

Steady state growth space study of *Lactococcus lactis* in D-stat cultures

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Abstract Growth space of *Lactococcus lactis* subsp. *lactis* IL1403 was studied at constant growth rate using D-stat cultivation technique. Starting from steady state conditions in a chemostat culture ($\mu = 0.2 \text{ h}^{-1}$), the pH and/or temperature were continuously changed in the range of 5.4–6.4 and 26–34°C, respectively, followed by the return to the initial environmental conditions. Based on substrate consumption and product formation yields and expression changes of 1,920 genes, it was shown that changes of physiological state were not

dependent on the direction of movement (from pH 6.3 to 5.4 or from 5.4 to 6.3), showing that quasi steady state values in D-stat corresponded to the steady state values in chemostats. Relative standard deviation of growth characteristics in triplicate D-stat experiments was below 10%. Continuing the experiment and reestablishing initial growth conditions revealed in average 7% difference (hysteresis) in growth characteristics when comparing chemostat steady state cultures prior and after the change of environmental conditions. Similarly, shifts were also seen at gene expression levels. The large amount of quantitatively reliable data obtained in this study provided a new insight into dynamic properties of bacterial physiology, and can be used for describing the growth space of microorganisms by modeling cell metabolism.

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Introduction

We have entered the era of synthetic biology with high throughput DNA sequencing (genetic) and omics methods, which in principle should make comprehensive quantitative characterization of molecular physiology of cells possible. However, cultivation methods where cells are in defined physiological states (Hoskisson and Hobbs 2005) and thus quantitatively

reliable data on cell metabolism could be obtained, have found little use in practice. Until present, multidimensional growth space has been systematically determined, and studied in batch cultures (Le Marc et al. 2005, see also <http://www.bioprocessors.com/>). However, batch experiments do not supply information about the growth space dynamics. For obtaining data on dynamic properties of the bacteria, they have to be cultivated preferably in continuous cultures in defined physiological states. Defined physiological states could be most readily realized in chemostat cultures. In spite of that, chemostat has not been used in bacterial physiology studies as widely as expected because the method is quite complex, laborious and time-consuming.

Due to its industrial importance, *L. lactis* is one of the best-studied microorganisms. However, a limited number of studies about growth dynamics—especially about dynamics of change of physiological state of cell on the change of growth conditions, could be found in the literature. Effects of pH, oxygen and dilution rate on the growth of *L. lactis* in chemostat have been studied by Thomas et al. (1979), O’Sullivan and Condon (1999), Even et al. (2001, 2003), Jensen et al. (2001), Dressaire et al. (2008). Dressaire et al. have made the first attempt to characterize the growth of *L. lactis* at the whole genome level using global transcriptome profiling.

We have previously shown that A-stat (chemostat with smooth change of dilution rate) is reproducible, informative and efficient cultivation method for studying physiology of cells at different specific growth rates in (quasi) steady state (Adamberg et al. 2009), provided that the change of growth conditions does not disrupt the quasi steady state of growing cells. Results of the *L. lactis* D-stat cultivation experiments (Kasemets et al. 2003) with smooth change of pH and/or temperature are reported in this paper. The rates of pH and temperature change sufficient for maintaining the quasi steady state growth of the culture during the transitions were determined, and the results obtained showed that D-stat cultures are reproducible tools for scanning the growth space of growing cells. In addition to the more conventional growth characteristics (Y_{xs} , Y_{lact} etc.) measured, also changes in transcriptome patterns of *L. lactis* IL1403 were investigated in different growth conditions. To our knowledge, this is the first

attempt made to characterize quasi steady state growth in multidimensional growth space of *L. lactis*.

Materials and methods

Bacterial strain and culture medium

The cultivated strain *Lactococcus lactis* subsp. *lactis* IL1403 was kindly provided by Dr. Ogier from INRA (Jouy-en-Josas, France). Inoculum was prepared using a lyophilized stock culture stored at -80°C which was pre-grown twice on the cultivation medium. The composition of the cultivation medium used was as follows (g L^{-1}): limiting substrate glucose—5, alanine—0.24, arginine—0.125, asparagine—0.21, aspartate—0.21, cysteine—0.13, glutamate—0.2, glutamine—0.1, glycine—0.175, histidine—0.15, isoleucine—0.33, leucine—0.66, lysine—0.44, methionine—0.125, phenylalanine—0.275, proline—0.225, serine—0.52, threonine—0.225, tryptophan—0.05, tyrosine—0.08, valine—0.33, K_2HPO_4 —3, KH_2PO_4 —2.5, NaCl —2.9, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —0.2, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ —0.05, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ —0.016, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ —0.005, $\text{CoCl}_2 \cdot 5\text{H}_2\text{O}$ —0.003, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ —0.003, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ —0.003, $\text{FeSO}_4 \cdot 4\text{H}_2\text{O}$ —0.0014, pyridoxine—0.002, biotin—0.001, folic acid—0.001, niacin—0.001, pantothenic acid—0.001, riboflavin—0.001 and thiamine—0.001.

Cultivation system

The D-stat cultivation system consisted of a 1.25 L Biobundle bioreactor (Applikon, Schiedam, the Netherlands) controlled by an ADI 1030 biocontroller (Applikon) and a cultivation control program “BioXpert NT” (Applikon). The system was equipped with pH, pO_2 and temperature sensors. Two variable speed pumps (feeding and out-flow) were controlled using “BioXpert NT” control software. The bioreactor was set on a balance, which output was used as the control variable to ensure constant culture volume (300 ± 1 mL). Similarly, the inflow was determined by measuring the mass of fresh culture medium added. Cultivations were carried out under anaerobic conditions (N_2 -environment) with an agitation speed of 300 rpm at 34°C and pH 6.4, unless otherwise stated.

pH of the culture was controlled using 2 M NaOH and temperature with a heating blanket.

D-stat cultivation

The D-stat cultivation technique has been previously described by Kasemets et al. (2003). The principle is similar to chemostat where the culture is initially “stabilized” in a steady state at a chosen dilution rate (*D*). After a steady state is obtained, smooth change(s) of environmental parameter(s) (e.g. pH, temperature, substrate concentration) is (are) started while keeping the dilution rate constant. In our experiments, 300 mL of cultivation medium was inoculated with 5 mL of an

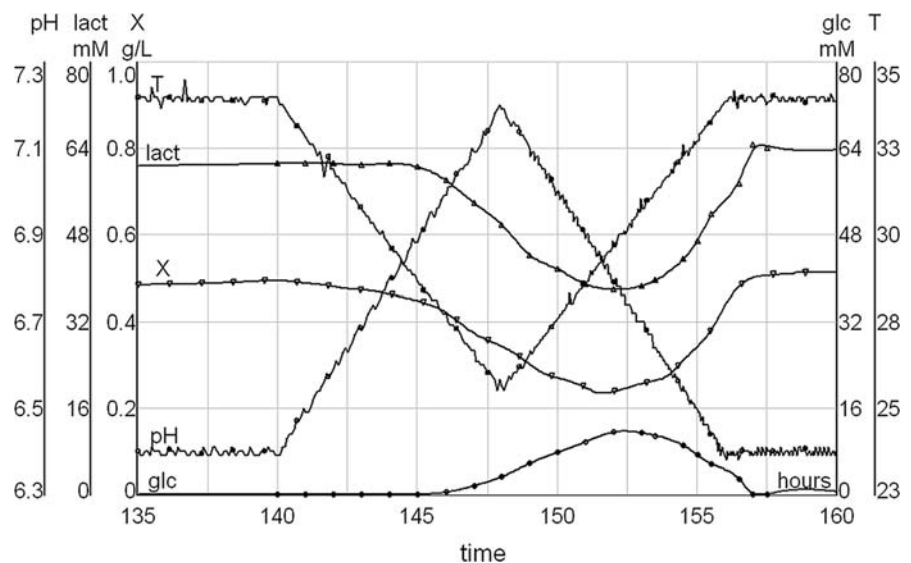
overnight grown culture. When optical density (OD₆₀₀) had reached 1.5–2, dilution rate was slowly increased up to the desired dilution rate where the culture was stabilized. Constant dilution rate *D* = 0.2 h⁻¹ was maintained throughout all experiments. After pumping through at least five working volumes of fresh medium for obtaining a steady state chemostat culture (OD = const.), smooth change of temperature and/or pH was switched on (Figs. 1, 3) at the changing rates presented in Table 1. After reaching the desired pH or temperature, the change of environmental conditions were reversed, and pH and/or temperature were changed back to the initial values, either immediately (Fig. 1) or after a period during which the

Table 1 Overview of *L. lactis* IL1403 D-stat experiments at dilution rate 0.2 h⁻¹

	Parameter(s) changed	Acceleration rate	Range	Units
Expt. 1	pH	0.1 U h ⁻¹	6.4–7.2–6.4	pH
	Temp.	1°C h ⁻¹	34–26–34	°C
Expt. 2	pH	0.1 U h ⁻¹	6.4–5.6–6.4	pH
	Temp.	1°C h ⁻¹	34–26–34	°C
Expt. 3	pH	0.025 U h ⁻¹	6.4–5.6–6.4	pH
	Temp.	0.25°C h ⁻¹	34–26–34	°C
Expt. 4	pH	0.02 U h ⁻¹	6.3–5.4–6.3	pH
Expt. 5	pH	0.0125 U h ⁻¹	6.1–5.4–6.1	pH
Expt. 6	pH	0.02–0.006 U h ^{-1(a)}	6.4–5.6–6.4	pH
Expt. 7	pH	0.02 U h ⁻¹	5.4–6.1–5.4	pH
Expt. 8	Temp.	0.25°C h ⁻¹	34–27–34	°C

^a Acceleration of pH was decreased gradually based on biomass formation and lactate production

Fig. 1 *Lactococcus lactis* IL1403 D-stat exp. 1 with simultaneous two-parameter change (pH and temperature). glc—glucose concentration in bioreactor (mM); lact—lactate concentration in bioreactor (mM); T—temperature (°C), X—biomass concentration in bioreactor (g L⁻¹)



culture was “stabilized” in chemostat (Fig. 2). Stability of the culture growth characteristics in chemostat was used to validate quasi steady state growth of the bacteria during the change of pH and/or temperature.

Analytical methods

The concentrations of organic acids (lactate, acetate and formate), ethanol and glucose in the culture media were analyzed by liquid chromatography (Alliance 2795 system, Waters Corp., Milford, MA), using a BioRad HPX-87H column (Hercules, CA) with isocratic elution of 0.005 M H₂SO₄ at a flow rate of 0.6 mL min⁻¹ and at 35°C. UV (210 nm; model 2487;

Waters Corp.) and refractive index (RI) detectors (model 2414; Waters Corp.) were used for detection and quantification of the substances. Detection limit for the analytical method was 0.1 mM. Samples from culture media were centrifuged (14,000g, 4 min), supernatants were collected and analyzed immediately or stored at -20°C until analysis. Amino acid concentrations were determined from the same sample with an amino acid analyzer (UPLC; Waters Corp.) according to the manufacturer’s instructions. Empower software (Waters Corp.) was used for the data processing.

Biomass concentration was calculated by measuring the optical density at 600 nm using a biomass

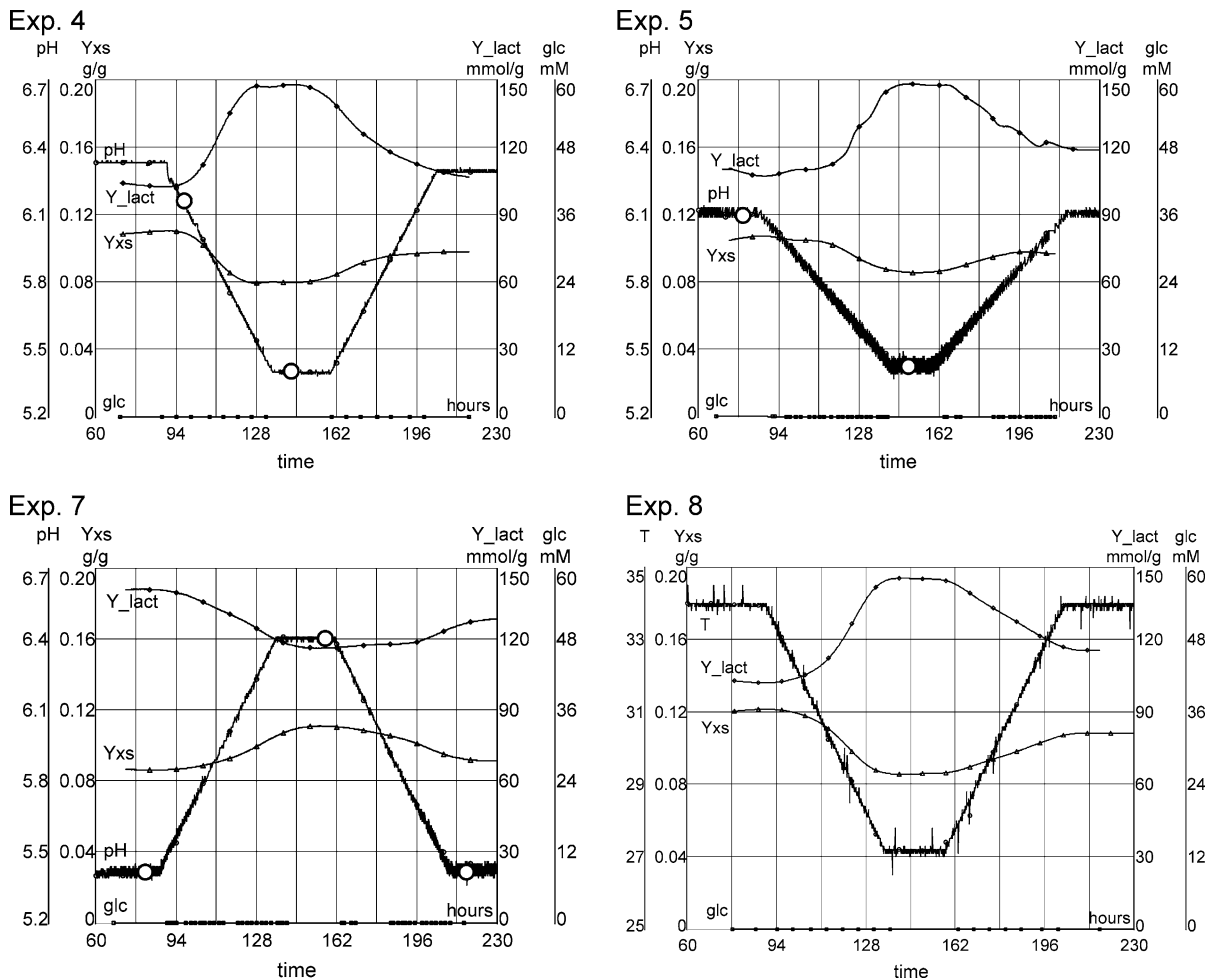


Fig. 2 *Lactococcus lactis* IL1403 D-stat experiments with the change of pH or temperature. Descriptions of the experiments can be seen in Table 1. Circles indicate sampling points for transcriptome analysis which in each experiment were

compared to one another. glc —glucose concentration in bioreactor (mM); Y_{lact} —lactate yield based on biomass production (mmol lact gdw^{-1}); T —temperature (°C); Y_{xs} —biomass yield based on glucose consumption (g gdw^{-1})

conversion factor $K = 0.30 \pm 0.02 \text{ g L}^{-1} \text{ OD}_{600}^{-1}$ ($K = X/\text{OD}_{600}$, where X is in g dry weight [gdw] L^{-1}). Dry weight of bacteria was determined gravimetrically by centrifuging biomass from 15 mL of culture, washing it multiple times with distilled water and drying at 100°C for 24 h.

Global transcription profiling

Microarrays used in this study were purchased from Eurogentec (Seraing, Belgium) and contained 1,920 open reading frames (ORF) of *L. lactis* spotted in duplicates on standard $2.5 \times 7.5 \text{ cm}$ glass slides.

Samples of biomass were fixed with RNa protect solution (QIAGEN, Valencia, CA)—the samples of culture media were mixed with RNa protect solution (1:1 ratio), incubated for 5 min at room temperature and centrifuged for 10 min at $8,000g$. Supernatant was discarded and the pellet stored at -80°C . Total RNA was extracted with RNeasy Mini Kit (QIAGEN) and genomic DNA was removed using RNase-Free DNase Set (QIAGEN). After addition of $500 \mu\text{L}$ lysozyme (10 mg lysozyme in 1 mL TE pH 8; Amresco Inc., Solon, OH), three freeze-thawing cycles were carried out followed by incubation at 37°C for 1 h. $20 \mu\text{L}$ of proteinase K solution (20 U proteinase K in 1 mL; Amresco Inc.) was added and incubated for 1 h at 37°C before RNA extraction.

cDNA was synthesized from $15 \mu\text{g}$ of total RNA at 46°C overnight using the following reagents per reaction: $1 \mu\text{g}$ random primers (Random decamers; Invitrogen, Carlsbad, CA), $6 \mu\text{L}$ five times concentrated first strand buffer (Invitrogen), $3 \mu\text{L}$ 0.1 M DTT (Invitrogen), $0.9 \mu\text{L}$ dNTPs (final concentrations: dATP 0.5 mM , dCTP 0.5 mM , dGTP 0.5 mM , dTTP 0.3 mM , aminoallyl-dUTP 0.2 mM), $1 \mu\text{L}$ RNase Inhibitor (Bioron, Ludwigshafen, Germany), $2 \mu\text{L}$ Superscript III (Invitrogen). RNA strands were hydrolyzed in the samples by adding $4.5 \mu\text{L}$ 1 M NaOH and incubating at 70°C for 10 min, after incubation the samples were neutralized with $4.5 \mu\text{L}$ 1 M HCl. cDNA was purified with MinElute PCR purification kit (QIAGEN) and labeled with Cy3 (CyTM3 Mono Reactive Dye Pack, Amersham, Buckinghamshire, UK) or Cy5 (CyTM5 Mono Reactive Dye Pack, Amersham). Staining was carried out in $10 \mu\text{L}$ of NaHCO_3 (pH 9) at room temperature in dark for 1 h and the samples were purified again with MinElute PCR purification kit. Subsequently, the following

hybridization master mix was used (per slide): $20 \times \text{SSC}$ $16.3 \mu\text{L}$, 100% formamide $32.5 \mu\text{L}$, 10% SDS $0.65 \mu\text{L}$. The hybridization was carried out at 42°C for 16 h.

Analysis of gene expression data

Microarray slides were scanned using an Agilent DNA Microarray Scanner (Santa Clara, CA). Spot intensities and corresponding background signals were quantified with Genepix Pro (version 6; Axon Instruments [http://www.moleculardevices.com/pages/software/gn_gene_pix_pro.html]). Spots which had a signal-to-noise ratio less than three or intensities of both, 635 and 532 nm channels lower than 500 units were filtered. Further analysis was carried out in R environment (version 2.6.1; R Development Core Team [<http://www.r-project.org/>]) using KTH package (KTH Microarray Center [<http://www.biotech.kth.se/molbio/microarray/dataanalysis/index.html>]). Flagged spots and background were extracted before “printTipLoess” normalization.

Calculation of growth characteristics

Growth characteristics of bacteria were calculated based on OD of the culture, total volume of medium pumped out from bioreactor (L) and lactate or glucose concentrations in culture medium (mol L^{-1}) as follows:

$$\mu = \{[d(V_{\text{out}})]/(V \times dt)\} + \{[d(\text{OD})]/(dt \times \text{OD})\}$$

$$Q_{\text{glc}} = \{[\Delta S_{\text{glc}} \times d(V_{\text{out}})]/(V \times \text{OD} \times K \times dt)\} - \{[d(\Delta S_{\text{glc}})]/(dt \times \text{OD} \times K)\}$$

$$Y_{\text{XS}} = \mu/Q_{\text{glc}}$$

$$Q_{\text{lact}} = \{[\text{lact} \times d(V_{\text{out}})]/(V \times \text{OD} \times K \times dt)\} - \{[d(\text{lact})]/(dt \times \text{OD} \times K)\}$$

$$Y_{\text{lact}} = Q_{\text{lact}}/\mu,$$

where μ is specific growth rate (h^{-1}); lact is concentration of produced lactic acid (mmol L^{-1}); Q_{glc} is specific glucose consumption rate ($\text{g glc gdw}^{-1} \text{ h}^{-1}$ or $\text{mmol glc gdw}^{-1} \text{ h}^{-1}$); ΔS_{glc} is amount of consumed glucose (g glc L^{-1} or mmol glc L^{-1}); Y_{XS} is biomass yield calculated on glucose consumption ($\text{gdw g glucose}^{-1}$); Q_{lact} is specific production rate of lactate ($\text{mmol lact gdw}^{-1} \text{ h}^{-1}$); V is bioreactor volume (L); Y_{lact} is lactate yield based on biomass

production ($\text{mmol lact gdw}^{-1}$); OD is optical density at 600 nm; V_{OUT} is total volume of medium pumped out from bioreactor (L); K is biomass conversion factor (see above for equation) and t is running cultivation time (h).

Growth space visualization

3-D growth space was visualized using Datafit program (version 9.0; Oakdale Engineering [<http://www.curvefitting.com>]) with polynomial fit [$Y_{\text{XS}} = a + b/\text{pH} + c/\text{pH}^2 + d \times \ln T + e \times (T)^2$] and quasi steady state data points (the chosen regression represented our data points the best).

Results

D-stat cultivation experiments with continuous change of one parameter (pH or temperature) or simultaneous change of two parameters (pH and temperature) were carried out, and growth characteristics of *L. lactis* IL1403 in different pH and/or temperature conditions at dilution rate 0.2 h^{-1} were studied (see Table 1). Thus continuous moving in two-dimensional bacterial growth sub-space was performed. As can be seen on Figs. 1 and 2, environmental conditions were changed in D-stat experiments back and forth, where initial growth conditions were restored in the end of the experiments. Special attention was paid to make sure whether the initial physiological states (steady states) were reinstated after the back and forth change of growth conditions.

Experiments with two-parameter change (exp. 1, 2, 3 in Table 1; Fig. 1) showed a possibility for culture characteristics to return to their initial values after initial optimal growth conditions were reestablished. However, it has to be noted that the culture was not in quasi steady state throughout the whole experiment (see transient increase of residual glucose concentration on Fig. 1). This indicated that changing rates of pH and temperature, 0.1 U h^{-1} and 1°C h^{-1} respectively, were too fast to enable the culture to adapt to the changing environmental conditions, and maintain steady state growth in the glucose limited state. Hence, single parameter change experiments with slower changing rates (see Table 1) were carried out subsequently to make sure that the bacteria were in the

glucose limited quasi steady state during the full course of the experiments.

No glucose was observed in the culture media during the transition from the initial stabilization value of pH (pH 6.1–6.4) until pH 5.4–5.6 (exp. 4–6; Table 1), indicating maintenance of quasi steady state growth in these conditions (for exp. 4 and 5, see also Fig. 2). To prove the maintenance of the steady state, D-stat experiments were switched into chemostat at the lowest pH values and kept in constant environmental conditions for over five generations. Results obtained in latter chemostat showed that production rates of biomass and main growth by-products were maintained constant, varying only in the range of measurements error ($<5\%$) (Fig. 2). In addition, Y_{lact} and Y_{XS} values at pH 5.4 in exp. 7 (initial chemostat) and quasi steady state values at pH 5.4 in exp. 4 and 5 (after the first transition) were similar in the range of standard deviation. Furthermore, it was observed that growth characteristics were not dependent on the direction of pH movement (from pH 6.3 to 5.4 in exp. 4 or from 5.4 to 6.3 in exp. 7), showing that quasi steady state values in D-stat were identical to steady state values in chemostat. Hence, it can be assumed that the bacteria were in (quasi) steady state during the entire transition. Maintenance of quasi steady state growth during the change of temperature from 34 to 27°C was proved also in exp. 8.

Growth characteristics

For the comparison of four D-stat cultivations with pH changing rates below 0.02 U h^{-1} (quasi steady state growth; exp. 4–7), biomass yield per consumed glucose (Y_{XS}) and lactic acid yield per biomass produced (Y_{lact}) were calculated. Y_{XS} was $0.108 \pm 0.003 \text{ gdw g glucose}^{-1}$ at optimal pH, and decreased to $0.084 \pm 0.003 \text{ gdw g glucose}^{-1}$ at pH 5.4. Small relative standard deviation (6%) of the Y_{XS} values determined in four experiments showed very good reproducibility of D-stat method. The value of Y_{lact} decreased with the decrease of pH from $144 \pm 11 \text{ mmol gdw}^{-1}$ at optimal pH to $106 \pm 14 \text{ mmol gdw}^{-1}$ at pH 5.4 with the average standard deviation value of 9%. Similarly, changes in Y_{lact} and Y_{XS} were observed in the experiments with simultaneous pH and temperature change (exp. 1, 2, 3 in Table 1) as well. However, biomass yield (Y_{XS}) coefficients values at pH 5.6 and temperature 26°C were about 15% lower in

comparison with those observed in the case of only changing pH or temperature—see exp. 4–7. This can be explained by applying higher adaptive stress on the metabolism in the case of simultaneous pH and temperature change in double-parameter experiments than in the single-parameter experiments, and by the fact that changing rates used were higher ($a_{\text{pH}} = 0.025 \text{ U h}^{-1}$ and $a_{\text{T}} = 0.25^\circ\text{C h}^{-1}$) in the double-parameter experiments.

All described cultivations could be considered as homolactic—lactate production comprised more than 90% of all products observed, and the share of lactate slightly increased in higher stress conditions.

Transcriptome

To characterize the effects of changing pH on the physiological state of the bacteria more thoroughly, global transcriptome measurements were carried out at different quasi steady state growth conditions. Samples taken for comparison are shown in Fig. 2 and the list of measured genes which expression changed more than 1.8 times in different environmental conditions may be found in supplementary materials (<http://www.tftak.eu/?id=69>).

Forty-one genes which expression changed more than 1.8 times were observed as the result of the first pH change, i.e. as the result of moving away from the initial chemostat conditions in exp. 4, 5 and 7 (Fig. 2 exp. 4, 5 and 7). The most remarkable change at transcriptional level took place in arginine (urea cycle) metabolism, where expression of genes *arcA*, *arcC1*, *arcC2*, *arcD1*, *argE* and *argR* increased 2- to 6.25-fold on lowering the pH. When moving from acidic conditions to optimal pH values, expression levels of the same genes decreased (in exp. 7) as a response to the change of pH—independent of the movement direction in growth space. It must be noted that the concentration of arginine in the culture media was below the detection limit (0.01 mM) during the whole experiment. Citrate cycle genes *citC*, *citD* and *citE* were down-regulated when lowering the pH and up-regulated when the pH was returned to the initial value. Arginine and citrate formation pathways are well-known as taking part in the regulation of survival against acid stress by increasing internal pH or generating additional metabolic energy (Konings 2002). In the case of *lysQ*, which encodes a lysine/histidine specific transport permease protein (Vitreschak et al. 2004), a

significant fivefold up-/down-regulation was observed while pH was decreased/increased, respectively. As the overall consumption of lysine and histidine were less than the error of the measurement (5%), we can only assume that shifts in the expression of the genes responsible for lysine/histidine transport were necessary to adapt to the varying pH conditions. Moreover, based on homology analysis, genes *yxbE* and *yxbF*, which are known to encode universal stress proteins, were strongly up-regulated at lower pH conditions. All other detected major changes in transcriptomes concerned hypothetical or poorly studied proteins (see supplementary materials).

Hysteresis

As can be seen from Fig. 2, biomass concentration in chemostat in the beginning of the experiments and after the return to the initial conditions in the end of the experiments differed by $8 \pm 3\%$, indicating hysteresis in the physiological states of the culture. Production of lactate per biomass and glucose consumption per biomass was lower in the end of the experiments (exp. 4–8) by 6–13%, in comparison with the beginning of the experiments. Microarray analysis revealed that the expression levels of certain arginine and citrate metabolism genes—*arcA*, *arcC1*, *arcC2*, *arcD1*, *argE*, *argR* and *citC*, *citD*—changed during the first pH shift. However, instead of recovering their expression after moving back to initial environmental conditions, these genes remained expressed at the level attained during the first pH shift (exp. 7 in Fig. 2). See also supplementary materials for more information about transcription differences during the back and forth changes of the growth conditions (hysteresis).

It was shown in three additional parallel chemostat experiments with *L. lactis* that no changes, neither in biomass concentration, nor in the levels of main metabolites were observed at optimal growth conditions during 70 generations (data not shown). However, as mentioned above, after reducing of the changing rates used in the experiments 4–8 which led to the remarkable prolongation of the D-stat experiments, and the time while the bacteria were exposed to the changed environmental conditions (mild stress), hysteresis of the gene expression and growth parameters were observed in our experiments. Although significant, further characterization and

study of the hysteresis was out of the scope of the present study, and was postponed to the future.

Discussion

The results obtained showed that D-stat cultivation method made it possible to reproducibly scan relatively large growth space areas of *L. lactis* in reasonable time in quasi steady state at fixed dilution rate. 3D graph describing the dependence of biomass yield (based on glucose consumption) on temperature and pH for the bacteria is shown on Fig. 3. The differences between modelled surface and actual data points were less than 10%. The region of the optimal growth conditions in the studied range is indicated by the lightest area on the surface on Fig. 3. Simultaneous change of pH and temperature led to a more noticeable change of growth parameters in comparison with a single parameter change, as expected.

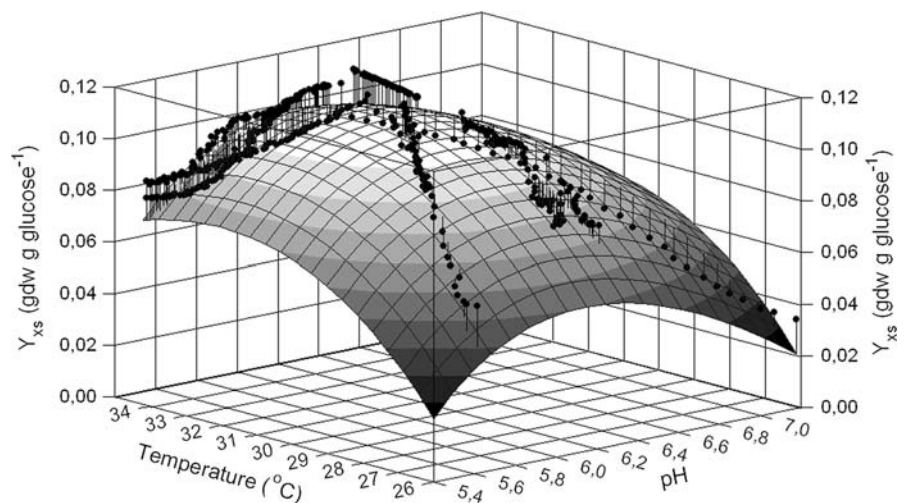
Important issues in planning and carrying out D-stat (changestat) experiments are duration of cultivation experiments, range of growth conditions scanned and choice of changing rates, which should enable to maintain quasi steady state. Duration of the cultivation experiments might be a crucial factor in prolonged experiments. It has been shown previously that extended chemostats (100–1,000 generations) could be used for studying culture adaptation and selecting mutated strains with special characteristics (Francis and Hansche 1973; Maharjan et al. 2007). The observation that bacteria can be found in

different physiological states in the same environmental conditions before and after applying mild stress in D-stat experiments during 60 generations was made in the present study. The data obtained in our experiments indicated that the changed environmental conditions applied in the D-stat experiments might lead to the onset of the adaptive mechanisms in shorter time than in the constant environmental conditions in chemostat.

The hysteresis of physiological states of the D-stat cultures observed seemed to be phenomenologically similar to the temporary application of the sub-lethal stress after which the vitality of microorganisms have been improved and maintained even after the cessation of stress conditions (Sánchez et al. 2007). Even though sub-lethal stress is still not sufficiently studied at molecular level, three general hypotheses for the explanation of this phenomenon have been formulated: (1) some stress response proteins are expressed in stress conditions and maintained expression of these genes leads to the increase of the survival even long after the cessation of the stress; (2) mutations take place during the sub-lethal stress, or (3) some mutated sub-populations could survive with more ease than the main population. Taking into account these possibilities, more detailed analysis of stress and hysteresis should be carried out in the future, investigating especially possible mutations—e.g. sequencing genomes of the culture during the cultivation.

Another important factor in carrying out changestat experiments is the changing rate of environmental parameters. It has been previously shown that the

Fig. 3 *Lactococcus lactis* IL1403 quasi steady state growth surface describing the dependence of biomass yield (based on glucose consumption) on temperature and pH for the bacteria. The surface is based on all D-stat experiments. Y_{xs} —biomass yield calculated on glucose consumption ($\text{gdw g glucose}^{-1}$). Black dots represent quasi steady state data points from exp. 1–8



farther the culture is moved from optimal growth conditions, the lower changing rates have to be applied in order to maintain quasi steady state (Adamberg et al. 2009; O'Sullivan and Condon 1999; van der Sluis et al. 2001). Therefore, identification of physiological parameters, which would indicate the (forthcoming) loss of quasi steady state, is a critical issue in carrying out changestat experiments in practice, especially when developing and applying adaptive algorithms. The most suitable indicators of physiological states are the parameters, which could be measured online—e.g. optical density, titration rate, gas production rate etc. In addition, substrate consumption and product formation can be used. Indeed, it has been shown that online measurements of carbon dioxide evolution could be successfully used for the elucidation of the response of the metabolism to the change of the growth conditions (Vemuri et al. 2006). Additionally, indicator molecules of overflow metabolism (acetate, ethanol) in microorganisms having respirofermentative growth like *E. coli* and yeast could be used for adaptive control (Jobé et al. 2003). However, the changes of these metabolic characteristics can also be a “normal” quasi steady state physiological response of the cell metabolism to the change of the environmental conditions. Therefore, the most reliable, though time-consuming way to check the maintenance of steady state growth in changestat experiments is the “chemostat control”, i.e. halting the changing rate and continuing the experiment in chemostat. If all physiological parameters (production or consumption rates of metabolites per biomass etc.) remain constant, it can be concluded that the changestat (D-stat, etc.) culture was in a quasi steady state before the acceleration was stopped. This kind of control was also routinely used in our experiments.

A method for scanning the growth space of microorganism was tested in this paper. Use of D-stat cultivation method allowed obtaining a large amount of steady state data in multidimensional quasi steady state growth space of *L. lactis* in reasonable time and effort. It was possible to study reproducibly and quantitatively adaptive responses of growth characteristics as well as gene expression at transcriptome level while changing the pH and temperature in D-stat cultures.

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