Carbohydrate starvation response in *Lactococcus lactis*

Anna Roffel
Supervisor Prof.dr.Jan Kok

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Abstract

Lactococcus lactis is part of the lactic acid bacteria. It is used a lot as an industrial bacterium for the production of milk and cheese. During the production process *L. lactis* slowly runs out of a carbohydrate source to use as energy, has to switch glycolysis pathways and then choose another different pathway when complete carbohydrate starvation has been achieved. This thesis briefly looks at the processes the *L. lactis* cell goes through during carbohydrate starvation, how it tries to combat the increasingly harsh living circumstances and if its stress-response is comparable to the stringent response of *Escherichia coli*.

Contents

1 Introduction

1.1 *Lactococcus lactis*

In the present day many branches of the food industry are utilizing different species of bacteria in their production processes. These industrial bacteria are subsequently able to initiate or speed up a production process to increase production efficiency. One of these industrial species is *Lactococcus lactis*, a spherically shaped, gram-positive member of the lactic acid bacteria (LAB). The LAB share the common trait of producing lactic acid as a primary metabolic end product of carbohydrate fermentation. All members of the LAB have also aquired the 'generally recognized as safe' status. Now widely used for milk fermentation in the dairy industry, *L. lactis* was originally found on plants and in the gastrointestinal tracts of ruminants. It is believed that they maintain a dormant state on plants and proliferate in animal gastrointestinal tracts after being swallowed by them (Bolotin *et al.*, 2001).

1.2 The current thesis

Considering that *L. lactis* is not only widely used as a starter culture in the dairy industry, but also contributes to shelf life, flavour and often nutritional value of dairy products, it is considered a suitable target for genetic engineering to improve its ability to produce lactate in an efficient manner. However, during the fermentation process the bacteria will gradually create an environment unfavorable to them with low lactose concentrations and low pH values due to rising concentrations of lactate (Loubiere *et al.*, 1997).
These limiting conditions are also abundant in nature where bacterial species have varying ways of dealing with these stimuli. A stress-response is triggered, making some species like *B. subtilis* form durable endospores where they remain dormant until living conditions return to a favorable state and necessary nutrients are replenished (Naclerio *et al.*, 1996). Other species like *L. lactis* and *E. coli* do not form these spores, but instead retain a viable but non-culturable state where the cells are not able to form colonies on solid medium, but do retain a form of metabolic activity. This state differs from the endospores that show no metabolic activity while dormant. Ganesan *et al.* (2007) discovered that *L. lactis* cells in this viable but non-culturable state could last up to 3,5 years.

It may be argued that this stress-response could still raise a problem for the dairy industry. This viable state may still be metabolically active, but it is not protected by a protein coat like endospores are, and may therefore still not be able to survive in the limiting growth conditions of the dairy industry cultures. However, a study by Hartke *et al.* (1994) shows that *L. lactis* cultures during carbohydrate starvation (0,1% glucose) developed enhanced resistance against low pH, oxidative and osmotic stress, temperature increase and rising ethanol concentrations. The aim of this thesis is to gain more insight into the metabolic and molecular processes that *L. lactis* undergoes during carbohydrate starvation to obtain the aforementioned enhanced resistances. This will be done by discussing a few of the processes that occur in *L. lactis* before and during carbohydrate starvation, as well as the starvation response of *E. coli* to find possible connections between the two species.

2 Carbohydrate metabolism

2.1 Natural energy source

Before the processes of starvation in *L. lactis* can be discussed, the metabolic pathway for carbohydrates under regular growth conditions will be discussed first for comparison. This pathway is more commonly known as glycolysis. It should be noted that *L. lactis* is capable of making its metabolics function with both aerobic and anaerobic reactions, but only the anaerobic pathway will be discussed here as that is the one most utilized by *L. lactis* and the one pertaining to fermentation in the dairy industry. Previously mentioned was that *L. lactis* is used in the dairy industry by fermenting lactose as a carbohydrate source from milk into lactate. Lactate is not the only product resulting from this reaction; however, it is usually the main product making up around 90 % of all produced metabolites in this pathway (Loubiere *et al.*, 1997).

Albeit it using lactose from milk as a carbohydrate source while being used as a starter culture, the preferred carbohydrate source of *L. lactis* is glucose. That being said, *L. lactis* can utilize many other mono- or disaccharides as its nutrient source for glycolysis. Its growth rate is highest on glucose, because it first has to convert the other sources to glucose as well.

2.2 Glycolysis mechanism

The first step in glycolysis for *L. lactis* is the cell taking up extracellular mono- or disaccharides through phosphotransferase systems (PTS) located in its cell membrane using phosphoenolpyruvate (PEP) as an energy source for transport. They can also be taken up by non-PTS membrane permease (Rute Neves *et al.*, 2005; Kandler, 1983). Glucose is then phosphorylated into glucose-6-phosphate (G6P) and again converted into fructose-bisphosphate (FBP) by firstly transferring one phosphate group to a different location on the molecule using the enzyme phosphoglucose isomerase, and afterwards using phosphofructokinase to transfer the phosphate group of one ATP molecule as donor to create FBP. FBP-aldolase then divides the FBP molecules into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). This DHAP is then also converted into GAP by an isomerase reaction. GAP-dehydrogenase and phosphoglycerate kinase react with GAP to form phosphoglyceric acid (3-PGA). This reaction yields one molecule of ATP and one molecule of NADH. 3-PGA is consequently converted into 2-PGA, which is then converted into the previously mentioned PEP, the energy source of the PTS transporters.

When PEP is lastly converted into pyruvate, this intermediate product can go one of several ways. This is also where distinction between homolactic fermentation and heterofermentation begins. As a homofermentative process, pyruvate can be converted into lactate while regenerating one molecule of NAD^+. Under normal growth circumstances *L. lactis* grows homofermentatively, meaning that a large amount of pyruvate will be converted into the single metabolic end product lactate (Eckhardt *et al.*, 2013). Pyruvate can be metabolized through two different routes when the heterofermentative pathway is used. When pyruvate is converted by pyruvate formate lyase it will generate acetyl-CoA and formate. The energy carrier molecule acetyl-CoA can then be converted into ethanol while releasing two molecules of NAD^+, or it can be converted into acetate, releasing one molecule of ATP. The production of more than one final metabolite is where the term heterofermentative originates.

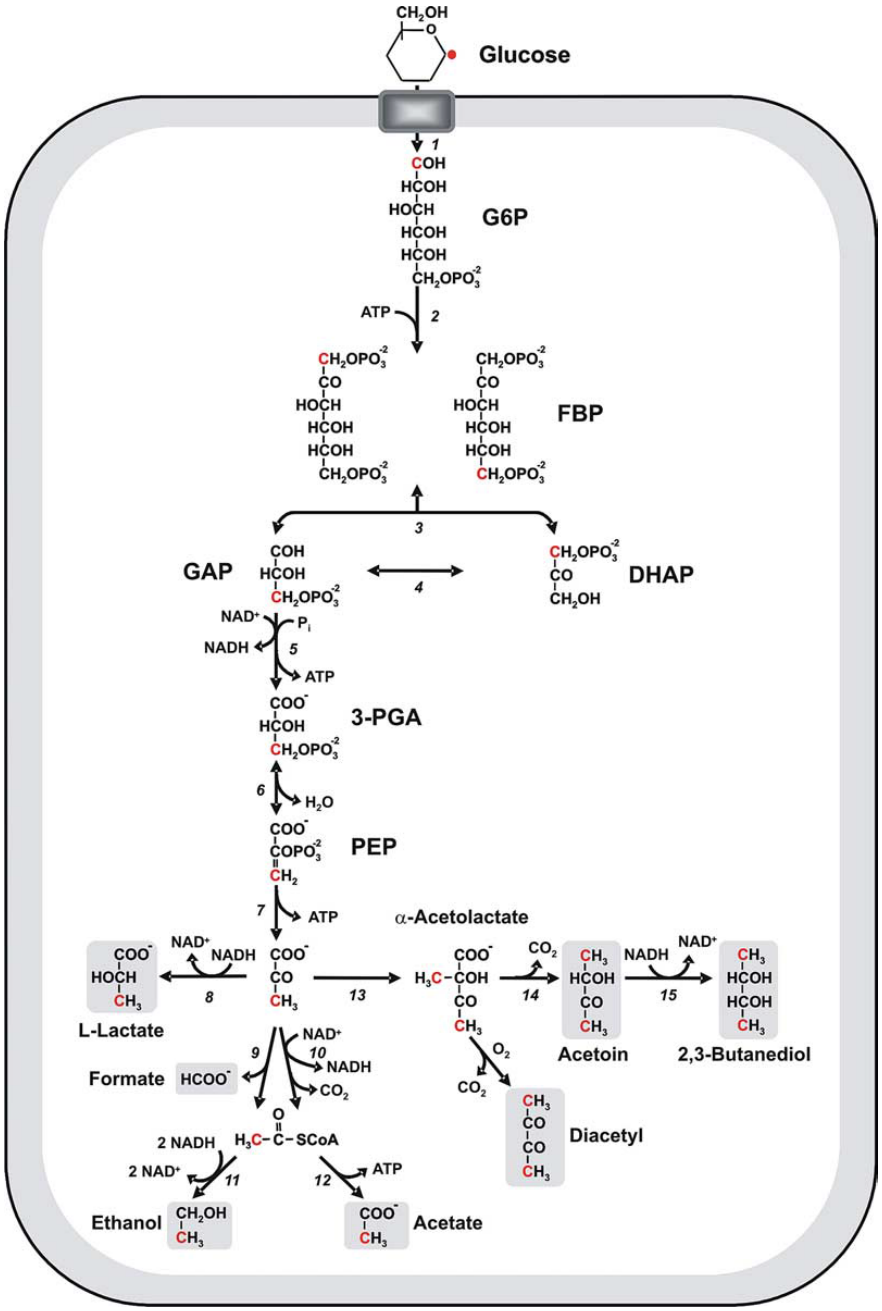

Figure 1: Schematic overview of glycolysis in *L. lactis* (Rute Neves *et al.*, 2005). The reactions shown in this image are as follows: 1. Converting glucose into glucose-6-phosphate (G6P) by the phosphotransferase system (PTS); 2. Converting G6P into fructose bisphosphate (FBP) by phosphoglucose isomerase and phosphofructokinase; 3. Converting FBP into dihydroxyacetone phosphate (DHAP) and glyceralde-3-phosphate (GAP) by FBP-aldolase; 4. The isomerization of GAP and DHAP by triosephosphate isomerase; 5. Converting GAP into phosphoglyceric acid (3-PGA) by GAP-dehydrogenase and phosphoglycerate kinase; 6. Converting 3-PGA into phosphoenolpyruvate (PEP) by phosphoglycerate kinase and enolase; 7. Converting PEP into pyruvate by piruvate kinase. Pyruvate is then converted into the shown products by using the following enzymes: 8. lactate dehydrogenase; 9. pyruvate-formate lyase; 10. pyruvate dehydrogenase; 11. alcohol dehydrogenase; 12. phosphotransacetylase and acetate kinase; 13. acetolactate synthase; 14. acetolactate dehydrogenase; 15. butanol dehydrogenase. The unlabeled product of reactions 9 and 10 is acetyl-CoA.

The second heterofermentative option for pyruvate, being not as common as the other pathways, is the conversion into 2,3-butanediol and diacetyl. The production process of all heterofermentative byproducts of fermentation together is called mixed-acid fermentation. During mixed-acid fermentation energy is produced more efficiently than during homofermentation, so *L. lactis* tends to use heterofermentation during sub-optimal growth conditions when nutrients are scarce.

3 Physiological processes during starvation

Thus in the case of slow lactose depletion *L. lactis* shows a change from homolactic fermentation to the energy-wise more efficient mixed-acid fermentation, continuously producing metabolic end products that make their environment undergo harsh changes. These changes include lowering the environmental pH and depleting the carbohydrate sources. However, what would happen if lactose was fully depleted from the medium? *L. lactis* does not have an intercellular manner of storing carbohydrates, yet it does have to obtain its energy from a different source if it is to maintain its metabolic activity (Stuart *et al.*, 1999). During starvation the levels of PEP and 3-PG as discussed in the glycolysis pathway are relatively high, continuing to mediate the transport of glucose or lactose into the cell with the PTS transporter. This also allows the cells to rapidly take up any mono- or disaccharides whenever they should become available again in the environment (Stuart *et al.*, 1999; Rute Neves *et al.*, 2005).

In the meantime ATP levels collapse during starvation, yet some ATP is slowly being formed by converting PEP into pyruvate, one of the major intermediates of glycolysis. *L. lactis* takes on a viable but non-culturable (VBNC) state, continuing to transport and metabolize nutrients. When the unfavorable environment caused by low pH, unfavorable osmotic conditions and high temperatures during pasteurization passes a certain threshold, *L. lactis* starts showing resistance to these conditions during its transition phase from logarithmic growth to the stationary phase (Duwat *et al.*, 2000). The cells will begin to show increased resistance to the unfavorable conditions of its environment (Hartke *et al.*, 1994), with resistance peaking while they are in the stationary phase. They do not remain static in this resistant but non-growing phase; the cells still adjust themselves in size to minimize energy requirements or they decrease their ribosome count to slow down protein synthesis for more efficient use of energy (Ganesan *et al.*, 2007).

3.1 Viable but Non-Culturable state

The existence of this viable but non-culturable state was at first somewhat controversial, for researching these cells in laboratories was challenging due to the cells not growing on agar. However, research conducted by Ganesan *et al.* (2007) discovered different ways of indicting the existence of the VBNC state.

Firstly, it was noticed that during carbohydrate starvation the amino acids serine and methionine were being produced while arginine and glutamine were being depleted from the medium. When adding more branch-chain amino acids to the medium it was discovered that the intercellular ATP concentrations would rise and the cells would produce new metabolites, indicating that branced-chain amino acids aided the survival of *L. lactis* cells that were undergoing carbohydrate

starvation. Moreover, using transcription analysis they discovered that genes responsible for glycolysis and cell division were being suppressed, while genes for autolysis and cell wall metabolism were also not explicitly induced during starvation. Collectively, this indicates that even though its preferred method of gaining nutrients was not being actively used, *L. lactis* was still maintaining transcription and metabolic activity with a different source of energy, producing different metabolites during a nonculturable state.

This research was not the only one indicating that higher concentrations of arginine in the medium were beneficial during starvation. Stuart *et al.* (1999) grew *L. lactis* in medium with lactose, when the cells used homolactic fermentation to produce lactate. One batch of these cells was grown without arginine in the medium and one batch was grown in medium containing arginine supplements. It was observed that cells with more arginine in their environment were able to retain higher ATP levels, higher cell counts and a longer survival time. More research on this was performed by Brandsma *et al.* (2012), who also tested growth of *L. lactis* during starvation on medium without and medium containing arginine. It was discovered that arginine-metabolizing cells had a viability of up to three times longer than cells that were not metabolizing arginine.

3.2 Arginine metabolism

It thus seems that arginine improves viability of *L. lactis* cells during starvation, but is arginine being metabolized during just this starvation period or is it also metabolized during normal growth? Chou *et al.* (2001) tried adding a large concentration of arginine to the growth medium while the cells were experiencing regular growth utilizing glycolysis. Using transcription analysis, this showed that the cells did not start metabolizing the arginine until the pH gradient of the environment started to drop and the cells started advancing to the starvation response. The first enzyme in the metabolization pathway of arginine (arginine deiminase pathway) was not being induced by excess concentrations of arginine, but rather by the lowering pH levels. This may be an early indicator that this pathway assists in resistance against the lowering of the pH in the cell environment.

Taking the above mentioned statements into account it can be said that during carbohydrate starvation, *L. lactis* switches from glycolysis as its main energy source to the arginine deiminase (ADI) pathway. However, this is also one of the main characteristics to distinguish between *L. lactis* ssp. *lactis* and ssp. *cremoris*, as the latter does not possess the ADI pathway. Firstly, at the beginning of starvation, *L. lactis* degrades a certain amount of the proteins it utilizes for cell growth to form a pool of amino acids that are subsequently used for the synthesis of proteins relevant to the starvation processes (Kunji *et al.*, 1993). There are two main membrane proteins that facilitate the importing of arginine across the cell membrane. The first protein is GltS, a substrate-binding ABC transporter for arginine and ornithine (Eckhardt *et al.*, 2013). The second protein is an antiporter called the ArcD1/D2 complex, where arginine is transported into the cell with help from the concentration gradient of ornithine, which is being transported out of the cell. Using a concentration gradient instead of ATP for this transport is beneficial to the cells under starvation stress, as they do not have to supply the transporter with ATP from their already low ATP reserves. The excess arginine that is transported into the cell is metabolized through the arginine deiminase (ADI) pathway to produce ATP (Stuart *et al.*, 1999).

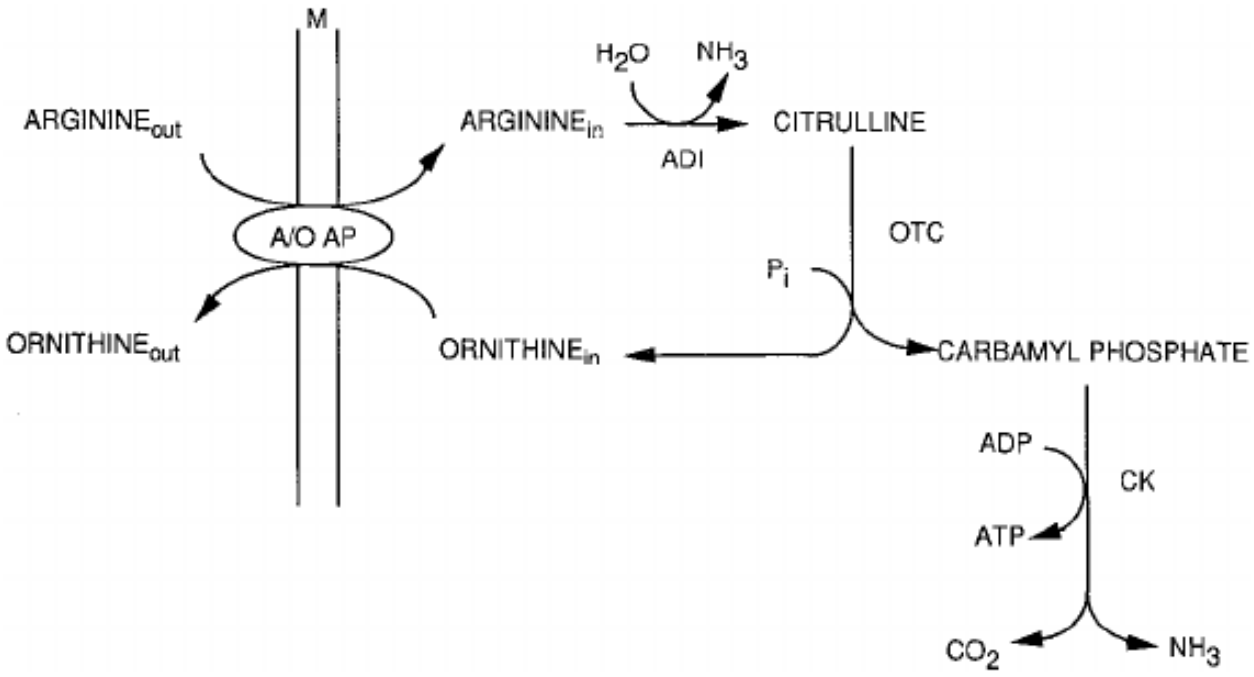

Figure 2: Schematic overview of the arginine deiminase pathway in *L. lactis* (Christensen *et al.*, 1999). The pictured abbreviations mean the following: 1. M: cytoplasmic membrane of *L. lactis*; 2. A/O AP: arginine/ornithine antiporter; 3. ADI: arginine deiminase; 4. OTC: ornithine transcarboxylase; 5. CK: carbamate kinase.

When arginine enters the ADI pathway it is first converted into citrulline, and afterwards into both ornithine and carbamyl phosphate. Ornithine maintains the concentration gradient needed for arginine transport, while carbamyl phosphate can then be deaminated into carbon dioxide and ammonia, where one molecule ADP is converted into ATP (Brandsma *et al.*, 2012). The key enzymes used in this pathway are firstly arginine deiminase (ADI), secondly ornithine transcarbamoylase (OTC), and lastly carbamate kinase (CK) (Chou *et al.*, 2001). Even though the ADI pathway isn't as efficient as the glycolysis pathway, producing 1 mole ATP for each mole arginine instead of 4 mole ATP for 1 mole glucose (Brandsma *et al.*, 2012), the production of ammonia during usage of this pathway may be used intentionally by the cells to combat the acidic environment, since ammonia reacts with water molecules to form ammonium hydroxide: a weak base. However, there are more ways in which *L. lactis* may combat starvation.

3.3 Ribosome stalling

Another method that *L. lactis* may use when protecting itself from carbohydrate starvation is a method called ribosome stalling. Ribosomes in *Lactococcus lactis* are made up of two subunits. In bacterial cells, these subunits are a larger 50S subunit and a smaller 30S subunit (Starosta *et al.*, 2014). When these two subunits of a ribosome lock around a strand of RNA and subsequently have amino acids transferred to them by tRNA, a protein is synthesized. It was previously mentioned that during starvation *L. lactis* decreases its ribosome count for more efficient energy consumption. This would imply that excess RNA coding for new ribosomes (rRNA) is degraded by ribonucleases to provide energy for the cell.

Ribosome stalling is a name for the mechanism of linking the two ribosomal subunits together without locking around a strand of RNA so they become inactive. This inactivity however is also viewed as a manner of protecting ribosomes from ribonucleases to save them until the period of starvation has passed (Eckhardt, 2013).

The ribosomal mechanisms for transcription have best been identified in *Escherichia coli*, where the *rrn* promoter region contains promoters that enhance the binding of ribosomes. During ribosome stalling in *E. coli* the ribosome modulation factor (RMF) will first attach to both the large and small subunit and hold them together. Then as a second step the hibernation promotion factor (HPF) binds to the complex, completing the dimerization of the ribosome. This state is also called the 100S state of the ribosome not only due to the dimerization of the two ribosomal subunits forming a 80S ribosome, but also because of the binding of both dimerization factors. The 100S form of a ribosome is translationally inactive (Ortiz *et al.*, 2010). This mechanism could also be applicable to *L. lactis* due to some homology found in the dimerization factors. *L. lactis* posesses the YfiA protein, which is homologous with the HPF of *E. coli*. When Puri *et al.* (2013) did research on the function of YfiA, they found that ribosome dimerization did not take place in the stationary growth phase when the YfiA gene had been deleted from *L. lactis*, suggesting that YfiA has an important role in ribosome stalling. It is possible that *L. lactis* does indeed apply ribosome stalling like *E. coli* to protect ribosomes during starvation, but that does not necessarily indicate that both bacterial species adopt the same methods to obtain these results.

4 Similarities to *Escherichia coli*

In the previous section about ribosome stalling a homologous protein between *L. lactis* and *E. coli* was discussed. Stress responses of *E. coli* have been documented in more detail on the molecular level than stress responses of *L. lactis*, while they both enter a VBNC state. To be able to better genetically engineer *L. lactis* for the dairy industry it is beneficial to know as many things as possible about the molecular processes during a stress response.
The so-called stringent response that *E.coli* employs as an answer to nutrient limitations in its environment will now be discussed (Starosta *et al.*, 2014). Some similarities between *E. coli* and *L. lactis* may be discovered to see if there could also be a stringent response taking place in *L. lactis*.

4.1 Stringent response

The stringent response of *E. coli* is a stress response to varying nutrient limitation or other stress factors like heat shock in the environment. Stringent factor relA is associated with a fraction of the ribosomes in the cell. This factor activates whenever an uncharged tRNA enters the ribosome, indicating the absence of an amino acid and therefore indicating nutrient limitation in the environment (Starosta *et al.*, 2014). RelA then synthesizes the alarmones guanosine pentaphosphate and guanosine tetraphosphate, which are often written down as (p)ppGpp. This synthesis uses cellular ATP and GTP reservoirs as its energy source.

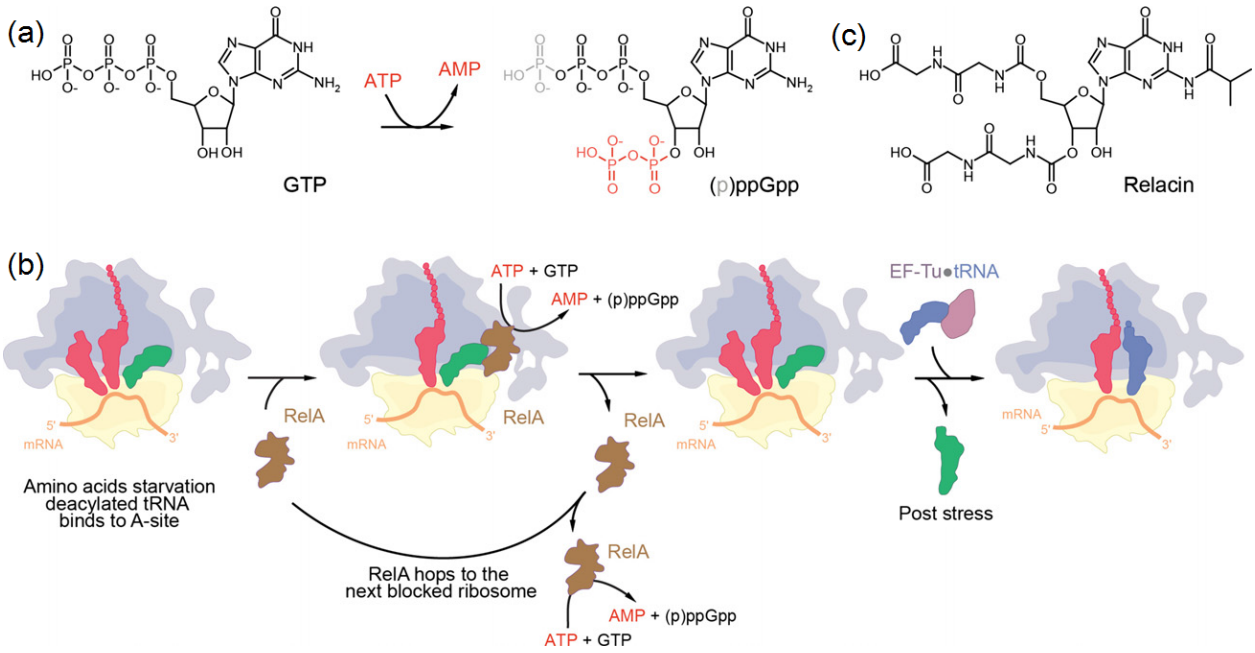

Figure 3: (a): (p)ppGpp being synthesized using GTP as a phosphate donor. (b): Activation of relA after sensing an uncharged tRNA. (c): Structure of relacin, inhibitor of relA. EF-Tu: Elongation Factor Thermo Unstable. This is a prokaryotic elongation factor that catalyzes the binding of a charged tRNA to the ribosome (Starosta *et al.*, 2014).

Accumulation of both of these alarmones takes place at a quick pace, making it an effective way of alerting the cell. These alarmones then bind to the polymerase molecules in the cell, halting translation, transcription, and other intermediate factors that come with those processes, like tRNAs. On the contrary, enzymes that pertain to amino acid synthesis are induced at a higher rate during this response. Another member of the relA protein family exists with the same function: spoT. This protein can sythesize (p)ppGpp like relA, but can also hydrolyze the alarmones again after the stress reponse has passed. When a cell has high concentrations of (p)ppGpp during nutrient limitations or other environmental stress factors, it is more viable than cells without this response, as the cells with (p)ppGpp prioritize the synthesis of the nutrients they require at that time over their cell growth (Potrykus *et al.*, 2011).

Shortly summarizing, when *E.coli* senses a limitation in its environment, stringent factor relA will start interacting with ribosomes to produce the alarmone together with spoT. Rallu *et al.* (1996) state that the consequences of (p)ppGpp synthesis work in a pleiotropic way by directly tampering with the expression profile of the cell through altering specificity of the ribosomes, and indirectly by inducing a stress response factor that also alters the expression profile.

4.2 Other homologous genes

Because the stress response of *E. coli* has been mapped out in more detail than that of *L. lactis*, homologues between the two could be used to furhter map out the stress response of *L. lactis*. When talking about ribosome stalling, the promoter region *rrn* was named as the binding site for promoters encouraging ribosome binding for protein synthesis. Puri *et al.* (2013) have noted that *L. lactis* also posesses at least one of these *rrn* promoters. It is speculated that this promoter region

is under the regulation of the alarmones (p)ppGpp during the stringent response because of GTP being its initiating sequence for transcription. GTP is necessary for the synthesis of (p)ppGpp, and those alarmones being synthesized is a clear indication of a stress response. If the alarmones would indeed be synthesized through this promoter region it is a possibiliy that the alarmones halt synthesis of any other proteins after being synthesized themselves. It is not entirely clear how this *rrn* promoter region is being regulated yet, but there have been speculations. The alarmone synthesis might interfere with the binding of the polymerase and the DNA of *rrn* by destabilizing their connection, or it might interfere with the polymerase itself by binding to it so it will form a closed complex and cannot bind to the DNA.

Furthermore, Eckhart (2013) reported that *L. lactis* has both the relA and the spoT genes, the genes that cause (p)ppGpp synthesis during the stringent response in *E. coli*. It is also not yet known how those genes regulate the cell metabolism in *L. lactis*, yet that may become clearer if it is indicated that the system could be similar to the stringent response of *E.coli*. Rallu *et al.* (1996) stated that they found multiple heat shock proteins in *L. lactis* that had been highly conserved, such as dnaJ, groELS, and grpE-dnaK. These genes are also used by *E. coli* as genes encoding for chaperones which are used for the refolding of proteins after heat shock or for targeting denatured proteins for degradation.

5 Discussion

Thus far it has been discussed in this thesis how *Lactococcus lactis* normally metabolizes its carbohydrates by glycolysis, but that it starts changing to the arginine deiminase pathway whenever carbohydrate starvation takes place. This starvation reponse also causes the cell to develop resistance against many stress-inducing factors like the acidification of the environment due to lactate production, or the heat due to pasteurization. When *L. lactis* is under stress from carbohydrate starvation the cells enter a viable but non-culturable state in which they still produce metabolites that differ from the ones they produce during glycolysis.

Producing ammonia and ammonium hydroxide in the arginine deiminase pathway might be a way for *L. lactis* to combat the acidification of its living environment. It is beneficial for *L. lactis* to stay capable of producing lactate under acidic circumstances, because acidification of the environment during milk fermentation by *L. lactis* is an important step in the production process. Not only does it help flavor production (Brandsma *et al.*, 2012), but it also competes with pathogens that are then repelled by the acidic medium (Cretenet *et al.*, 2011). However, over-acidification during long periods of storage is not beneficial for the customer and neither for the producer of the product. Perhaps manually inducing a state of carbohydrate starvation after a certain amount of storage time will assist *L. lactis* in having longer lifespans and will stop the product from over-acidifying. This may even give the product some additional probiotic effect. Another way of preventing over-acidification would be to 'train' bacterial starter cultures beforehand so that they are pre-adapted to being stored and their lactose metabolism starts off more slowly.

These research suggestions would be somewhat easier to bring into practice if the molecular processes that trigger a stress response in *L. lactis* would be known in more detail, like the stringent response of *E.coli*. It was indicated that *L. lactis* and *E. coli* do have some homologous proteins,

like yfiA as a homologue to HPF during ribosome stalling, and the relA and spoT genes that both *L. lactis* and *E. coli* possess that trigger the stringent response in *E. coli*. Yet despite the proteins being homologues of each other, it can not be indicated yet if they also operate in the same way in both species of bacteria. *L. lactis* is a gram-positive bacterium while *E. coli* is gram-negative. This makes the transportation of nutrients already different between the species. Instead of comparing these bacterial species, *L. lactis* could also be compared to another well-studied gram-positive species: *Bacillus subtilis*. Unfortunately, while *B. subtilis* is gram-positive, it is also a sporulating species of bacteria. *L. lactis* continues running its metabolism after environmental stress, but *B. subtilis* develops a sturdy layer of protein around itself and goes into something not unlike hibernation, where it will only start becoming metabolically active again once the stress-triggers in the environment have disappeared and there are enough nutrients. (Naclerio *et al.*, 1996).

Despite everything, it is not impossible for *L. lactis* and *E. coli* to have the same mechanisms during a stress response and that the response of *L. lactis* could be classified under another stringent response, but more information will have to be found to indicate that. A few homologous proteins and a similar process such as ribosome stalling are not yet enough to prove this potential stringent response in *Lactococcus Lactis*.

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