Protein turnover forms one of the highest maintenance costs in *Lactococcus lactis*

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Protein turnover plays an important role in cell metabolism by regulating metabolic fluxes. Furthermore, the energy costs for protein turnover have been estimated to account for up to a third of the total energy production during cell replication and hence may represent a major limiting factor in achieving either higher biomass or production yields. This work aimed to measure the specific growth rate (μ)-dependent abundance and turnover rate of individual proteins, estimate the ATP cost for protein production and turnover, and compare this with the total energy balance and other maintenance costs. The lactic acid bacteria model organism *Lactococcus lactis* was used to measure protein turnover rates at μ = 0.1 and 0.5 h⁻¹ in chemostat experiments. Individual turnover rates were measured for ~75% of the total proteome. On average, protein turnover increased by sevenfold with a fivefold increase in growth rate, whilst biomass yield increased by 35%. The median turnover rates found were higher than the specific growth rate of the bacterium, which suggests relatively high energy consumption for protein turnover. We found that protein turnover costs alone account for 38 and 47% of the total energy produced at μ = 0.1 and 0.5 h⁻¹, respectively, and gene ontology groups Energy metabolism and Translation dominated synthesis costs at both growth rates studied. These results reflect the complexity of metabolic changes that occur in response to changes in environmental conditions, and signify the trade-off between biomass yield and the need to produce ATP for maintenance processes.

INTRODUCTION

Recently, protein turnover rates have been recognized as an important factor that provides growth advantage both in terms of higher maximal specific growth rate (μ) and biomass yield between different strains (Hong et al., 2012), and in laboratory evolution experiments (González-Ramos et al., 2013). In addition, protein synthesis and degradation are firmly related and therefore represent an important control factor for metabolic regulation (Schwanhäusser et al., 2013). Individual protein turnover rates have been determined in mammalian cells, yeast and bacteria by measuring the incorporation rate of fluorescent tags (Khmelinskii et al., 2012), affinity tags (Belle et al., 2006) or isotopic labels into the proteome (methods reviewed by Hughes & Krijgsveld, 2012; Tötschel et al., 2013). The metabolic incorporation of isotopic labels is currently the most widely used method for cell cultures and conducted using either labelled ammonium (Helbig et al., 2011; Martin et al., 2012), carbon (Cargile et al., 2004) or amino acids (Gerth et al., 2008; Maier et al., 2011; Schwanhäusser et al., 2011). Protein turnover rates are predominantly measured during the logarithmic or stationary phase of batch growth and are assumed to be constant during this phase of growth. Only a few studies report protein degradation rates determined from a steady physiological state (Helbig et al., 2011; Pratt et al., 2002). Although overall protein turnover has been estimated at different temperatures (Tötschel et al., 2012), no study to date has compared individual protein degradation rates determined under different environmental growth conditions (e.g. growing on different substrates or...
specific growth rates) to gain insight into the energetic costs of cellular maintenance. Whilst the growth rate dependence of mRNA turnover rates has been described (Dressaire et al., 2013), analogous studies for protein turnover rates are missing.

*Lactococcus lactis* has become an important model microorganism due to its importance in the food industry and its increasing use as a cell factory for the production of therapeutic recombinant proteins and DNA vaccines (Bahey-El-Din, 2012; Bermúdez-Humarán et al., 2011, 2013; Buonaguro et al., 2011). The physiology, metabolism and genetics of *L. lactis* have been studied intensively, and a number of genetic modification technologies have been developed. As *L. lactis* has lost its ability for oxidative phosphorylation in the absence of haem, it has a relatively simple energy metabolism. During anaerobic growth on glucose energy metabolites are recycled via substrate-level phosphorylation in glycolysis and additional energy can be produced via the arginine deiminase (ADI) pathway. However, not all of the energy produced is used for biomass formation. The energetic costs for biomonomer transport, *de novo* synthesis and polymerization have been estimated previously (Adamberg et al., 2012; Karr et al., 2012; Russell & Cook, 1995; Wodke et al., 2013). Whilst the biomass composition is required to calculate the energetic costs for biomass formation, it is rarely determined, yet often taken from the literature. Moreover, dynamic changes in the biomass composition are usually neglected. Nevertheless, it is well accepted that in addition to biomonomer transport, biosynthesis and polymerization, there are additional costs for cell maintenance. Pirt (1965) defined maintenance energy as ‘the energy consumed for functions other than the production of new cell material’. In more recent studies, maintenance energy has been divided into non-growth components and physiological or growth-related maintenance (van Bodegom, 2007; Taymaz-Nikerel et al., 2010). Whilst global-scale analytical and modelling methodologies are advancing rapidly under the framework of systems biology, only a few studies have quantified various cellular maintenance costs (Karr et al., 2012; Wodke et al., 2013). A number of reviews have discussed various components of maintenance and its dependence on specific growth (van Bodegom, 2007; Russell & Cook, 1995); however, a quantitative understanding of maintenance as a function of specific growth and its dependence on specific growth technologies have been developed. As *L. lactis* has lost its ability for oxidative phosphorylation in the absence of haem, it has a relatively simple energy metabolism. During anaerobic growth on glucose energy metabolites are recycled via substrate-level phosphorylation in glycolysis and additional energy can be produced via the arginine deiminase (ADI) pathway. However, not all of the energy produced is used for biomass formation. The energetic costs for biomonomer transport, *de novo* synthesis and polymerization have been estimated previously (Adamberg et al., 2012; Karr et al., 2012; Russell & Cook, 1995; Wodke et al., 2013). Whilst the biomass composition is required to calculate the energetic costs for biomass formation, it is rarely determined, yet often taken from the literature. Moreover, dynamic changes in the biomass composition are usually neglected. Nevertheless, it is well accepted that in addition to biomonomer transport, biosynthesis and polymerization, there are additional costs for cell maintenance. Pirt (1965) defined maintenance energy as ‘the energy consumed for functions other than the production of new cell material’. In more recent studies, maintenance energy has been divided into non-growth components and physiological or growth-related maintenance (van Bodegom, 2007; Taymaz-Nikerel et al., 2010). Whilst global-scale analytical and modelling methodologies are advancing rapidly under the framework of systems biology, only a few studies have quantified various cellular maintenance costs (Karr et al., 2012; Wodke et al., 2013). A number of reviews have discussed various components of maintenance and its dependence on specific growth (van Bodegom, 2007; Russell & Cook, 1995); however, a quantitative understanding of maintenance as a function of specific growth rate is still lacking. Recently, energy generation and consumption have been quantified in the small bacteria *Mycoplasma pneumonia* (Wodke et al., 2013) and *Mycoplasma genitalium* (Karr et al., 2012). For fast-growing bacteria, both protein turnover and ion leakage are considered to be the main energy-consuming maintenance processes (Russell & Cook, 1995; Taymaz-Nikerel et al., 2010).

In the current study, protein turnover rates were measured in *L. lactis* subsp. *lactis* IL1403 to understand the role of protein turnover from the perspective of physiology and this knowledge was used to elucidate the proportion of protein resynthesis cost from total ATP expenditures under steady-state conditions at two specific growth rates. More specifically, we aimed to determine whether there was a correlation between protein concentrations and turnover rates, and which enzymes or pathways were the most costly for the cell to express. This is the first study that uses this knowledge to simultaneously increase both protein turnover rates and specific growth rate (i.e. metabolic flux rates). The ATP costs for proteome turnover were in good accordance with the energetic calculations, forming 38 and 47 % of the total energy production and 44 and 75 % of the total maintenance expenditures at μ=0.1 and 0.5 h⁻¹, respectively. Specific growth rate-dependent measurement of protein abundance and turnover rates, combined with mRNA and flux levels, could shed additional light on future studies of metabolic regulation; however, this approach was beyond the scope of the current paper.

**METHODS**

Strain and cultivation conditions

**Strain/medium.** *L. lactis* IL1403, acquired from INRA, France, was used in all experiments and grown with the following chemically defined base medium (g l⁻¹): d-glucose, 4.5; l-alanine, 0.0782; l-arginine, 0.1852; l-asparagine, 0.12; l-aspartate, 0.0723; l-cysteine, 0.0656; l-glutamate, 0.0703; l-glutamine, 0.2; l-glycine, 0.0578; l-histidine, 0.0597; l-isoleucine, 0.1018; l-leucine, 0.2072; l-lysine, 0.1576; l-methionine, 0.0407; l-phenylalanine, 0.086; l-proline, 0.0917; l-serine, 0.1634; l-threonine, 0.0758; l-tryptophan, 0.0164; l-tyrosine, 0.0295; l-valine, 0.1072; biotin, 0.305; choline chloride, 9.8; l-pantothenate, 0.65; folic acid, 1.21; niacinamide, 0.325; pyridoxine hydrochloride, 0.642; riboflavin, 0.326; thiamine hydrochloride, 0.51; vitamin B₁₂, 0.98; adenine, 0.025; hypoxanthine-Na, 0.025; MgSO₄·7H₂O, 0.2; FeSO₄·4H₂O, 0.0014; CaCl₂, 0.05; MnSO₄·5H₂O, 0.016; ZnSO₄·7H₂O, 0.0005; CoSO₄·5H₂O, 0.003; CuSO₄·2H₂O, 0.003; (NH₄)₂MoO₄·24H₂O, 0.003; NaCl, 2.9; K₂HPO₄, 3; KH₂PO₄, 2.5. Glucose, amino acids, buffers and minerals were dissolved separately and mixed together after autoclaving in the above-mentioned order to avoid precipitation. Vitamins, nucleotides, cysteine and lysine were added by filter sterilization. Inoculum was prepared from a glycerol stock culture stored at −80 °C. Stock culture was pre-grown twice on the base medium and 2 % (v/v) of overnight-grown culture was inoculated into the bioreactor.

Experiments/switch of medium.** The cultivation system (described in Lahtvee et al., 2011) was run in chemostat mode. Briefly, this system consisted of a Biobundle 1.25 l reactor (Applikon) controlled by an ADI 1030 bioctrlontroller (Applikon), together with the cultivation control program BioXpert (Applikon). Chemostat experiments were carried out in triplicate at each of two dilution rates (0.1 and 0.5 h⁻¹). All cultivation experiments were performed at 34 °C, pH 6.4, in an anaerobic (N₂) environment with an agitation speed of 300 r.p.m. and a working volume of 300 ml. In all chemostat experiments, the lysine concentration in the medium was reduced to a level which did not affect the growth parameters (production yields), but resulted in a joint limitation of glucose and lysine (both concentrations were under the limit of detection). Hence, 0.05 and 0.03 g lysine l⁻¹ were used in the chemostat experiments at dilution rates of 0.1 and 0.5 h⁻¹, respectively. To measure protein degradation rates, the rate of medium inflow was kept constant whilst switching from isotopically unlabelled medium (light) to a medium with identical composition aside from the use of [³⁵C]⁻[¹⁵N]lysine (heavy) (Cambridge Isotope Laboratories). Rapid turnover of lysine in the cultivation environment was required to ensure a rapid switch from
light (unlabelled) lysine to heavy lysine. Samples for proteome analysis were collected from the steady physiological state before the medium switch (0 h), and at 0.5, 1.5, 5 and 10 h following the switch to isotopically labelled heavy medium. Note that the 10 h sample was collected only in the case of the dilution rate of 0.1 h⁻¹. Although WT L. lactis is not auxotrophic for lysine, 97% of lysine in the proteome was labelled in L. lactis IL1403 after roughly nine generations in separate batch cultivation experiments (data not shown).

**Analytics**

**Biomass concentration, composition and extracellular metabolites.** The biomass concentration was monitored by measuring the OD₆₀₀ and the dry weight (DW) was determined gravimetrically. The OD₆₀₀ and DW correlation constant K was 0.31 ± 0.02, and was not found to be specific growth rate dependent.

Samples of culture medium were centrifuged (14 000 r.p.m., 4 min); supernatants were collected and stored at −20 °C until analysis. Levels of glucose, lactate, formate, acetate and ethanol in the culture medium were measured using liquid chromatography (LC; Alliance 2975 system; Waters) with a HPX-87H column (Bio-Rad) and isotropic elution of 5 mM H₂SO₄ at a flow rate of 0.6 ml min⁻¹ at 35 °C. A refractive index detector (model 2414; Waters) was used to both detect and quantify each substance with a detection limit of 0.1 mM. The concentrations of free amino acids were determined using an AccQ-Tag (Waters) method supplied by the manufacturer. Briefly, all samples and an amino acid standard mixture (Waters) were subject to pre-column AccQ-Tag Ultra derivatization and analysed with the Acquity UPLC (ultra performance liquid chromatography) system (Waters). The compounds within derivatized samples were separated using reverse-phase chromatography and detected with a UV detector; and the outflow was fed into an electrospray ionization (ESI) mass spectrometer (LTQ Premier; Waters) operated using the following parameters: negative ionization mode, desolvation gas 700 l h⁻¹ and 200 °C, capillary voltage 2500 V, sample cone 30 V. For each amino acid, we analysed the ratio of heavy to light (H/L) chromatographic peaks using Empower or MassLynx software (Waters). To determine the amino acid composition of the protein content, a biomass sample was hydrolysed with 6 M HCl for 20 h at 120 °C in an N₂ environment with an Eldex Hydrolysis/Derivatization WorkStation (Eldex Laboratories). From this hydrolyte, amino acids and their H/L protein ratios were determined as described above. The H/L ratios of intracellular free amino acids were measured from snap-frozen culture samples which were pelleted by centrifugation (14 000 r.p.m., 4 °C), washed twice with 0.85 % NaCl solution followed by extraction of the intracellular metabolites with a 70 % ethanol solution at 80 °C. This extraction solution was freeze-dried at 0.14 mbar to complete dryness and the free amino acids were determined as described above. Due to the leakage of intracellular metabolites into the extracellular environment, this method cannot be used to determine the concentration of intracellular amino acids; however, it should correctly represent the H/L isotope ratios. The cellular protein content was determined both from an amino acid analysis of dry biomass and measured directly using the Lowry method (Lowry et al., 1951), where BSA was used as a standard.

**Proteome analysis.** L. lactis steady-state culture was harvested in a 2 ml vial (−10⁻³ gDW⁻¹), pelleted by centrifugation at 14 000 r.p.m. for 20 s and flash frozen in liquid nitrogen. Samples were stored at −80 °C until further processing. To lyse the cells, the cell pellets were washed with PBS, suspended in 100 µl SDS lysis buffer (4 % SDS, 100 mM Tris/HCl, pH 8 and 100 mM DTT) and heated to 95 °C for 15 min. Cell lysates were cleared by sonication with ultrasound for a few pulses and centrifuged (10 min at 14 000 r.p.m.) in a tabletop centrifuge. The protein concentration was determined with a tryptophan fluorescence calibration curve at an excitation wavelength of 295 nm and emission wavelength of 350 nm. Protein samples (5 µg) were digested with trypsin according to a FASP (filter-aided sample preparation) protocol (Wisniewski et al., 2009). Prior to LC-MS-MS analysis, peptides were purified with C18 StageTips (Rappsilber et al., 2007). LC-MS-MS analysis was performed on an Agilent 1200 series nanoflow system (Agilent Technologies) connected to a LTQ Orbitrap mass spectrometer (Thermo Electron) equipped with a nano-electrospray ion source (Proxeon). Purified peptides were loaded onto a self-packed fused silica emitter (150 mm × 0.075 mm; New Objective) packed with ReproSil-Pur C18-AQ 3 µm particles (Dr Maisch) at a flow rate of 0.7 µl min⁻¹. Peptides were separated with a 240 min gradient from 2 to 40 % B (A, 0.5 % acetic acid; B, 0.5 % acetic acid/80 % acetonitrile) using a flow rate of 200 nl min⁻¹ and sprayed into an LTQ Orbitrap mass spectrometer operated with a 180 °C capillary temperature and 2.2 kV spray voltage. Full mass spectra were acquired in profile mode with a m/z range of 300–1900 at a resolving power of 60 000 (full width at half maximum). Up to five data-dependent MS-MS spectra were acquired in centroid mode in the linear ion trap for each Fourier transform MS full-scan spectrum (normalized collision energy 35 %, maximum injection time 150 ms, fill value 5 × 10⁶). Each fragmented ion was dynamically excluded for 60 s.

Raw data files were analysed with the MaxQuant software package (version 1.2.7.4). The resulting peak lists were searched using the Andromeda search engine (built into MaxQuant) against the L. lactis database (downloaded on 28 June 2012 from http://www.genome.jp/kegg/genes.html). Andromeda searches were performed with full tryptic specificity with a maximum of two missed cleavages and a mass tolerance of 0.5 Da for the fragment ions. Carbamidomethylation of cysteine was set as a fixed modification, and both methionine oxidation and protein N-terminal acetylation were set as variable modifications. The required parameter of false discovery rate was set to 1 % for both the peptide and protein levels, and the minimum required peptide length was set to 6 aa. In addition, the ‘Match between runs’ option was utilized with an allowed time window of 1.5 min.

The concentration of each protein (molecules gDW⁻¹) was calculated by normalizing the MS intensity of each protein to the total sample intensity and multiplying this normalized intensity with the measured protein content in the sample (Arike et al., 2012; Wisniewski et al., 2012). To estimate the total abundance of the measured proteome, the MS intensities of individual proteins were correlated with measured absolute mRNA levels (data not shown). Based on received correlation, protein concentrations were estimated for unmeasured proteins. As a result, ~91 % of the total proteome was quantified. In the energy calculations, the remaining 9 % was taken into account as a protein with median molecular mass and median degradation rate. All proteins quantified, together with their property values used in the following analysis, are listed in Table S5 (available in the online Supplementary Material). The MS proteomics data have been deposited in the ProteomeXchange Consortium (http://proteomcentral.proteomeexchange.org) via the PRIDE partner repository (Vizcaíno et al., 2013) and can be retrieved using the dataset identifier PXD000494.

**Metabolic flux analysis (MFA).** To calculate the carbon and ATP balances and intracellular fluxes we made use of a curated stoichiometric model of L. lactis (Adamberg et al., 2012). Briefly, this simplified metabolic model is based on data from the BioCyc database, and takes into account reactions from the central carbon metabolism (glycolysis, pentose phosphate pathway and pyruvate metabolism), amino acid metabolism and biomonomer synthesis pathways. Substrate consumption (glucose, amino acids and nucleotides), product formation and biomass composition values were used as input to the model. This model allows the calculation of a unique set of intracellular fluxes without using optimization.

**Calculation of protein degradation rates.** From the moment when the unlabelled lysine substrate is replaced by heavy lysine, the fraction of unlabelled amino acid begins to decrease because lysine is
incorporated continuously into the biomass as the bacteria grow. However, the fraction of unlabelled lysine is also influenced by the turnover rate of each protein. The rate of change in the fraction of unlabelled lysine can be described with the differential equation:

\[
\frac{df_a}{dt} = f_a(t)(d + k_{d1}) - f_{p,i}(t)k_{d1} - f_{p,i}(t)d
\]  

(1)

Parameters \(f_a\) and \(f_{p,i}\) are unlabelled fraction of lysine in its pool and in protein \(i\) at time \(t\). \(k_{d1}\) is the turnover rate of protein \(i\). The specific growth rate \(\mu\) of bacteria is equal to the dilution rate \(d\) at steady state in continuous culture experiments.

The change in the fraction of labelled lysine in protein \(i\) is equal to the amount of synthesized labelled protein minus the amount of labelled protein degraded or left in the system through dilution. Under steady state, the total amount of protein is constant and therefore the rate of protein synthesis is equal to the sum of the dilution rate and protein turnover rate. The fraction of metabolic label in the proteins being synthesized depends on the fraction of label in the metabolic lysine pool, whilst the fraction of label in degraded or diluted proteins is a function of the lysine fraction in the protein.

The free lysine pool \(f_a\) is defined as the amount of lysine available to bacteria for the synthesis of proteins. This free lysine pool is common for all proteins in the cell. The fraction of unlabelled lysine in its pool is a function of time and can be defined as:

\[
f_a(t) = e^{-at}
\]  

(2)

where coefficient \(a\) describes the rate at which unlabelled lysine is replaced by heavy lysine in its free amino acid pool. This rate is affected by the rate of introducing labelled lysine into the cell and also by the protein degradation rate, because light lysine can be reused for the synthesis of new proteins. The higher the protein degradation rates, the higher coefficient \(a\). This concept is very similar to the dynamic labelling experiments described by Hong et al. (2012).

The integration of equation (1) gives a solution for \(f_{p,i}\):

\[
f_{p,i}(t) = \frac{e^{-(d+k_{d1})t}}{d+k_{d1}-a} \left[ e^{(d+k_{d1})t} - a \right]
\]  

(3)

Equation (3) contains two unknown parameters \(a\) and \(k_{d1}\), that are calculated in the present study by fitting to experimental data. Note that parameter \(a\) is the same for the entire proteome, whilst \(k_{d1}\) is specific for every protein. This means that the fitting of experimental data must be performed for all proteins at once, i.e. the system in equation (4) must be solved for \(n\) proteins:

\[
\begin{align*}
\frac{df_a}{dt} &= \frac{e^{-(d+k_{d1})t}}{d+k_{d1}-a} \left[ e^{(d+k_{d1})t} - a \right] \\
\vdots & \vdots \\
\frac{df_a}{dt} &= \frac{e^{-(d+k_{d1})t}}{d+k_{d1}-a} \left[ e^{(d+k_{d1})t} - a \right] \\
\frac{df_a}{dt} &= \frac{e^{-(d+k_{d1})t}}{d+k_{d1}-a} \left[ e^{(d+k_{d1})t} - a \right]
\end{align*}
\]  

(4)

All proteins with at least three corresponding H/L chromatographic peak ratios were selected for the fitting procedure. Altogether, 634 and 562 proteins were found to be suitable for the fitting procedure conducted for experiments at dilution rates of 0.1 and 0.5 h\(^{-1}\), respectively. The number of data points used whilst fitting was 5010 and 3756 for experiments at dilution rates of 0.1 and 0.5 h\(^{-1}\), respectively.

Careful study of equation (3) reveals symmetry between \(a\) and \((d+k_{d1})\). This means it is impossible to distinguish between the situation of a high value of \(a\) and a low \((d+k_{d1})\) or vice versa for individual proteins. However, because we find a global fit to the system in equation (4) using a large dataset, this problem can partially be overcome. Using the extra data, we tested to see if this situation existed by performing a sensitivity analysis. The optimal value for \(a_{opt}\) found in the original optimization was fixed at different levels (0.5\(a_{opt}\), 2\(a_{opt}\), 4\(a_{opt}\)), whilst new protein degradation rates \(k_{d1}\) were found in subsequent fitting procedures. An F test was performed to compare total residual sum of squares between different models. The model with the \(a_{opt}\) value displayed the smallest sum of squared residuals at a statistically significant level \(a<0.001\).

The data-fitting procedure was performed using Mathematica 8.0 (Wolfram Research). Fitting was performed in two stages. First, the most abundant proteins with summarized abundance of at least 50% of the total proteome were selected. Parameters in the system in equation (4) were found by fitting with experimental data. This resulted in turnover rates for a select set of proteins together with the rate at which unlabelled lysine is replaced by heavy lysine in its metabolic pool \(a\). In the second stage of the fitting procedure, the remaining proteins were fitted to equation (3) using \(a\) from the results of the first stage. This dual-fitting approach was chosen because the algorithm used cannot incorporate weights. This means that all proteins have equal significance in the fitting process regardless of their abundance. However, the rate at which labelled lysine is incorporated into the cell is controlled more by abundant proteins. Therefore, dropping the less abundant proteins from the first fitting stage actually improves the precision of the results. A secondary reason was that the fitting procedure was split into two steps was to reduce the calculation time. Remarkably, only 39 and 22 proteins cover at least 50% of the entire proteome for experiments conducted at dilution rates of 0.1 and 0.5 h\(^{-1}\), respectively.

The quality of the resulting fit was good, especially in the experiment with a dilution rate of 0.5 h\(^{-1}\), with the median coefficient of determination \(R^2\) being 0.9962. For the experiment with a dilution rate of 0.1 h\(^{-1}\), the quality of fit was lower with the median \(R^2\) being just 0.8262 and 0.9413 for the upper quartile. The poorer quality fit at the lower dilution rate can be associated with slower incorporation of heavy lysine into the proteins which leads to a lower precision in the quantification of heavy proteins.

The rate at which unlabelled lysine is replaced by heavy lysine in its metabolic pool \((a)\) was calculated to be of the same order of magnitude as the dilution rate, 0.067 ± 0.006 and 0.471 ± 0.053 h\(^{-1}\) for experiments conducted at dilution rates of 0.1 and 0.5 h\(^{-1}\), respectively. These values were also validated by measuring directly the incorporation of heavy lysine into its metabolic pool, whilst the extracellular lysine concentration was under the limit of detection. Measured data showed no significant difference from calculated values \((a=0.040 \text{ and } 0.459 \text{ h}^{-1} \text{ for experiments conducted at dilution rates of 0.1 and 0.5 h}^{-1}, \text{ respectively})\) and, therefore, the calculated values were used in the following calculations.

Some of the calculated turnover rates had disproportionally large confidence intervals which indicates that their values could not be calculated precisely using this dataset. As there is no obvious distribution of calculation errors, we applied a non-parametric limit of four median values to remove proteins with large calculation errors. This filter affected 160 and 30 proteins for experiments conducted at dilution rates of 0.1 and 0.5 h\(^{-1}\), respectively. As an example, 132
of 160 proteins had protein half-lives <5 min. After non-parametric filtering, 474 and 532 proteins with turnover rates remained. In the next step we found proteins whose turnover rate confidence intervals intersected the zero value. The turnover rate of these proteins was considered to be zero.

Energy calculations. Both the amount of ATP generated during substrate consumption per gram of dry biomass produced and the ATP cost for biomass formation were calculated with the aid of a MFA model that was built in-house (see above and Adamberg et al., 2012). We defined the difference between the ATP generated and the ATP consumed for biomass production to be the energy expenditure for maintenance. Cell maintenance could be compared with measured protein degradation and resynthesis rates. The ATP consumption associated with protein polymerization has been estimated to be 4.306 ATP equivalents for each amino acid added to the protein chain (Stephanopoulos et al., 1998), whilst the cost of protein degradation by the proteasome has been estimated to be two ATP molecules per peptide bond (Burton et al., 2001; Menon & Goldberg, 1987). In this work, we presume that the ATP-dependent proteasome degrades proteins into peptides with 10 aa and further degradation is carried out by ATP-independent peptidases. These assumptions provide an estimated turnover cost of 4.506 molecules of ATP per peptide bond.

RESULTS AND DISCUSSION

Physiology of L. lactis

Three replicate chemostat cultivations of L. lactis IL1403 were carried out at each of two different dilution rates (0.1 and 0.5 h\(^{-1}\)) using chemically defined medium (Fig. 1a) to study its physiology. After obtaining steady-state culture in each chemostat cultivation, medium containing unlabelled lysine was switched with an identical medium containing heavy \([^{13}C_6^{15}N_2]\)lysine at the same flow rate. After the isotopic label switch, we followed the incorporation of heavy lysine into the protein content (Fig. 1b). Contrary to
Using absolute proteome quantification, the intracellular concentration of 911 proteins at \( \mu = 0.1 \) h\(^{-1} \) and 964 proteins at \( \mu = 0.5 \) h\(^{-1} \) were estimated to contribute 91\% of the entire \( L.\) lactis proteome (see Methods). Protein turnover rates, together with confidence limits, were calculated for 471 and 529 proteins at \( \mu = 0.1 \) and 0.5 h\(^{-1} \), respectively. We used an algorithm within MaxQuant (Cox & Mann, 2008) to calculate both the H/L ratios and amount of heavy lysine incorporated into the biomass. The latter quantity was confirmed by conducting quantitative amino acid measurements of both the unlabelled and labelled lysine concentrations in the biomass. In addition, the amino acid analysis confirmed that lysine was not used as a substrate for the production of other proteinogenic amino acids because labelled nitrogen was not incorporated into other amino acids. Protein turnover calculations were conducted using the model presented in Fig. 1(c), which takes into account amino acid recycling. Typically, protein turnover rates are calculated based only on the decrease of unlabelled protein.

### Table 1. Physiological data from \( L.\) lactis chemostat experiments (mean \pm SD of three biological replicates)

<table>
<thead>
<tr>
<th>Specific growth rate, ( \mu ) (h(^{-1} ))</th>
<th>( \mu = 0.1 )</th>
<th>( \mu = 0.5 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu = 0.1 )</td>
<td>0.10 \pm 0.00</td>
<td>0.14 \pm 0.03</td>
</tr>
<tr>
<td>( \mu = 0.5 )</td>
<td>0.20 \pm 0.00</td>
<td>0.55 \pm 0.02</td>
</tr>
<tr>
<td>( \mu = 0.1 )</td>
<td>0.09 \pm 0.00</td>
<td>0.14 \pm 0.00</td>
</tr>
<tr>
<td>( \mu = 0.5 )</td>
<td>0.18 \pm 0.01</td>
<td>0.56 \pm 0.02</td>
</tr>
<tr>
<td>( \mu = 0.1 )</td>
<td>0.09 \pm 0.00</td>
<td>0.14 \pm 0.00</td>
</tr>
<tr>
<td>( \mu = 0.5 )</td>
<td>0.18 \pm 0.01</td>
<td>0.56 \pm 0.02</td>
</tr>
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*Although present in the medium, glutamate, aspartate and glycine (latter only at \( \mu = 0.1 \) h\(^{-1} \)) were produced during the cultivation of \( L.\) lactis.*
content in the cells (Helbig et al., 2011), thus neglecting the unlabelled amino acids which are liberated from degraded proteins. As additional unlabelled amino acids enter into the intracellular amino acid pools, they can influence considerably the rate of incorporation of heavy amino acids into proteins. Therefore, we measured the H/L ratio of the free intracellular pool of lysine at the same time points used to collect samples for proteome analysis. The observed increase in the free lysine H/L ratio was compared with theoretical values obtained from the model used to calculate protein turnover (refer to Methods for more information).

Protein turnover rates for individual proteins were found to range from 7 h⁻¹ to close to zero (lowest turnover rates which could be measured adequately within the current experimental timespan were 0.028 and 0.055 h⁻¹ for chemostat experiments at μ=0.1 and 0.5 h⁻¹, respectively; Table S2). The median rates of protein turnover were determined to be 0.122 and 0.913 h⁻¹ (corresponding to 340.5 and 45.5 min) for μ=0.1 and 0.5 h⁻¹, respectively (Fig. 3a). These values are higher than the specific bacterial growth rate and indicate a relatively high energetic cost for protein turnover. We found an acceptable correlation (R²=0.44) between individual protein turnover rates at μ=0.1 and 0.5 h⁻¹, meaning that the majority of protein turnover rates increased with the increase in specific growth rate (Fig. 3b, Table S2). No significant correlation was detected between protein turnover rates and protein abundance or length.

Furthermore, protein turnover rates were grouped according to the gene ontology (GO; according to Bolotin et al., 2001), and group medians and distributions were compared (Fig. 3c). Although intergroup variations were found to be high, we observed a number of trends. Groups such as Energy metabolism, Translation, Regulatory functions, etc., had protein turnover rates close to the median values at both specific growth rates studied. Lower protein turnover rates belonged to groups such as Cell envelope, Cellular processes, and Transport and binding. The higher protein turnover rates were found for Biosynthesis of cofactors, prosthetic groups and carriers, Fatty acid and phospholipid metabolism, and Purines, pyrimidines, nucleosides and nucleotides (Fig. 3c). An interesting exception was fructose bisphosphate aldolase (FbaA), which is a glycolytic enzyme within the group of Energy metabolism. The proteins within Energy metabolism typically had median protein halflives; however, FbaA had more than twofold higher turnover rates than the group median (turnover rates 2.8 and 1.8 h⁻¹ at μ=0.1 and 0.5 h⁻¹, respectively). The main substrate for FbaA, fructose bisphosphate, is a well-known allosteric regulator of the L. lactis central carbon metabolism (Neves et al., 2005; Oh et al., 2011; Thompson, 1978) and high turnover rates of this enzyme may indicate the need for rapid regulation of the fructose bisphosphate pool at the (post) translational level.

### Energetic burden of protein synthesis and turnover

The dataset gathered in this study forms one of the largest datasets of protein turnover rates published so far, covering between 70 and 87% of cellular protein mass at μ=0.1 and 0.5 h⁻¹, respectively. Individual protein abundances and turnover rates were used to calculate the energy cost for proteome synthesis and turnover (degradation and resynthesis). For proteins where the turnover rate could not be
calculated, the median protein turnover rate was used instead. These proteins formed 30 and 13% of the total protein abundance at $\mu = 0.1$ and 0.5 h$^{-1}$, respectively. Based on the absolute concentration of proteins (molecules gDW$^{-1}$), protein length and the amount of ATP equivalents required for polymerization of an amino acid into a protein chain, we calculated the degradation, synthesis and resynthesis costs for all proteins (Table S2). At both specific growth rates studied, protein synthesis costs for biomass duplication were in the range of $18.2 \pm 0.4$ mmol ATP gDW$^{-1}$. The additional ATP cost for protein turnover (degradation and resynthesis) was $47.5$ and $35.5$ mmol ATP gDW$^{-1}$ at $\mu = 0.1$ and 0.5 h$^{-1}$, respectively. This forms 44 and 75% of the total maintenance costs ($95.1 \pm 3.2$ and $47.7 \pm 0.9$ mmol gDW$^{-1}$ at $\mu = 0.1$ and 0.5 h$^{-1}$), and is a major ATP sink that may prevent bacteria from achieving a higher specific growth rate or yield. In total, protein synthesis (including synthesis and turnover costs) is also the most expensive cellular expenditure, comprising 49 and 69% of the total energy produced (ATP) at $\mu = 0.1$ and 0.5 h$^{-1}$, respectively.

Based on the abundance and turnover rates of individual proteins, we calculated the energy demand for the production of individual proteins and cellular protein groups. Altogether, the 15 most expensive proteins comprised 50% of the total energy spent for protein synthesis at $\mu = 0.1$ h$^{-1}$ (Table S2). At $\mu = 0.5$ h$^{-1}$, 24 proteins were responsible for half of the total protein synthesis costs. Energetically, the most expensive GO groups at the lower specific growth rate (0.1 h$^{-1}$) were Energy metabolism (28% of total protein synthesis costs), Transport and binding proteins, Amino acid biosynthesis, and Miscellaneous activities (25% of total protein synthesis costs) (Fig. 3a, b, c). The 15 most expensive proteins comprised 50% of the total energy spent for protein synthesis at $\mu = 0.1$ h$^{-1}$ (Table S2). At $\mu = 0.5$ h$^{-1}$, 24 proteins were responsible for half of the total protein synthesis costs.
Whilst energy generation in the ADI and acetate generation pathways was more efficient at lower specific growth rates, glycolysis was found to be the only pathway whose efficiency increased at the higher specific growth rate studied. These results are also in accordance with physiological observations where both the ADI and acetate generation pathways are downregulated at higher specific growth rates.

In this study, we demonstrate that protein turnover (degradation and resynthesis) cost is one of the highest maintenance expenditures in the L. lactis metabolism. This finding is in accordance with previous studies with yeast where it was demonstrated that differences in the rate of protein degradation may account for observed physiological differences between different strains and mutants (Canelas et al., 2010; González-Ramos et al., 2013; Hong et al., 2012). In addition to protein turnover, mRNA turnover, futile cycles, protein translocation, etc., are sources of ATP expenditure that could be categorized under maintenance costs. Based on median mRNA turnover rates at \( \mu = 0.1 \) and \( 0.5 \text{ h}^{-1} \) taken from Dressaire et al. (2013) and the mRNA polymerization cost of 0.4 ATP per phosphate bond (Karr et al., 2012; Stouthamer, 1973), we estimated mRNA turnover to account for \( \sim 2\% \) of the total maintenance expenditure at both specific growth rates studied. Only futile cycles and ion leakage or pH homeostasis could account for the majority of the remaining loss of ATP, which formed 56 and 26 \% of the total energy expenditure at \( \mu = 0.1 \) and 0.5 \text{ h}^{-1}, respectively. Although difficult to measure directly, those ATP costs increased by more than fourfold with decreasing specific growth rate, which is in accordance with an observed fivefold increase in the length of the cell cycle. As this cost also depends on the length of cell cycle, it can help to explain the increased ATP loss at lower specific growth rates. It has also been shown in both L. lactis and yeast that a decrease in specific growth rate is accompanied with a decrease in intracellular pH, which could be explained by increased energy demand for ion homeostasis at lower specific growth rates (O’Sullivan & Condon, 1999; Orij et al., 2012).

### Table 2. Efficiency of various energy generation pathways for L. lactis at two specific growth rates (\( \mu = 0.1 \) and 0.5 \text{ h}^{-1})

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Efficiency [mmol ATP (mmol ATP)^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One-time synthesis of biomass</td>
</tr>
<tr>
<td></td>
<td>0.1 h^{-1}</td>
</tr>
<tr>
<td>ADI pathway</td>
<td>328.4</td>
</tr>
<tr>
<td>Acetate synthesis</td>
<td>46.7</td>
</tr>
<tr>
<td>Glycolysis</td>
<td>76.1</td>
</tr>
</tbody>
</table>

*Energy was not generated under the corresponding conditions."
Increased protein degradation rates with increasing specific growth rate are also in accordance with recently published mRNA degradation rate data for *L. lactis* (Dressaire et al., 2013). Although we could not detect a significant correlation between individual mRNA and protein degradation rates at $\mu=0.1$ or $0.5$ h$^{-1}$, the median trend for both datasets was similar. A lack of correlation between individual protein and mRNA degradation rates has been observed earlier for mammalian cells (Schwanhäusser et al., 2011).

To our knowledge this is the first study where extensive specific growth rate-dependent proteome turnover data from a steady physiological state were acquired in an attempt to understand the ATP requirements for protein turnover of both individual proteins and various protein groups together with a comparison of the total ATP generation and consumption profiles. The main finding is that protein degradation rates are specific growth rate dependent. The proteome content was observed to be relatively constant when the intracellular metabolic fluxes increased by a mean of 3.3-fold with an increase in specific growth rate from 0.1 to 0.5 h$^{-1}$. This indicates that the catalytic activities of enzymes increased (as demonstrated previously by Adamberg et al., 2012 and Valgepea et al., 2013), which could result in higher protein degradation rates at higher specific growth rates. In addition, under energy-rich conditions (at higher specific growth rate values), an increase in the protein turnover rate improves the robustness of cells to potential perturbations in food supply because cells are able to regulate their protein content more rapidly compared with slow-growth conditions. The latter indicates that protein turnover is a dynamic parameter, which may be dependent on the availability of free energy. Knowledge regarding the mechanisms by which micro-organisms regulate their protein stability may provide an opportunity to design strains with both reduced turnover and higher production yields.

**CONCLUSION**

Knowledge of the rate of protein turnover plays an important role in the interdisciplinary field of systems biology. Large-scale quantitative datasets are emerging that include information about the abundance and degradation rates of individual proteins, their associated mRNA levels, together with metabolite concentrations and pool sizes, and kinetic parameters for individual enzymes. This knowledge will help us better understand how cells actually regulate their metabolism and adjust their associated fitness mechanisms. Genome-scale modelling at both the single-cell and population levels which aims to describe cellular growth aids in our quest to construct efficient microbial cell factories. Specific growth rate-dependent data of protein degradation rates together with energetic burden calculations demonstrate the complexity of both cellular regulatory mechanisms and how they choose their fitness under conditions where the intracellular energy demand fluctuates.

This work shows that protein turnover rates display similar trends to specific growth and metabolic flux rates, and therefore can be influenced by a change in environmental conditions. In addition, the observed increase in biomass yield with an increase of specific growth rate for *L. lactis* is mainly caused by decreased ATP demand for maintenance where the ATP cost for protein turnover is one of the most important factors.

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