnown	–	3/6
Lactate dehydrogenase related 2-hydroxyacid dehydrogenase	116333957	Pyruvate metabolism	–	3/6
Aryl-alcohol dehydrogenase related enzyme	116334825	Energy metabolism	–	2/6
Phosphoglycerate mutase	116334603	Glycolysis/gluconeogenesis	–	2/6
Glutathione reductase	116333096	Glutathione metabolism	–	1/6
Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	116334209	Aminoacyl-tRNA biosynthesis	–	1/6
Phosphotransacetylase	116333321	Pyruvate metabolism	–	1/6
Glycerol-3-phosphate dehydrogenase	116333292	Glycerophospholipid metabolism	–	1/6
S-ribosylhomocysteinase	116333010	Cysteine and methionine metabolism	–	1/6
Acetoin dehydrogenase complex, E1 component, alpha subunit (*pI* = 5.5)	116334012	Glycolysis/gluconeogenesis	–	1/6
Bifunctional acetaldehyde-CoA/ alcohol dehydrogenase	116332794	Glycolysis/gluconeogenesis	–	1/6
(b) Proteins that are overexpressed in 75-day-old clones				
Glucose-6-phosphate 1-dehydrogenase	116332772	Pentose phosphate pathway	+	3/6
Acetoin dehydrogenase complex, E1 component, alpha subunit (*pI* = 5.8)	116334012	Glycolysis/gluconeogenesis	+	2/6
Acetoin/pyruvate dehydrogenase complex E2 component, dihydrolipoamide succinyltransferase	116334010	Glycolysis/gluconeogenesis	+	2/6
Acetate kinase	116333800	Pyruvate metabolism	+	2/6
Dihydrolipoamide dehydrogenase	116334009	Pyruvate metabolism	+	2/6
Aldo/keto reductase	116334411	Energy metabolism	+	2/6
Cell division initiation protein	116334044	Cell division	+	2/6
Translation elongation factor P	116333618	Translation	+	2/6
Pyrroline-5-carboxylate reductase	116333191	Arginine and proline metabolism	+	1/6
Alcohol dehydrogenase	116333656	Energy metabolism	+	1/6
NAD(FAD)-dependent dehydrogenase (*pI* 4.8)	116334141	Energy metabolism	+	1/6
NAD(FAD)-dependent dehydrogenase (*pI* 4.7)	116334141	Energy metabolism	+	1/6
Phosphoglycerate dehydrogenase-like protein	116334222	Amino acid transport and metabolism	+	1/6
50S ribosomal protein L4	116334268	50S ribosome assembly	+	1/6
UDP-glucose pyrophosphorylase	116333293	Pentose and glucuronate interconversions	+	1/6
DNA-binding response regulator	116332709	Two-component system	+	1/6
Oxidoreductase	116333485	Energy metabolism	+	1/6
Ribosome-associated protein Y	116333275	Stress-response protein	+	1/6
Elongation factor Tu	116333991	Protein biosynthesis	+	1/6
CTP synthetase	116333149	Nucleotide metabolism	+	1/6
Recombinase A	116333847	Homologous recombination	+	1/6
NADH dehydrogenase, FAD-containing subunit	116332990	Energy metabolism	+	1/6
Nucleoside phosphorylase	116334039	Cysteine and methionine metabolism	+	1/6
Maltose phosphorylase	116333027	Starch and sucrose metabolism	+	1/6

[a]GenInfo Identifier (gi) number of NCBI nr database.
[b]Positive protein identifications in six separately cultivated 75-day-old clones.

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subunit was found to be expressed, with different *pI* values, in both 1-day-old cells and 75-day-old-clones; this finding indicates that this enzyme underwent a mutation or postranslational modification in the 75-day-old clones that caused a divergence in the *pI* values.

Further proteomics analysis confirmed the activation of specific *L. brevis* metabolic pathways during starvation. Although the 75-day-old clones have been recultivated after growth in starvation conditions, the overexpression of the enzymes involved in amino acid metabolism was preserved. Nucleoside phosphorylase, an integral component in cysteine and methionine metabolism,[22] and pyrroline-5-carboxylate reductase, which is involved in arginine and proline metabolism,[26] continued to be highly overexpressed in the 75-day-old clones.

CONCLUSIONS

The application of CAF−/CAF+ technology after in-gel tryptic digestion, as outlined in the current study, provides a powerful method for high-sensitivity *de novo* peptide sequencing using MALDI-TOFTOFMS. This approach to *de novo* peptide sequencing was used to detect 36 differentially expressed proteins in *L. brevis* in 75 days of nutrient deprivation. The procedure enabled accurate identification of changes in amino acid transport, translation and ribosomal structure, stress response, energy production and conversion and metabolic pathways, mostly in carbohydrate-, amino acid-, lipid-, and nucleotide-corresponding proteins (validated through MS/MS spectra obtained in both negative and positive ion mode). Furthermore, the mutations were identified as sequence discrepancies between our samples and the sequences in the NCBI nr database (derived from CAF−/CAF+ applied technology, peptide sequence readings were confirmed from both positive and negative ion modes). The MS experiment was additionally confirmed by *L. brevis* DNA sequencing (Supplementary Fig. S1 and Table S1 in the Supporting Information). Differentially expressed proteins (Table 1) identified exclusively by peptide sequence reading provided promising results for CAF−/CAF+ implementation in a standard proteomics workflow (e.g. biomarker and mutation discovery and biotyping). The practical performance of a reliable *de novo* sequencing technique in routine proteomics analysis is emphasized.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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