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Optimisation of Cell Bioenergetics in Food-Associated Microorganisms

Diego Mora and Stefania Arioli
University of Milan, Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Milano, Italy

1. Introduction

Microorganisms display a considerable versatility, with mechanisms that govern cell bioenergetics and a large number of redox active molecules being used as electron donors or acceptors. We will not review the basis of microbial bioenergetics here, but instead focus attention on the metabolic systems that microorganisms have evolved to optimise the efficiency of cell catabolism and cell energy homeostasis. The mechanisms that act in the regulation of cell bioenergetics belong to the complexity of biological systems in which large networks of metabolic pathways interact to govern the life and responsiveness of cells towards environmental fluctuations. During growth, all microorganisms determine considerable changes in the environmental concentration of nutrients, organic acids and other molecules generated by cell catabolism. As a consequence, microorganisms are constantly faced with different environmental stimuli and stresses. The natural habitats of some microorganisms may fluctuate erratically, whereas others which are more predictable offer the opportunity to prepare in advance for the next environmental change. In this context, microorganisms may have evolved the bioenergetic machinery to anticipate environmental fluctuations by adapting to their temporal order of appearance. Food matrixes represent an example of 'predictable' fluctuating environments, generated by anthropic activities and able to drive the speciation of several microorganisms. The nutrient's richness, and specifically the abundance of mono- and disaccharides that characterise several food matrixes (such as milk and grape juice), have allowed the speciation of lactic acid bacteria (LAB) and yeasts with a high fermentation capacity. The bakers' yeast *Saccharomyces cerevisiae* degrades sugars to two-carbon components – in particular, ethanol – even in the presence of excess oxygen, thus using a fermentation metabolism instead of the energetically favourable respiration metabolism (2 mol versus about 32 mol of ATP per mol of glucose respectively). *S. cerevisiae* alcoholic fermentation has been exploited for several millennia throughout the world in a variety of food processes of crucial importance for humans, such as the making of beer, wine and bread. Moreover, LAB species have partially lost the genetic information need in order to carry out a respiratory metabolism on behalf of a homofermentative pathway in which lactic acid is the primary product, or a heterofermentative pathway in which lactic acid, CO_2, acetic acid and/or ethanol are produced (Kandler, 1983). The seemingly simplistic metabolism of LAB has been exploited throughout history for the preservation of foods and beverages in nearly all societies, and dates back to the origins of agriculture. The domestication of LAB strains passed down through various

culinary traditions and the continuous passage of food stuffs has resulted in modern-day cultures that are able to carry out these fermentations (Makarova *et al.*, 2006).

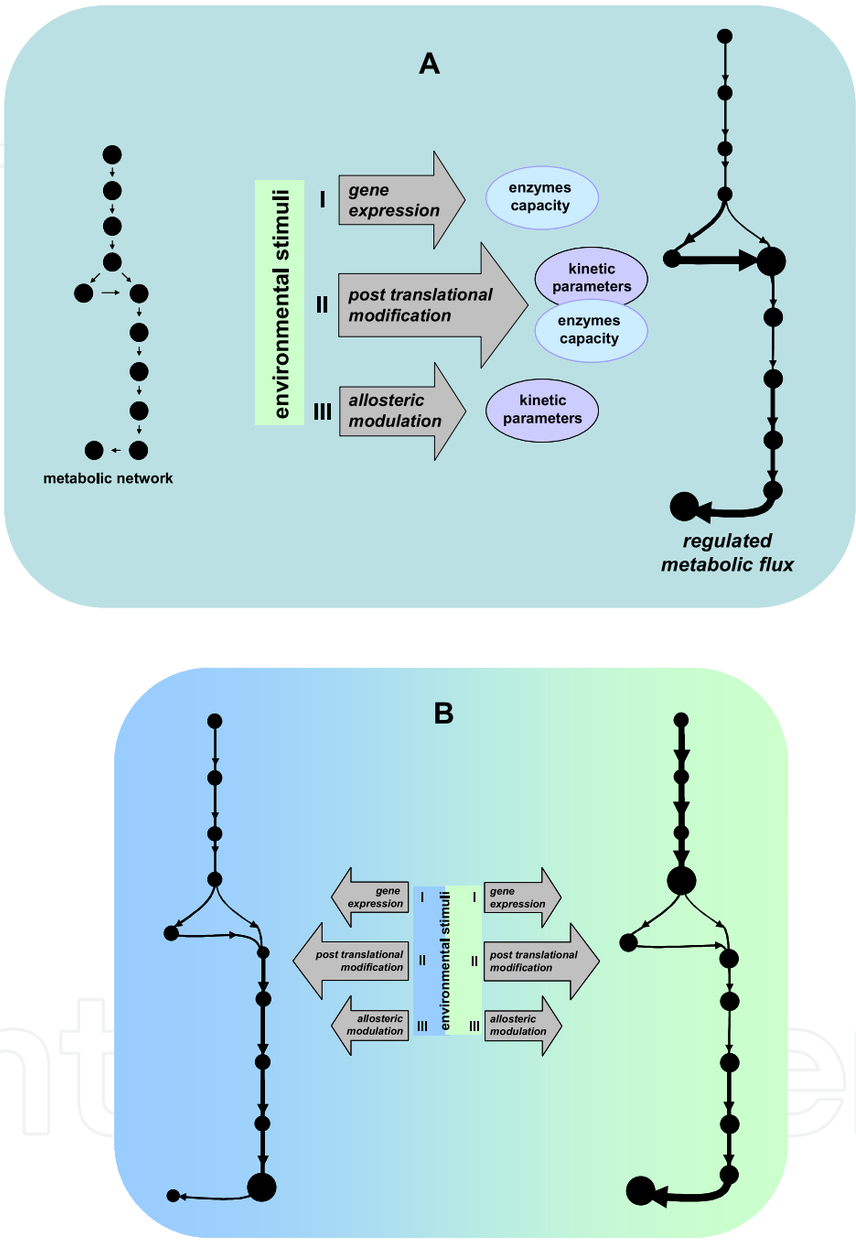

Fig. 1. View of the overlapping regulatory mechanisms modulating metabolic fluxes. A) Example of a metabolic network and schematic representation of the three layers of cellular regulatory mechanisms. The metabolic flux and metabolite pools' concentrations are subjected to the three layers of regulation. The regulation mechanisms act as a response to environmental stimuli. B) Different environmental stimuli (blue and green areas) affect the metabolic fluxes thereby determining the accumulation or depletion of intermediate metabolites.

Both LAB and *S. cerevisiae* have definitely evolved their energetic metabolism to reach maximum fitness in a defined environmental niche characterised by a high carbohydrates concentration. Milk, the proposed evolutionary environmental niche for the LAB *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and the "domesticated" strains of *Lactococcus lactis* (Bolotin *et al.*, 2004; van de Guchte, 2006; Passerini *et al.*, 2010), and the man-made niches, grape juice, has driven the evolution of the domesticated strains of *S. cerevisiae* (Martini, 1993, Fay & Benavides, 2005). These two are environments in which mono- and disaccharides resources are both large and dense. In these environmental contexts, fast sugar consumption, lactic acid or ethanol production, accumulation and tolerance, and the ability to propagate without oxygen are some of the 'winning' traits, and they have apparently evolved and become specialised to perfection in these fermenting microorganisms. In other words, energetic limitation is an important factor for organisms in their natural environment and therefore the ATP-production pathways have been under strong selection pressure during evolution (Pfeiffer *et al.*, 2001). Similarly, we can hypothesise that all mechanisms acting in the regulation and optimisation of the ATP-production pathways are subjected to the same selection pressures.

The complexity of the understanding of how metabolic fluxes are modulated arises from multiple overlapping regulatory mechanisms and metabolic feedback into regulatory networks (Figure 1). The *in vivo* capacity of an enzyme to govern and modulate a metabolic flux is a function of its abundance and kinetic properties. Both abundance and the kinetic properties of enzymes are governed by three layers of cellular regulatory mechanisms: i) *gene expression*, acting on enzyme abundance, ii) *post-translational modification*, modulating enzyme abundance and kinetic parameters, and iii) *allosteric modulation*, exclusively affecting the kinetic parameters. Moreover, the *in vivo* metabolic flux depends also on the *in vivo reactant concentrations* (Gerosa & Sauer, 2011) which are function of thermodynamics and reaction kinetics, i.e. parameters that a cell may modulate only indirectly.

This chapter examines the mechanisms regulating the primary metabolism by using as model organisms the dairy species *L. lactis* among prokaryotes, and the bakers' yeast *S. cerevisiae* among eukaryotes. Moreover, some enzymatic activities and metabolic pathways are described and their physiological role is revisited, taking into consideration the optimisation of the cellular bioenergetics as a result of an environment-dependent selection pressure.

2. The regulation of the energetic metabolism in lactic acid bacteria

Despite the wide use of LAB in food production and the role of some species for their health benefits for the human gastro intestinal tract, the regulatory mechanisms that govern the main energetic metabolism of these bacteria have still not been completely disclosed. Most of the studies have been carried out on the 'domesticated' *L. lactis* species, a member of the LAB widely used in the industrial manufacture of milk-fermented products. The most important industrial application of *L. lactis* is based on its energetic metabolism, which leads mainly to the production of high amounts of lactic acid. Anaerobic glycolysis is the principal energy-generating process of *L. lactis*, it is thus considered exclusively as a fermenting microorganism. Nevertheless, in aerobic conditions and in presence of an exogenous source of heme, *L. lactis* may be able to carry out oxidative phosphorylation (Duwat *et al.*, 2001). This cofactor-dependent respiration capacity has also been discovered in other LAB species (Lechardeur *et al.*, 2011). Although named and used for their capacity to produced lactic

acid, numerous LAB can be induced through a respiratory metabolism, thereby improving the population size and its survival. It follows that *L. lactis* is currently industrially produced as biomass using a heme-dependent respiration, while in the manufacture of fermented milk and cheeses the homolactic fermentation has a key role in the food matrix's transformation or preservation as a consequence of the sizable production lactic acid. Due to the relatively recent timing of studies on the respiratory behaviour of *L. Lactis*, most of the work carried out in order to elucidate the intricate regulation of the energetic metabolism in this species has focused on lactic fermentation. A detailed description of the dynamics of metabolic pools were obtained through *in vivo* measurements, and kinetic analysis by using cell extracts and the techniques of nonlinear systems modelling (Voit *et al.*, 2006). The monitoring of the glycolytic intermediates made, at first glance, intuitive sense. During homolactic fermentation, glucose was taken-up by lactococcal cells and converted into glucose 6-phosphate and then fructose 1,6-biphosphate. The latter is converted into trioses, which ultimately form lactate (Figure 2).

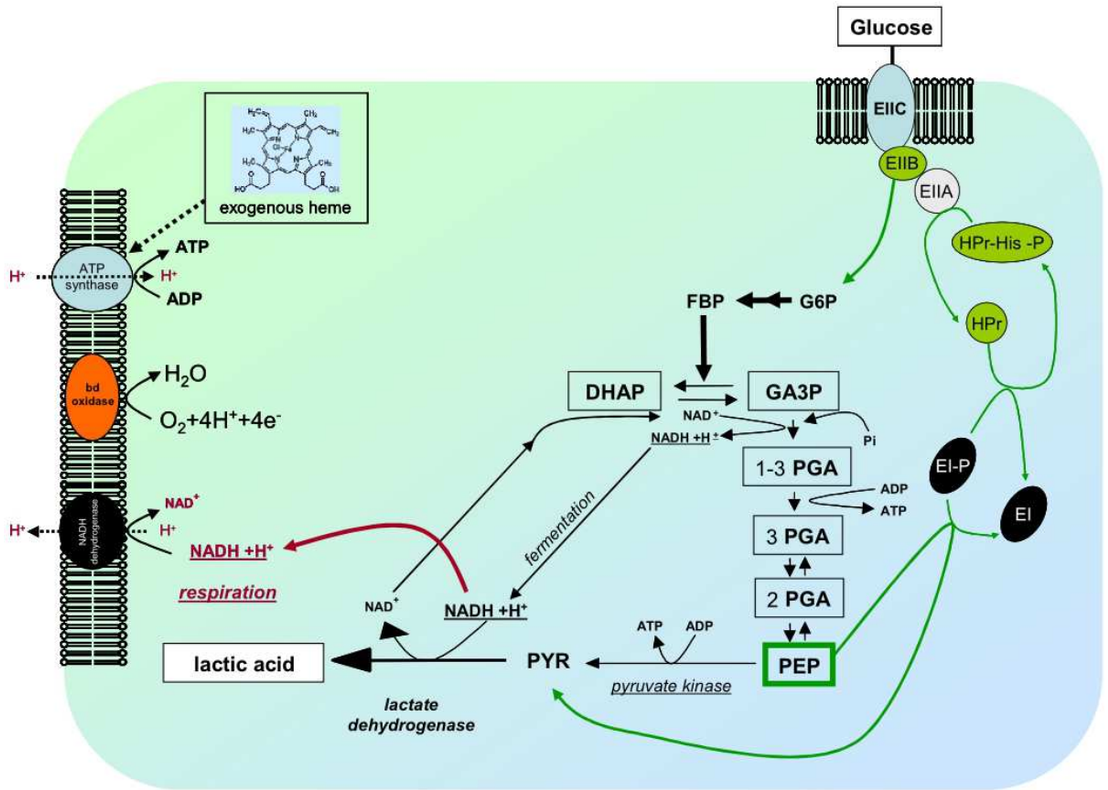

Fig. 2. Simplified representation of glycolysis, homolactic fermentation and heme-dependent respiration in *L. lactis*. The Black arrows show the metabolic fluxes. The red arrow shows the regeneration of NAD^+ occurring during the heme-dependent respiration. Glucose-6-phosphate (G6P), dihydroxyacetone phosphate (DHA-P), glyceraldehyde-3-phosphate (GA3P), 1,3-biphosphoglycerate (1-3PGA), 3-phosphoglycerate (3PGA), 2-phosphoglycerate (2PGA) phosphoenolpyruvate (PEP), pyruvate (PYR). The green arrows show the role of PEP as a phosphate donor in the glucose uptake, PTS-dependent phosphoenolpyruvate phosphotransferase system (PTS). EIIC, EIIB, EIIA, HPr, HPr-His-P, EI, and EI-P are components of the PTS system.

While it is usually assumed that the accumulation of intermediates in a linear pathway is disadvantageous because their storage is chemically costly, in *L. lactis* it a strongly persistent accumulation of trioses (3-phosphoglycerate and phosphoenolpyruvate) at relatively high concentrations (6-20 mM) after glucose consumption was observed (Voit *et al.*, 2006). The reason for the accumulation of trioses in the glycolytic pathway was identified when the overall primary metabolism was considered (Figure 2) together with the nutritional characteristics of the environments where many homofermentative bacteria, including *L. lactis*, live. These environments are characterised by the availability of glucose, which fluctuates widely between high concentrations and extended periods of starvation. As long as glucose is available, the glycolytic pathway is efficiently fed so as to obtain energy production and population growth. During glucose starvation, it becomes crucial to be well-prepared for future carbohydrate availability, when the cell must use them as fast as possible in order to restart the flux of the glycolytic pathway and grow. The maintenance of the high concentration of trioses is, therefore, necessary because glucose transport across the membrane depends upon phosphoenolpyruvate (PEP) as phosphate donor through a PTS system (Figure 2) (Voit *et al.*, 2006). As such, it can be speculated that *L. lactis* and other homofermentative LAB have evolved regulatory mechanisms to be able to control the level of PEP in order to bridge normal periods of starvation.

The maintenance of the 'necessary' concentration of PEP (PEP holding pattern) during starvation periods requires a fine tuning of downstream reactions in the pathway. If pyruvate kinase is closed too rapidly, unnecessary amounts of materials are stored in the form of trioses. Otherwise, if pyruvate kinase is deactivated too slowly, the glycolytic flux is accelerated towards the production of lactate. In other words, this regulatory mechanism has evolved to use the phosphotransferase system rather than ATP for glucose phosphorylation, thereby having most of the glycolytic process short-circuited through the PTS system. The main ecological advantage of such metabolic control is that cells use the first available glucose directly in order to produce pyruvate and than lactate, thereby acidifying the local environment when potential competitors attempt to take up glucose (Voit *et al.*, 2006).

Beside the PEP holding pattern, a further interesting metabolic control mechanism developed by *L. lactis* is represented by the 'feed-forward activation', which is quite rare in metabolic systems. The observation of a transient high concentration of fructose 1,6-biphosphate (FBP) during glucose consumption led to the hypothesis of a regulatory role for this glycolytic intermediate. It was suggested that FBP represents a strong activator of the pyruvate kinase (PK), thereby facilitating the very quick conversion of PEP into pyruvate and lactate while glucose is available. On the other hand, the reduction of glucose availability and, therefore a drop in FBP concentration, allows the decrease of PK activity until an effective stop when glucose is no longer available. The specific activation of PK by FBP has also affected the tuning of PEP concentration. This complex regulation of the energetic metabolism was strictly driven by environmental and ecological constraints. In a more general view, the 'PEP holding' strategy and the FBP 'feed-forward activation' represent an adaptive prediction of environmental changes (in this case related to the availability of carbohydrates). The increasing concentration of PEP during glucose starvation represents a metabolic anticipation of the next environmental stimulus (i.e. new

glucose availability). The anticipation of environmental change is considered an adaptive trait because pre-exposure to the stimulus – which typically appears early in the ecology – improves the organism's fitness when it encounters a second stimulus (Mitchell *et al.*, 2009). In the regulatory mechanisms described above, carbohydrates-availability and carbohydrates-starvation represent two consecutive and predictable environmental stimuli for fermentative domesticated LAB. In *L. lactis* the FBP 'feed-forward activation' represents a clear example of the relevance of allosteric regulatory mechanisms (Figure 1) on the modulation of the energetic metabolism.

A study done to explain the ability of *L. lactis* to grow, retain an active metabolism and survive at low pH highlights the complexity and the interplay of the overlapping regulatory mechanisms that operate in the regulation of the energetic metabolism. Culturing the microorganisms at low environmental pH sees the biomass yield diminished and the energy dedicated to maintenance increased as a response to the organic acid inhibition and cytoplasmic acidification (Even *at al.*, 2003). The request for energy for maintenance in acid conditions resulted in an increase in glucose consumption and the glycolytic rate with a significant reduction of biomass yield relative to ATP production. The adjustment of the metabolic flux in response to a low environmental pH was determined by an increase in the enzymes' capacity and by a specific modulation of the enzyme activities of the glycolytic pathway. A transcription profile and regulation analysis were effective in evaluating the contribution of each layer of regulatory mechanisms in the observed phenomena, highlighting the primary contribution of translational regulation to the increased concentration of glycolytic enzymes in acidic conditions, and confirming that the translation apparatus of *L. lactis* was optimised under acid stress conditions (Even *et al.*, 2003). In this case, the decrease of intracellular pH due to the acidity of the extracellular environment determines an important decrease in enzyme activity that was compensated for by an increase in the enzyme capacity through the efficiency of the translation machinery. In this context, it should be underlined that the enzyme concentration results from the rate of protein synthesis, corrected by dilution coefficient, which is affected by protein turnover (normally negligible except under conditions of stress) and the rate of cell division (at each cell division, the enzymes cellular content will be halved). It follows that cells growing at different rates will have substantially different rates of protein synthesis, even though the specific activities may remain similar (Even *et al.*, 2001). More recently, the primary role of allosteric regulatory mechanisms in controlling the glycolytic flux of *L. lactis* has been questioned, underlining the predominant regulatory role of the enzymes' concentration. This statement was supported by a new methodology whereby experimental measurements of fluxes and enzyme concentrations can be integrated into flux functions capable of predicting the 'fulsome' from the proteome (Rossel *et al.*, 2011). Nevertheless, by such an approach the understanding of the role of each layer of regulation can only be partially addressed. In this case, the approach of regulation analysis is more informative in delineating which regulatory layer is responsible for establishing fluxes through a given enzyme (Gerosa & Sauer, 2011).

Concerning the hierarchical (i.e. expression and post-translational modification) regulation of the energetic metabolism of LAB, the little information available are related to the catabolite control protein CcpA, the major regulator of the carbon metabolism in *L. lactis* and

other Gram-positive bacteria. CcpA belongs to the LacI/GalR family of bacterial regulator proteins, and the disruption of the *ccpA* gene reduces the carbon catabolite repression (CR) of several genes involved in carbohydrate metabolisms. CcpA-mediated regulation depends basically on three elements: i) a specific *cis*-acting DNA sequence, termed catabolite-responsive element (*cre*) which is present near the promoter region of genes affected by CR, ii) the HPr protein, a phosphotransferase protein of the PTS system, and iii) the concentration of glycolytic intermediates (such as FBP). A metabolite-activated kinase has been shown to phosphorylate HPr on residue serine 46. This phosphorylated form of HPr [HPr(Ser-P)] interacts with CcpA, and this interaction enhances the binding of CcpA to *cre* on the promoter region of genes, so affecting the level of their expression. Indirectly, the phosphorylation of HPr on serine residue, enhanced by high level of glycolytic intermediates (*e.g.* FBP), reduces the number of HPr molecules that can be phosphorylated on histidine residue so as to ensure the functionality of the PTS system in sugar uptake across the membrane (Figure 1). Besides the role of CcpA in the control of sugar metabolism (mainly the sugar uptake), it was demonstrated that the role of this protein in the transcriptional activation of the glycolytic *las* operon, encoding the enzymes phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase (Luesink *et al.*, 1998). Specifically, the disruption of the *ccpA* gene lowered the activity of pyruvate kinase and L-lactate dehydrogenase, resulting in the production of metabolites that are characteristic of a mixed-acid fermentation. It was, therefore, speculated that homolactic fermentation in *L. lactis* is maintained by CcpA-mediated repression of mixed-acid fermentation (Luesink *et al.*, 1998).

The regulatory function of CcpA on the energetic metabolism was further confirmed when its primary role in the regulation of aerobic and respirational growth of *L. lactis* was described (Gaudu *et al.*, 2003). CcpA was found to repress NADH oxidase activity, thus maintaining a correct NADH/NAD^+ ratio that directed the metabolism in favour of respiration. Moreover, it was proposed that a CcpA-mediated repression of the heme transportation system thereby prevented the oxidative damage provoked by precocious heme uptake at the start exponential growth. CcpA thus appears to govern a regulatory network that coordinates oxygen, iron and the energetic metabolism.

3. The regulation of the glycolytic pathway in *Saccharomyces cerevisiae*

The *S. cerevisiae* metabolism has been exploited by humans for several millennia through a variety of food processes in order to produce alcoholic beverages and leavened bread. Alcoholic fermentation began due to the presence of indigenous yeast in grapes, must, wort and dough, and with total ignorance regarding the existence of microorganisms and their fermentative role. In practice, humans started to apply microbiology before the role of yeast in beer, wine and bread production was formally proven by Pasteur in 1860 (Pasteur, 1860). Starting with the work of Emil Christian Hansen at the Carlsberg Laboratory in Copenhagen, in the early 1880s, the control of the *S. cerevisiae* metabolism became of crucial importance to enhance the efficiency of fermentation processes as well as the quality of the various products. Alcoholic fermentation is not the unique energetic metabolism in *S. cerevisiae* since it can use the more energetically favourable respiration, which sees a significant increase of ATP being produced per mole of glucose (Figure 3).

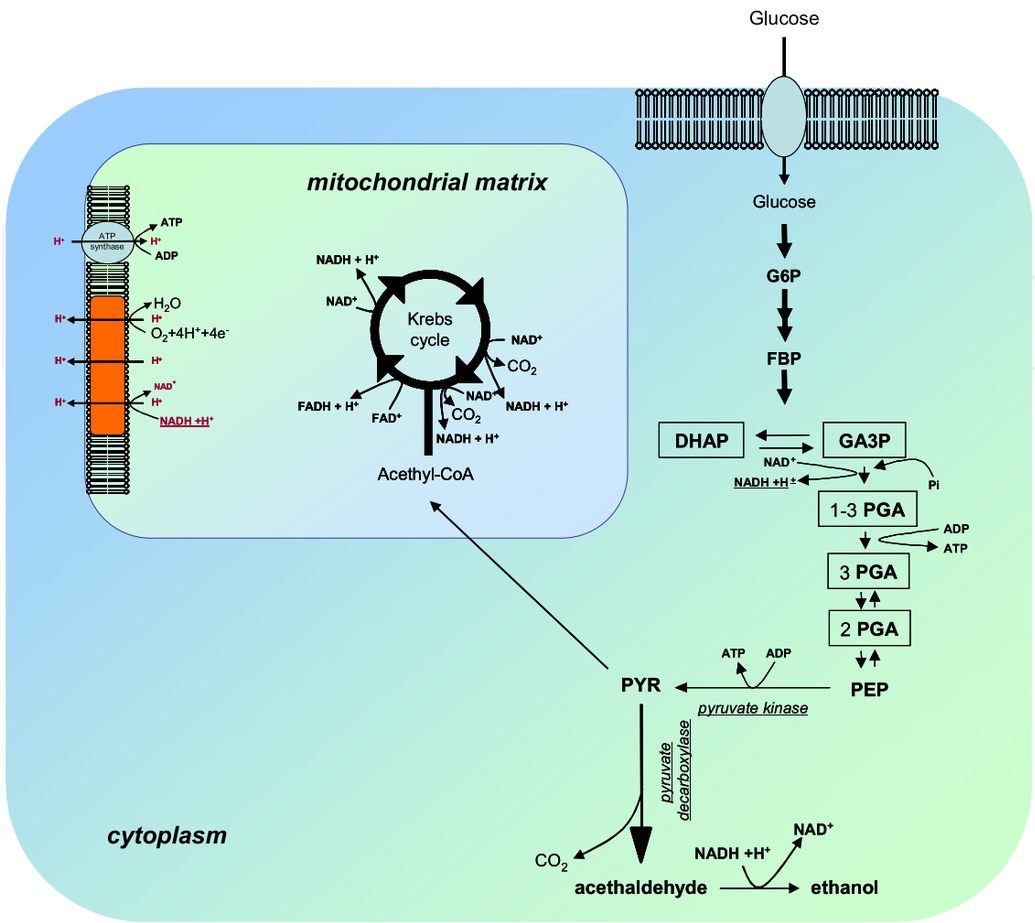

Fig. 3. Simplified representation of the glycolysis, alcoholic fermentation and respiration metabolism in *S. cerevisiae*. Black arrows show the metabolic fluxes. Glucose-6-phosphate (G6P), dihydroxyacetone phosphate (DHA-P), glyceraldehyde-3-phosphate (GA3P), 1,3-biphosphoglycerate (1-3PGA), 3-phosphoglycerate (3PGA), 2-phosphoglycerate (2PGA) phosphoenolpyruvate (PEP), pyruvate (PYR).

A fundamental characteristic of *S. cerevisiae* is the ability to ferment sugars, even in the presence of oxygen in aerobic conditions. This phenomenon is called the Crabtree effect, in honour of Herbert Grace Crabtree who first described the reversible switch between the glycolytic and oxidative metabolism in some cancer cells (Crabtree 1929). In more general terms, the duality of the *S. cerevisiae* metabolism allows this microorganism to use two different strategies for exploiting resources: the 'selfish' strategy and the 'cooperative' strategy. According to the 'selfish' strategy the individuals quickly consume resources and increase their own reproduction rate, whilst according to the 'cooperative' strategy the individuals exploit resources slowly but efficiently. A high rate of ATP production per unit of time is associated with a high reproduction rate and is considered to be a 'selfish' strategy (e.g., fermentation), whereas a high yield of ATP production (the number of units of ATP per unit of resource consumed) is associated with a low reproduction rate but with high biomass production, and is therefore considered to be a 'cooperative' strategy (e.g., respiration) (Pfeiffer, 2001). Given that, resource supply is one of the most important

ecological factors that drive the evolution of organisms, the presence in *S. cerevisiae* of two different metabolic strategies for exploiting resources (fermentation and respiration) represents an ecological advantage that has allowed this species to survive under different environmental conditions. The duality of the *S. cerevisiae* metabolism increases the complexity of the regulatory mechanisms interacting with each other to control the energetic metabolism under different environmental stimuli.

Despite *S. cerevisiae* has been extensively studied with regard to several of its characteristics, little information is available concerning the complexity of the regulatory mechanisms acting on the glycolytic pathway, i.e. the common pathway for fermentative and respiratory metabolism (Figure 3). Glycolysis is a highly conserved pathway from bacteria to yeast and humans, and presumably it has been under intense evolutionary pressure for its robust efficiency. It therefore represents an interesting model for investigating the correlation between the different levels of gene expression. As stated by the central dogma of molecular biology (DNA encodes mRNA and mRNA encodes proteins), a strong correlation was anticipated amongst mRNA concentrations, protein concentrations and metabolic fluxes. However, all attempts to verify these correlations – starting from the data on mRNA and protein levels, enzyme activities and *in vivo* fluxes – were far from perfect. A recent study developed a method to dissect the hierarchical regulation of *S. cerevisiae* glycolysis into contributions by transcription, translation, protein degradation and post-translational modification (Daran-Lapujade *et al.*, 2007). The authors propose the calculation of two coefficients, the hierarchical regulation coefficient ρ_h and the metabolic regulation coefficient ρ_m. ρ_h quantifies to what extent the local flux through the enzyme is regulated by a change in enzyme capacity which is affected by a cascade of gene expression, from transcription to post-translational modification. ρ_m quantifies the relative contribution of changes in the interaction of the enzyme with the rest of the metabolism to the regulation of the enzyme's local flux. While ρ_h can be measurable, ρ_m is calculated assuming that $\rho_h + \rho_m = 1$. It follows that a reaction that is purely regulated by a cascade of gene expression would have a ρ_h of 1, whereas a reaction that is solely metabolically regulated would have ρ_h of 0 and ρ_m of 1. A study by Daran-Lapujade compared different cultivation conditions in order to compare a fully respiratory metabolism with a fully anaerobic fermentative metabolism. Moreover, the anaerobic fermentative metabolism was studied by increasing the carbon fluxes in glycolysis by adding to the culture the non-metabolisable weak acid benzoic acid. The comparison of the three different cultivation conditions, carried out using a glucose-limited chemostat at the same dilution rate, highlights an increase of carbon fluxes (5- to 11-fold) in anaerobic rather than in aerobic cultures, with a further increase in the presence of benzoic acid. The dissection analysis revealed that in most cases the fluxes resulted from both hierarchical and metabolic regulatory mechanisms (ρ_h between 0.2 and 0.5). Surprisingly, the increase of glycolytic fluxes stimulated by benzoic acid revealed a dominant contribution of metabolic regulation because most of the reactions showed small ρ_h values and ρ_m values which were close to 1 (with the exception of the reactions governed by phosphofructokinase, fructose-bisphosphate aldolase, triose-phosphate isomerase and pyruvate kinase) (Daran-Lapujade, 2007). A further dissection approach was useful for analysing the contribution of transcription, mRNA degradation, translation, protein degradation or post-translational modification, to the hierarchical regulation of enzymes' capacities. The main conclusion was that fluxes through glycolytic enzymes were only marginally regulated by mRNA levels,

whereas most of the observed gene-expression regulation was exerted at the level of protein synthesis and/or degradation and the post-translational level. It was, therefore, speculated that in *S. cerevisiae*, the whole glycolytic regulation is an interplay of purely hierarchical regulation (ρ_h close to 1), purely metabolic regulation (ρ_m close to 1), cooperative regulation (ρ_m and ρ_m between 0 and 1) and antagonistic regulation (both ρ_h and ρ_m negative). The nature and the role of post-translational modification, which appeared to be relevant in the control of glycolytic fluxes, has not yet been investigated systematically for all glycolytic enzymes, even though phosphorylation seems to be the predominant mechanism of protein modification.

The ability of *S. cerevisiae* to switch from respiratory to fermentative metabolism is an important characteristic in the evolutionary and ecological context and for many of its industrial applications. In the natural – evolutionary – context, this ability may have helped this organism to quickly recover sugars and create a hostile environment for competing microorganisms. Concerning the industrial application of *S. cerevisiae*, yeast biomass starved of glucose during storage must rapidly adapt to a high sugar concentration when it is added to bread dough or wort. As has been reported, the shift from respiratory to fermentative metabolism resulted in a rapidly increase of the yeast glycolytic flux in order to compensate the differences in the ATP yield of the two metabolisms. The dynamics of glycolytic regulation during the adaptation of *S. cerevisiae* to fermentative metabolism have been investigated with the aim of understanding the time-dependent, multilevel regulation of glycolytic enzymes during the metabolic switch just described (van de Brink *et al.*, 2008). It was reported that within 45 min of the switch from respiratory to fermentative metabolism, the glycolytic flux increases eightfold without any changes in the glycolytic enzymes' capacities, thereby highlighting an increase of the enzymes activities via metabolic regulation (i.e. the regulation of activities by interaction with low-molecular-weight substrates, products and effectors). By prolonging the incubation during the fermentative metabolism under anaerobic, glucose-excessive conditions, a hierarchical regulation of enzymes was also observed. Specifically, the capacity of the kinases of the upper part of the glycolysis remained unaffected, whereas the enzymes' capacities of the lower part of the glycolysis increased, establishing a new homeostasis of glycolytic metabolites. The delay of the transcriptional regulation compared to the metabolic regulation of glycolytic enzymes observed after the metabolic switch was ascribed to the dramatic change in the rate of ATP production. While the glucose consumption rate increased more than 12-fold during the 2 hours after the switch, the rate of ATP decreased during the first 15 minutes as a result of the reduced ATP yield under fermentative conditions. It was, therefore, speculated that cells energy levels influence the induction of the enzymatic capacity in glycolysis. Due to the fact that an increased level of glycolytic enzymes was only observed 45 minutes after the metabolic switch, and given that the majority of the relevant transcripts were induced after 10 minutes, the step was severely affected by the cellular energetic status which was identified in the translation machinery.

4. Alkalising reactions and cell bioenergetics

Food associated bacteria, and in particular LAB, have been selected and used by humans in several food processes because of their ability to acidify milk or vegetables in order to obtain a more stable and safer food products. Acidification occurs in homofermentative LAB

through lactic acid production as the final product of their energetic metabolism. It follows that the growth of LAB determines a significant change in the environmental chemical composition, with a progressive decrease in sugar concentration and a simultaneous increase in lactic acid concentration thereby determining a decrease in environmental pH. Consequently, LAB during each fermentation process are faced with 'predictable' environmental changes, ending with the cessation of growth due to carbon source starvation and, mostly, with an environmental pH which is incompatible with the metabolic processes of the microorganism. The exposure to low pH for long period times determines the arresting of growth and a dramatic decrease of glycolytic fluxes, structural damage to the cell membrane and macromolecules (such as DNA and proteins), and a progressive loss in viability. Weak acids, such as lactic acid, have potent antimicrobial activity because the undissociated forms of weak acids pass freely through the cell membrane. Since the cytoplasmic pH is generally higher than that of the growth medium, inside the cell the weak acid dissociates by releasing a proton and leading to the acidification of the cytoplasm. Due to environmental constraints, LAB have developed through evolution a 'make-accumulate-consume' metabolic strategy (Pfeiffer *et al.*, 2001; Rozpedowska *et al.*, 2011) in order to have a faster sugar consumption, lactic acid production and accumulation. This strategy is aimed at rapidly monopolising sugars and creating an unfavourable environment so as to out-compete other microorganisms by the rapid secretion of fermentation products. In order to survive themselves, LAB have therefore developed a series of mechanisms to counteract low environmental pH and the negative effects of weak organic acids produced by their own metabolism. Several of these mechanisms have been extensively studied (Cotter & Hill, 2003) to understand how LAB protect themselves from the challenge posed by low-pH environments, such as food and gastric juice, and how they develop the strategies by which they can be aided or impeded. Nevertheless, the role of these mechanisms in the regulation of the energetic metabolism was barely investigated even though the loss of the activity of the relatively acid-sensitive glycolytic enzymes (which severely affects the ability to produce ATP) was well known. Indeed, even if LAB species are acid-tolerant bacteria, they cannot be considered to be acidotrophic, and the optimum pH of the highly conserved glycolytic enzymes is close to neutral-alkaline values (Hutkins & Nannen, 1993).

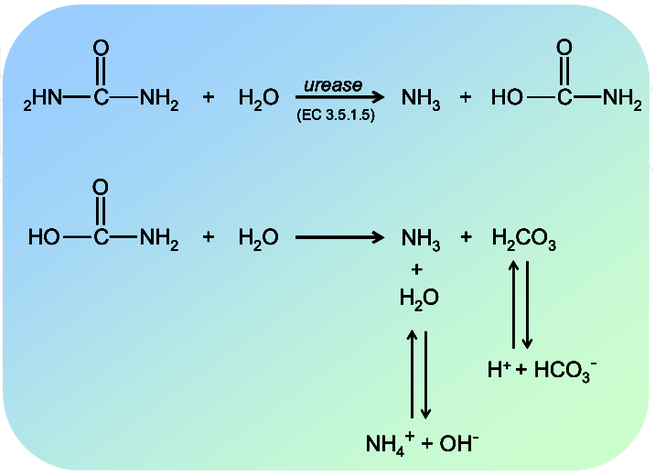

Fig. 4. Reaction catalysed by urease (EC 3.5.1.5) and the spontaneity of carbamate in ammonia and carbonic acid.

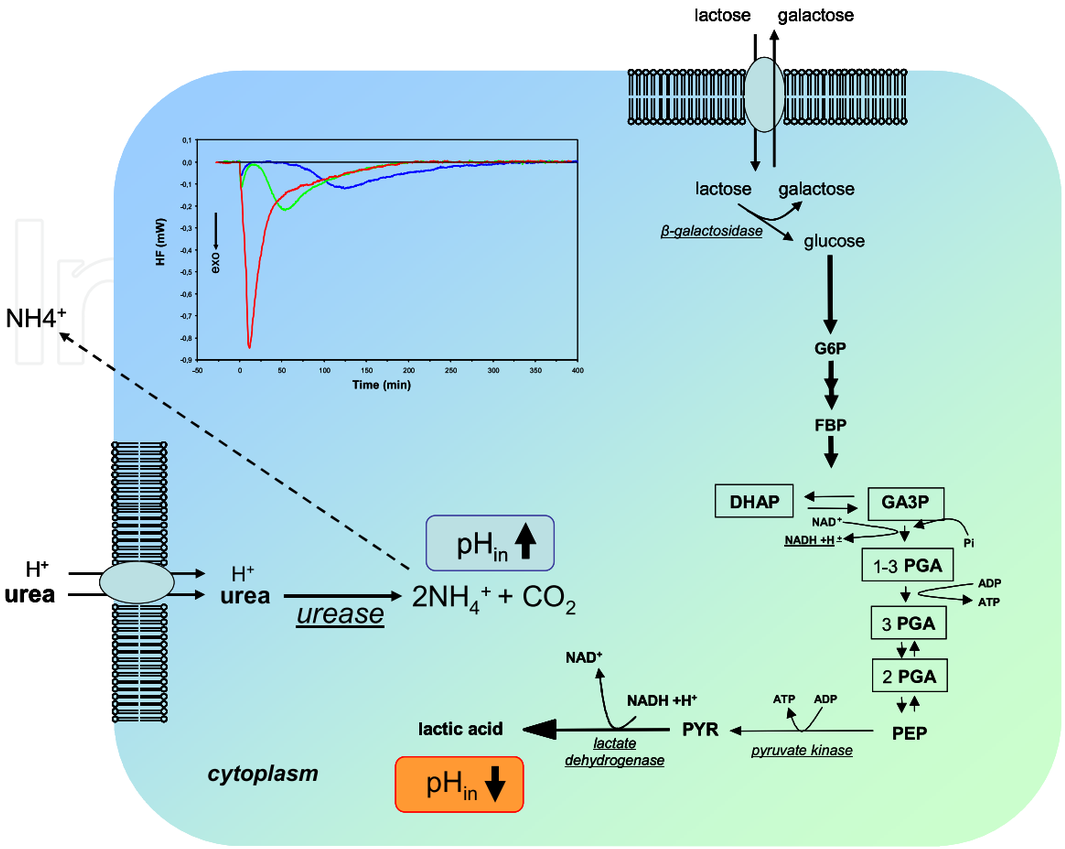

Fig. 5. Simplified representation of glycolysis, homolactic fermentation and urease activity in *S. thermophilus*. The inset represents the raw isothermal titration calorimetry data (heat flux versus time) of *S. thermophilus* lactose metabolism either alone (blue line) or in the presence of ammonia (green line) or urea (red line) (for the detailed experimental procedure see Arioli *et al.*, 2010).

Quite recently, the urease activity – an enzymatic reaction known as a stress response to counteract environmental acidic pH in several bacteria – has been described as a metabolic regulatory mechanism of the energetic metabolism in the dairy bacterium *Streptococcus thermophilus* (Arioli *et al.*, 2010). Urease is a multi-subunit urea amidohydrolase (EC 3.5.1.5) that catalyses the hydrolysis of urea to yield ammonia and carbamate, which spontaneously decomposes to yield a second molecule of ammonia and carbonic acid (Figure 4). The released carbonic acid and the two molecules of ammonia are in equilibrium with their deprotonated and protonated forms respectively, and the net effect of these reactions is an increase in intracellular (pH_{in}) and extracellular (pH_{out}) pH (Figure 5). Urea hydrolysis increases the catabolic efficiency of *S. thermophilus* by modulating the intracellular pH and thereby increasing the activity of β-galactosidase, glycolytic enzymes, and lactate dehydrogenase. Moreover, urease increases the overall change in enthalpy generated by the microbial metabolism as a consequence of an increased glycolytic flux (Figure 5).
In light of these considerations, urease activity – which is stimulated when environmental pH is weakly acidic (pH 5.8-6) (Mora *et al.*, 2005) – should be considered as a regulatory system that has evolved to optimise the activity of the glycolytic enzymes. These enzymes

are exposed to an increasingly acidic intracellular environment and must maintain cell energy homeostasis when the pH_{out} and pH_{in} decrease as a result of lactic acid production. Urease biogenesis is only important when the cells are actively growing, since it increases the fermentative capacity of *S. thermophilus* and leads to rapid growth and an increased acidification rate in milk (i.e. urease favour a cytoplasmic background suitable for a 'make-accumulate-consume' strategy). If we consider that energetic limitation is an important factor for organisms in their natural environment, we then expect that the properties of ATP-production pathways have been under strong selection during evolution (Pfeiffer *et al.*, 2001). Similarly, the regulatory mechanisms which act in optimising the efficiency of the ATP-production pathway should be under the same evolutionary selection. In this context, it is notable that eleven genes are necessary in order for the maintenance of an active urease, which accounts for 0.9% of the estimated core genome of *S. thermophilus*. This enzyme has been found in all the previously characterised *S. thermophilus* strains, and urease-negative mutants are not common in nature. The *S. thermophilus* genome has mainly evolved following divergent evolution from the phylogenetically related pathogenic streptococci bacteria. Loss-of-function mutations, counterbalanced by the acquisition of relevant traits (e.g. lactose utilisation) have resulted in a *S. thermophilus* genome that is well-adapted for dairy colonisation (Bolotin *et al.*, 2004). Because urease is not common in pathogenic streptococci (Mora *et al.*, 2005), its acquisition and maintenance within the *S. thermophilus* genome is likely to be dependent upon its contribution to the environmental fitness of this microorganism when linked to the environmental availability of urea. Urea is the major nitrogenous waste product of most terrestrial animals. Urea is produced in the liver, carried in the bloodstream to the kidneys and excreted in urine. Urea is also present in milk and in the secretions of the major and minor exocrine glands at concentrations approximately equivalent to serum, so a large proportion of circulating urea is translocated onto epithelial surfaces by secretory systems or else in tissue exudates. In this context, it is not surprisingly that urease is present in a high number of human pathogenic bacteria and represents an important factor in infection and disease (Burne and Chen, 2000; Mora *et al.*, 2005).

Since the activity of the bioenergetic machinery is modulated by the intracellular pH, the mechanism of metabolism regulation in other urease-positive bacteria, including human pathogens, should be further analysed. All of the metabolic reactions that result in the alkalisation of the cytosol of acidogenic organisms (such as those involved in the arginine deiminase (ADI) pathway, the citrate metabolism or else those involved in malolactic conversion) should be analysed in light of these novel findings. Indeed, and not surprisingly, all previous pathways act by subtracting protons from the cytoplasm and are strongly induced by an acidic environmental pH (Magni *et al.*, 1999; Cotter & Hill, 2003; Broadbent *et al.*, 2010). The conserved role of alkalising reactions across acidogenic bacteria is also supported by the data obtained for *L. lactis* IL1403-945 and *S. pneumoniae* SP292-945 in the presence of glucose and cellobiose as a carbon source (Arioli *et al.*, 2010). In both cases, the rate of ATP produced during the sugar catabolism was increased, alkalising with the ammonia the cytoplasm. Besides the selfish utility of urease for cells harbouring this enzymatic activity, the cooperative behaviour of urease in an ecological context in which different microbial species share the same environment was also underlined. Urea hydrolysis results in a rise of both pH_{in} and pH_{out} due to the rapid diffusion of ammonia outside the cell. It follows that in the presence of urea and a urease-positive microorganism, (or a urease-negative microorganism) sharing the same micro-environment, there will be benefits from the local transient increase of pH (Arioli *et al.*, 2010).

5. Conclusions and perspectives

The regulation and control of metabolic fluxes in microbes is based on our knowledge of regulatory networks topology, on input-output regulatory logics and metabolic feedback, and on the quantitative effect of control exerted by regulation events. No less important is our understanding of how metabolic regulatory circuits have evolved and what the significance of the impact of environmental constraints on the regulatory configuration will be. It has recently been described that microbes can 'learn' form exposure to a series of new environmental changes and rearrange some regulatory networks so as to predict the new environmental stimuli (Mitchell *et al.*, 2009). The ecological forces and the molecular mechanisms that govern this ability are not clear but it is evident that the regulatory networks that link environmental stimuli to microbial responses are complex and can evolve rapidly (Cooper, 2009). The origin of the adaptability of regulatory networks could be ascribed to microbial cell individuality and the underlying sources of heterogeneity. This heterogeneity is related to stochastic fluctuations in transcription or translation, despite a genetically homogeneous background and constant environmental conditions. Heterogeneity at single-cell level is typically masked in conventional studies of microbial populations, which are based on the average behaviour of thousands or millions of cells, but it has the potential to create variant subpopulations better equipped to persist during environmental perturbation (Avery, 2006). In other words, a population might enhance its fitness by allowing individual cells to make a stochastically transition amongst multiple phenotypes, thus ensuring that some cells are always prepared for erratic, unpredictable environmental fluctuations. It can be therefore be concluded that the regulatory mechanisms that act in the optimisation of the bioenergetics of food-associated bacteria should be analyzed by always taking into consideration the 'predictable' succession of environmental stimuli that have driven their domesticated speciation and evolution.

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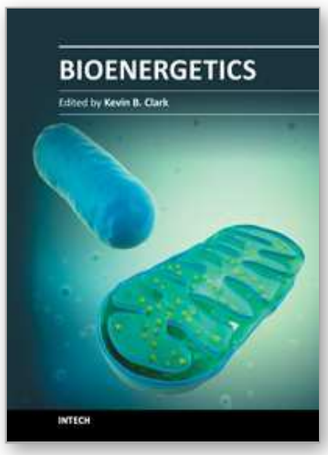

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