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# Studying the effect of High pressure processing on Listeria monocytogenes in food and developing a model to suppress its resistance.

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# Preface

This thesis, *Studying the effect of High pressure processing on Listeria monocytogenes in food and developing a model to suppress its resistance*, is written as the Master's Thesis of stud. techn. Tine Bergitte Heggernes. The thesis was issued at Nofima's researching department at Måltidets Hus, Stavanger and written by a student at the Department of Chemical Process Technology at the Norwegian University of Science and Technology in Trondheim, Norway. The thesis was written as the final project for the 5-year Master of Science Degree in the field of Chemical Engineering and comprises 30 ECTS credits.

The author of the study is hopeful that the information and results provided in this work can contribute to improve the high pressure processing technique on food at Nofima and elsewhere in the food industry.

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Tine Bergitte Heggernes

## Acknowledgements

I would like to express my gratitude first and foremost to my main supervisor Nadav Skjøndal-Bar at NTNU. Without your guidance, constructive feedback and professional advice during my work, this thesis would not have been possible.

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## Abstract

High pressure processing (HPP) is a food treatment technique, developed to increase the shelf life of food by inhibition of microbial growth, at the same time as quality degradation is avoided. By using high pressure, the components in the food is less affected than by the use of many traditional preservation methods. However, the ability of bacteria cells to recover is an important concern in food safety. The lack of knowledge in this field may impair the use of HPP treatment as an efficient food preservation method, making it essential to understand these adaption responses. Since there is not enough experience with high pressure processing, more knowledge is needed on its specific features and on how this technique affects bacteria. In particular it is of interest to know how high pressure affects the bacteria *Listeria monocytogenes* as this is a food-borne pathogen which can cause severe illness to those who get infected by it.

With the aim to learn more about the embedded mechanisms that allows bacteria to recover from pressure induced damages, a new strategy has been applied. This strategy includes the use of mathematical model simulations to get more insight in the behaviour and dynamics of cell recovery after HPP exposure. This approach includes three parts:

1. Creation of repair pathway models
2. Conduction of high pressure experiments
3. Simulating responses based on the experiments and developed models

This thesis has showed the method of how simulations can be used to find out more about bacteria repair. Pressure induce leakages in the cell envelope are being repaired by activating repair mechanisms in the cell wall, cytoplasmic membrane and envelope associated proteins. The genes responsible for reparation are those involved in the synthesis and turnover of peptidoglycan, phospholipids and proteins as well as repair chaperones. From the experiments it was verified that high pressure induce leakages in the cell. Based on the results, it is suggested that *L. monocytogenes* requires approximately two days to recover, though this assumption needs more verification. The mathematical simulations showed that the repair system is particularly sensitive to degradation reactions, meaning that they have the potential to be target elements for reduction of bacteria resistance towards high pressure.

# Sammendrag

Høytrykksprosessering av mat er en forholdsvis ny teknologi som har blitt utviklet for å øke holdbarheten til mat, ved hemming av mikrobiell vekst og/eller bedre kvaliteten til maten. Ved å bruke høytrykk kan komponentene i mat bli mindre påvirket enn ved andre tradisjonelle konserveringsmetoder. Bakteriers egenskaper til å bli levedyktige igjen etter prosessering er i midlertidig en utfordring for mattryggheten og dette kan svekke bruken av høytrykksprosessering som en effektiv konserveringsmetode. Det er dermed essensielt å få kunnskap om hvordan høytrykksprosessering påvirker bakterier og hvordan bakterier klarer å tilpasse seg høytrykk. Det er spesielt interessant å vite hvordan høytrykk påvirker den patogene bakterien *Listeria monocytogenes,* ettersom infeksjoner av denne type bakterier kan gi fatale konsekvenser for enkelte mennesker.

Målet med denne oppgaven var å lære mer om mekanismene som gjør at bakterier kan reparere seg igjen etter skader forårsaket av høytrykk. En ny strategi har blitt brukt for å få til dette. Denne strategien involverer bruk av matematiske modeller, hvor simuleringer av modellen kan gi mer innsikt om atferden og dynamikken hos en bakteriecelle når den repareres etter høytrykkseksponering. Prosedyren for å nå dette målet omfatter tre deler:

1. Opprettelse av reparasjonsmodeller
2. Utførelse av eksperimenter med høytrykksprosessering
3. Simulering av den bakterielle responsen, basert på eksperimentene og de utviklede modellene

Denne oppgaven har bidratt til å avdekke en metode hvor simuleringer brukes for å få mer kunnskap om den bakterielle reparasjonen etter eksponering for høytrykk. Lekkasjer i cellen oppstår som følge av trykkeksponering. Disse blir reparert ved å aktivere mekanismer i celleveggen, den cytoplasmiske membranen og proteiner tilknyttet cellekonvolutten. De genene som er ansvarlige for reparasjon er involvert i syntese og omsetning av peptidoglykan, fosfolipider og proteiner i tillegg til reparasjon «chaperones». Fra eksperimentene ble det bekreftet at høytrykk forårsaker lekkasjer i cellen. Basert på resultater fra forsøkene kan en anta at *L. monocytogenes* trenger rundt to dager for å bli levedyktig igjen, men denne antagelsen trenger videre verifisering. Matematiske simuleringer viste at reparasjonssystemet til *L. monocytogenes* er spesielt sensitiv for degraderingsreaksjonene og dermed har komponentene involvert i disse reaksjonene et potensial til å være målelementer i bakterienes reparasjonssystem.

# Abbreviations and Nomenclature

| | |
|---|---|
| BHI | Brain Heart Infusion |
| CFM | Confocal Fluorescence Microscopy |
| CIRCE | Controlling Inverted Repeat of Chaperone Expression |
| DIC | Differential Interference Contrast |
| DP | Damaged Protein |
| HP | High Pressure |
| HPP | High Pressure Processing |
| HCP | Heat Shock Protein |
| LTA | Lipoteichoic Acid |
| MP | Metabolite Pool |
| NAG | N-Acetylglucosamine |
| NAM | N-Acetylmuramic acid |
| ODE | Ordinary Differential Equation |
| PA | Phosphatidic Acid |
| Pbp | Penicillin binding protein |
| PG | Peptidoglycan |
| PI | Propidium Iodide |
| PMA | Propidium Monoazide |
| PtdChl | Phosphatidylcholine |
| PtdEtn | Phosphatidylethonlamine |
| PtdGro | Phosphatidylglycerol |
| PtdSer | Phosphatidylserine |
| SRP | Signal Recognition Particle |
| TCST | Two Component Signal Transduction |
| TP | Tagged protein |
| TSAYE | Tryptic Soy Agar supplemented with Yeast Extract |
| $\sigma^B$ | Sigma Factor |

## Table of Contents

# 1 Introduction

The main reason for processing food is to preserve or modify it. By doing so, the shelf life can be prolonged by inactivating microorganisms and enzymes, as well as changing the texture or palate of the food. In the past years, the requirements of food quality have become higher and people are more concerned about the health aspects of what they eat. Because of this, there has been a development of functional foods and new techniques requiring less processing too meet these new criteria. High pressure processing (HPP) is an example of such a technique. It is developed to increase the shelf life of food with inactivation of microorganisms at the same time that quality degradation is avoided. This requires a processing method which is both highly efficient in killing microorganisms at the same time as it is gentle to the food. HPP is a relatively new technology, and was first used for a commercial product in Japan in 1994 (Simonin, Duranton et al. 2012). Some knowledge is known related to the general effects of high pressure on bacteria, but there is less experience in the specific features of HPP. More research is required in order to find the optimal processing conditions for the inactivation of bacteria and to preserve food.

Exposure to high pressure is a stressful condition for bacteria that are not adapted to it, and most bacteria that can be found in food have atmospheric pressure as their optimum pressure level. More knowledge about how microorganisms react to these kinds of stresses is needed as the inhibition of bacteria is the main aim when processing food. Especially the bacteria *Listeria monocytogenes* is of great interest as it is a food-borne pathogen that can cause severe illness to those who get infected by it. These types of bacteria are especially of concern since they can grow during refrigerated in many pasteurized food products. It is useful to know how pressure affects this bacterium and how it responds to such disturbances. By knowing what kind of mechanisms the bacteria activates as an attempt to adapt to the changes and repair the induced damages, it is possible to get one step further in the elimination of pathogenic bacteria in food. By knowing more about how bacteria respond to environmental stresses and how they survive under high pressure, better methods to prevent their growth and survival can be found.

The bacterial response can be found by studying the change of gene expressions and mechanisms in the cell when it is presented upon stress. This requires a thorough research around the bacteria stress response system. By doing so, genes important for the bacteria survival can be identified and hence, target genes defined. A study of this manner improves our knowledge on how *L. monocytogenes* survives under high pressure. It can help with the development of a specific attack on genes or mechanisms as well as helping on the design of better high pressure processes.

The ability of bacterial cells to recover is an important concern in food safety. The lack of knowledge in this field may impair the use of HPP treatment as an efficient food preservation method, making it essential to understand these adaption responses. A method that can be used in order to increase the understanding of cell recovery after pressurization, is by simulating the event. In this thesis, such

a simulation has been done on the repair system of the bacteria. A hypothetical scenario is created where one pressure exposed bacterium is studied. When exposed to high pressure, a mechanism that repairs the damaged cell envelope of the bacteria, among other responses, is activated. It is assumed that some bacteria dies straight away as the loss of components from the permeable cell envelope has been too fatal to survive, even if it manages to reseal. Some bacteria are so rough that they maintain full integrity during pressurization and appear unaffected. These are not the bacteria that are under focus in this thesis. The relevant bacteria are those who are so affected by the pressure that they become permeable, but manage to reseal and start to grow again before the loss of components within the cell becomes fatal. Such bacteria represent one of the main limitations from the high pressure technique as the processed food can initially appear safe from bacteria, but then return to become hazardous. The research method that uses simulations to study the mechanisms, taking the bacteria from being so injured that it cannot grow anymore to turn back to the active log phase represents a new approach to reduce this possible hazard.

## 1.1 Scope of Thesis

The main scope of this thesis was to study the effect of high pressure on bacteria, and how genes are regulated in order to repair the pressure induced injuries in the cell envelope. New strategies should be examined in order to prevent a recovery of the bacteria and reducing the risk of food poisoning from *Listeria monocytogenes* following HPP treatment. A suggestion of key elements in the repair sequences, which can be used for further studies on reduction of the bacterium's resilience towards high pressure, was therefore also a desired outcome.

The problem definition was divided into the following three parts:

1. Creation of a simple cell envelope repair model by finding the interaction network of the genes and proteins responsible for repair.
2. Laboratory work using the high pressure machine at Nofima, Stavanger, to obtain required experimental data.
3. Creation of mathematical repair models, based on the interaction networks and experiments, which can be used to simulate the repair behaviour.

## 1.2 Outline

The report is organized as follows:

The first part, *Background*, consists of the background information that forms the theoretical basis being used in this thesis. The chapter gives information about high pressure as a processing method, the specific bacteria of interest, *Listeria monocytogenes*, and how the bacteria is damaged and possibly repaired after exposure to high pressure. Modelling of biological systems is also introduced.

The second part, *Materials and Methods*, gives an overview of the three steps involved in the procedure to develop a dynamic repair model of the cell envelope. These steps explains the pathway analysis, the experimental procedures and the methods used to mathematically express and simulate the developed models.

In the third part, *Results and Discussions,* the outcome of the three methods are presented and discussed. These results comprises the final models, the experimental results and the simulations of the models. Along are discussions about the outcomes from this work. These are possible explanations for the results and probable sources of errors that may have arisen during the work.

Lastly, the *Concluding Remarks* are given followed by suggestions for *Further Work*.

# 2 Background

Bacteria are able to grow in nearly all kinds of extreme environments. Some parts of the world are even so inhospitable that only bacteria are able to live here. Being able to grow under such conditions illustrates quite well their well-developed adaptability. Bacteria are simple organisms, but able to exchange genes with each other if necessary. Exchange of genes is not their principal solution for environment adaptation though. Often the necessary mechanisms already lies incorporated in the cell, they just need to be activated. This makes the genes and the mechanisms that these genes control, of great importance for the bacteria to be able to withstand all the stresses and challenges they are faced upon. Because the bacterium is so rough and so abundant they can thereof be as difficult to prevent from growing where they are not wanted. Hence, bacteria represent a great challenge for the food industry.

When bacteria senses undesired conditions, some can move away by using one or several flagella, to a more desired environment. If this is not an option, they can try to adapt to the environment instead by changing their own physiological function. To achieve this, the bacteria can use its response mechanisms embedded in the cell, for example by re-modelling a protein complex. This response is achieved by changing the patterns of gene expression for those genes that are involved in the bacterial defence mechanisms (Wright and Lewis 2007).

## 2.1 High Pressure Processing

High pressure processing is a possibility for gentler food processing as the technique aims to produce safe and high quality foods. This especially applies for the food industry, where the main application is for the extension of shelf-life or for the elimination of microbial pathogens in food. HPP is a relatively new processing method, especially compared with traditional methods such as heating, fermentation and drying. Today, this technology is considered as an alternative to traditional preservation methods and is applied in many different types of food segments. It is especially appropriate for liquid food, but the technique is of today's date mostly utilized in vegetable and meat products, as shown in Figure 2.1. However, there is still not enough experience in the specific features of HPP. How microorganisms react and responds to the high pressure that is applied to them is one of the areas that need to be investigated more closely. By knowing more of these aspects, an optimal design which produces safe and economically feasible HPP treated food can be obtained (Heinz and Buckow 2009).

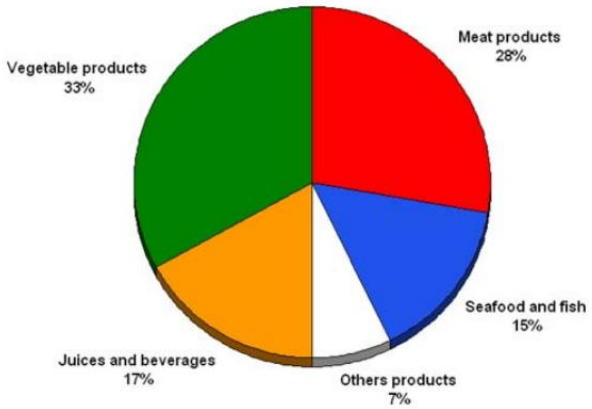



**Figure 2.1: Utilization of HPP preservation in different segments of food industry (Heinz and Buckow 2009).**

High Pressure Processing (HPP) is a pasteurisation technique that exposes food to a high level of hydrostatic pressure, lasting for a time range from a few seconds (eg. oscillating pumps) to several minutes (over 20 min). The process is usually performed at cold or room temperature, but it is possible to combine with high temperatures. The food can either be liquid or solid, packed or unpacked and the pressure can go up to 600 MPa in commercial equipment. This means that there are many different operating conditions to choose from (Farkas and Hoover 2000). Since the high pressure process is characterized by the three parameters pressure, exposure time and temperature, there is a broad variability for process design. Not many other processing techniques can say to operate with more than two parameters (Heinz and Buckow 2009).

High pressure is generated by increasing the free energy in the food, which is done by reduction of the mechanical volume (Heinz and Buckow 2009). The HPP treatment is usually a batch system, which operates similar to a thermal processing retort system. The processing cycle consists of filling the process vessel with the product and thereby water, closing the vessel and bringing the vessel to pressure process conditions. After the designated processing time, the vessel is decompressed by expanding it against a constraining liquid and removing the product. The method is illustrated in Figure 2.**2** for a better understanding of the system. This technique allows an instantaneously and uniform exposure throughout the mass of food which gives an even result, independent of package size, shape and composition (Farkas and Hoover 2000).

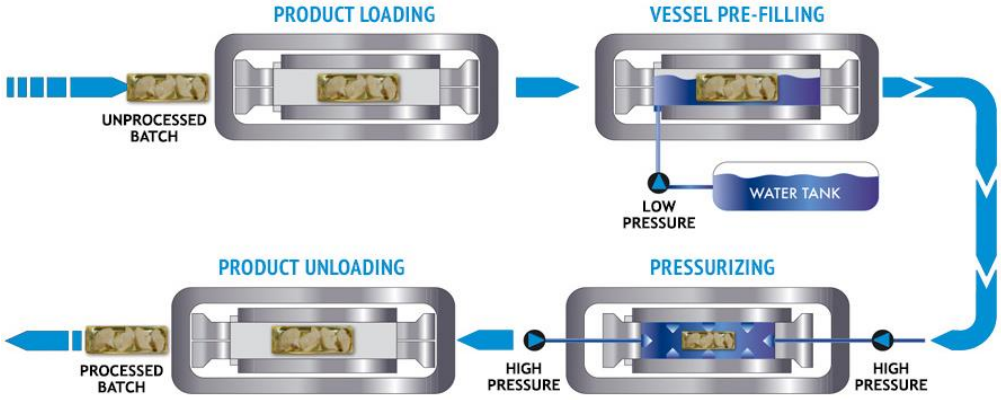



**Figure 2.2: The processing cycle of HP treatment. The vessel is loaded with the unprocessed batch and filled with water, brought up to a pressure level for a given time and lastly unloaded of the product (Hiperbaric 2013).**

HPP is known for being gentler to food than other processing techniques, like heating. This is because the applied pressures have appeared to have little effect on covalent bonds of low molecular mass compounds. This means that foods exposed to HPP treatment around room temperature does not change significantly as a direct result of the pressure treatment itself (Farkas and Hoover 2000). Since HP has limited effects on the covalent bonds, there are minimal changes in nutritional and sensory quality as colour and flavour compounds are of such low molecular masses.

HPP processing has proved to preserve nutritional value as well as the sensory properties in food. This means that the characteristics of a fresh product are kept, giving food with greater quality and extended shelf life. Having this said, there are also challenges with HPP and not all types of product are well suited for this preservation method. Food is a complex system and the compounds that are responsible for sensory properties coexist with enzymes, metal ions and other factors. Because of this, there are many other chemical and biochemical reactions that may occur which can affect either positively or negatively on the food quality (Oey 2008). Also, the greater the pressure level and time of application, the greater is the potential for changes in quality of foods. If the food is processed to tough, it will no longer appear fresh or raw. Therefore, the processing conditions cannot be generalised but need to be established for the different types of food (Farkas and Hoover 2000).

## 2.2 *Listeria monocytogenes*

The *Listeria* genus consist of six species; *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri* and *L. grayi*. They are Gram-positive and anaerobic soil bacteria, which belongs to the low G+C group of microorganisms, and do not make spores. Only two of the species are pathogenic; *L. monocytogenes* and *L. ivanovii*, with the first being the only one that can be dangerous to humans. The possible pathogenicity of the *L. monocytogenes* bacteria makes it very important to know more about it, especially how to prevent it from possible infectious conditions (Hain, Steinweg et al. 2006).

The natural habitat of *Listeria* is decaying plant material where they live as saprophytes, but it is in food products they become threatening to humans. The term listeriosis is a specific name from the bacteria infection caused by *L. monocytogenes* and it is estimated that 99% of all human listeriosis cases are caused by consumption of contaminated food products (Hain, Steinweg et al. 2006). *Listeria* can be present in all types of food as they are widespread in the environment as well as being very robust microorganisms. Among others, they have tolerance for high salt concentrations, a broad range of pH and ability to grow at wide temperature ranges, including low temperatures. (Abee, Schaik et al. 2004).

The occurrence of listeriosis is quite low. This is because it mainly affects people with a weaker immune system, such as elderly, pregnant women and new-borns. However, since the mortality rate of those who actually develop listeriosis is as high as about 20-30 %, *L. monocytogenes* is regarded as a serious human pathogen. The clinical symptoms shown on those who are *Listeria*-affected is often meningitis and other brain-related diseases, septicaemia, abortion, prenatal infection and gastroenteritis (infectious diarrhoea) (Hain, Steinweg et al. 2006). With this threat present in the food industry there a great interest in how to eliminate this problem and several experiments has been done in order to learn more about this bacterium. With the need of more knowledge, a lot has been discovered. At today's date, the complete genome sequence of *L. monocytogenes* is published, but there is still a lot that is unknown and needs more research (Glaser, Frangeul et al. 2001).

## 2.3 Impact of High Pressure on Cell Envelope

Even though bacteria have a highly advanced defence mechanism, it cannot withstand all types of stress. There are some conditions that inactivate or kill bacteria. Very high pressure, above 400 to 600 MPa can inactivate most vegetative bacteria including many infectious food-borne pathogens (Smelt 1998). There are many factors that affects the bacteria in such a way that it cannot adapt to these extreme pressures. In biological systems, pressure higher than 400 MPa can lead to a reversible and irreversible cleavage of intermolecular and intramolecular bonds. This means that the construction holding the cell together, as well as mechanisms controlling and conducting processes in the cell, are broken. In this way, structural changes in membranes together with the inactivation of vital enzymes are the key targets of microbial kill by high pressure (Heinz and Buckow 2009). Although the actual sites of pressure injury on bacterial cells are not well known, cell wall, cytoplasmic membrane, DNA, RNA and enzymes, both intracellular and membrane bound, are expected to be affected by pressure. The extent of damage is again depending on the level of pressure, type and strain of individual bacteria (Bozoglu, Alpas et al. 2004).

### 2.3.1 Stationary-Phase Adaptation and Pressure Resistance

Lethal effects on vegetative cells depends on the extent of cell membrane damage and inactivation of key enzymes. However, which growth phase the bacteria is in, also has a significant matter on the impact of stress, especially when it comes to the extent of membrane damage. Cells in the stationary phase are namely more pressure resistant than those in the exponential phase (Bartlett 2002). In exponential-phase cells, loss of viability has been correlated with a permanent loss of membrane integrity, while in stationary-phase cells; the effect has not shown to be as straightforward. Cell membranes has shown to become leaky during pressure treatment, but has resealed to a greater extent after decompression. The reasons for the differences between these phases are probably due to the synthesis of proteins that protect the bacteria cell against hostile conditions. Culture phase is therefore an important determinant when it comes to the kinetics of cell inactivation during pressure application. This particularly applies if the bacteria are going into stationary phase before they can protect themselves, as it would mean an additional step before protection. An extra step gives slower stress adaption and higher effect of inactivation (Casadei, Mañas et al. 2002).

### 2.3.2 Damage of Cell Wall

The bacteria cell wall mainly consists of peptidoglycan (PG) layers, where the structure of these layers determines the integrity and shape of the cell. Figure 2.3 shows the construction of the cell wall with lipoteichoic acid (LTA). Two fundamental components of the peptidoglycan include peptides and glucans (sugars). The basic unit of the sugars in PG are repeating units of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) which are linked together by glucosidic bonds into linear chains (Figure 2.4). NAG functions as a spacer molecule while NAM provides the site for peptide attachment to the glycan chain. The peptides are linked together by peptide bonds, but they also cross-link to other PG layers by bonding to neighbouring peptide chains.

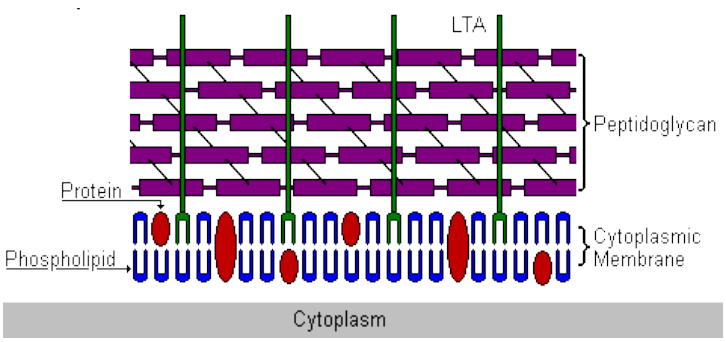



**Figure 2.3: Peptidoglycan and lipoteichoic acid (Fix 2014)**

These connections results in both horizontal and vertical bonds within the wall, creating a rather rigid cell wall structure. However, the cross-linked peptide chains are connected through hydrogen bonds, which are more susceptible to high pressure than the strong covalent bonds. The breakage of these hydrogen bonds are therefore assumed to be the site where leakage occur when the cell is exposed to high pressure (Quintela, Pittenauer et al. 1995, Barton 2005).

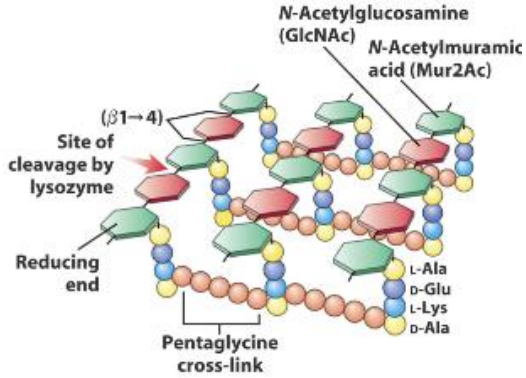



**Figure 2.4: Structure of the peptidoglycan constituents NAM and NAG (StudyBlue 2014).**

The role of a bacterial cell wall is to protect the cell against various influences. Protection against osmotic pressure is a good example of the importance of the cell wall. Since the bacteria cell generally contains more salt than the environment, osmosis will make water wanting to penetrate into the cell, which can lead to osmotic lysis. If the cell wall is exposed to high pressure, this physical barrier loses its ability to stop the intake of excessive water and thereby has the potential to kill the cell. Because of this, it is of great importance to keep the cell wall layers intact (Wonga, Vaillant-Barkab et al. 2012, Reuter, Hayward et al. 2013).

### 2.3.3 Damage of Cell Membrane

The membrane is the primary contact point between the cell and the environment. A natural function of the membrane is therefore to protect the cell and control the passage of components between the internal and external environment by being a selective barrier. This is only one of its many functions though. The membrane is actively involved in numerous physiological activities, including generation of energy, export of proteins for construction of molecular structures (such as the cell wall), sense and process signals, respond to temperature changes, flagella motor, cell division and interact with DNA (Barton 2005).

The cell membrane consists of lipids, mainly phospholipids, carbohydrates and a large content of proteins. The composition if these components is shown in
Figure 2.5. The lipids create the structure of the cell membrane while the proteins are responsible for biological activities and interactions inside and between the cells. These membrane proteins can

move freely within the lipid bilayer because of the fluidity of the membrane (Gardner and Bennet 1986).

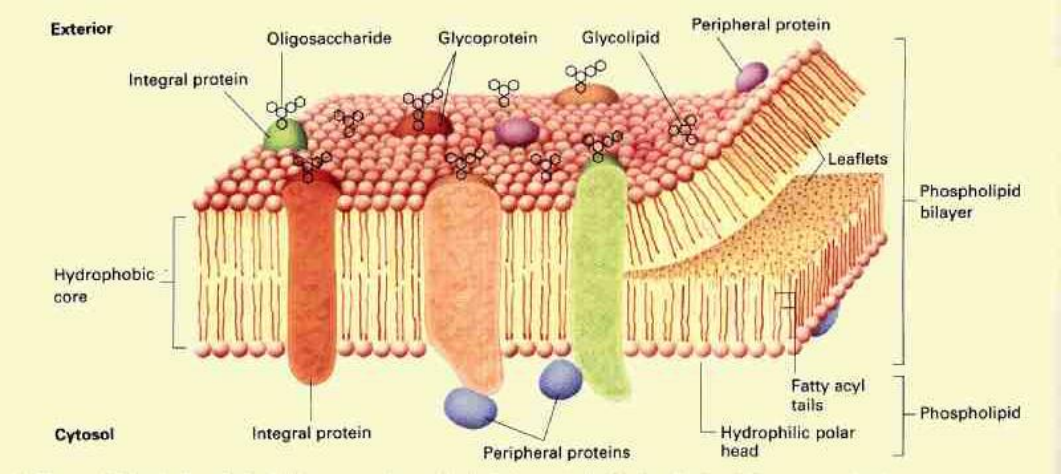



**Figure 2.5: Illustration of the cell membrane with all its components (Gardner and Bennet 1986)**

The cell membrane appears to be the most commonly affected component by most types of stresses. High pressure is no exception as the membrane has shown to be highly affected by HPP. In fact, the damage of the cell membrane and wall could be the main reason for cell inactivation or death (Bowman, Claudio et al. 2007). The degree of pressure resistance is related to the cell's ability to repair leaks after decompression (Farkas and Hoover 2000) as leakage of cell contents is often the consequence when bacteria are being exposed to high pressure. The leakage can come from an irreversible cleavage of intermolecular and intramolecular bonds, leading to structural changes in the membrane. When the structure is changed, there is a decrease of membrane fluidity. The pressure sensitive lipids are responsible for keeping the fluidity of the membrane, which is very important for maintaining cell homeostasis. One of the reasons why lipids are pressure sensitive, is because water may penetrate the lipid bilayer and disrupt bonds of hydrophobic parts. High pressure can therefore cause the normally fluid cell membrane to become waxy and relatively impermeable to nutrients (Mrozik, Piotrowska-Seget et al. 2004). Pressure induced misfoldings of transmembrane proteins can also be a cause of damage to the membrane. These proteins may be squeezed out of the structure because of their lost functionality. Loss of proteins leads to a loss of many important functions in the membrane (Casadei, Mañas et al. 2002, Wong 2012).

### 2.3.4 **Damage of Proteins**

Proteins, playing a major role in the metabolic activity of all living cells, are extremely susceptible to changes in the environment (Heinz and Buckow 2009). Pressure mainly affects the tertiary and quaternary structure of proteins since the covalent bonds in the primary and secondary structures are relatively unaffected. The reason why the higher structures are influenced comes from the presence of water. Water is incompressible, but at high pressure, it will force itself into the interior of the protein matrix. This penetration can lead to a loss of contact between groups in the non-polar domain and causes unfolding or reorientation of some parts of the protein (Knorr, Heinz et al. 2006).

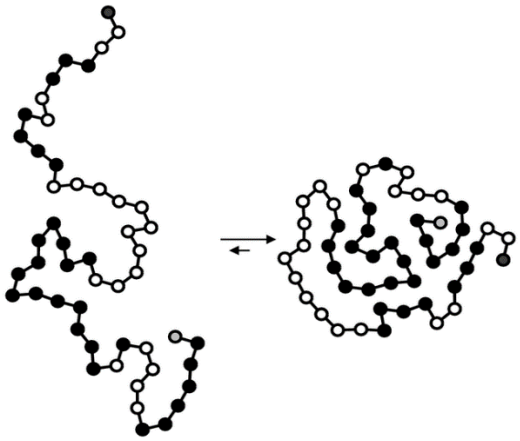


**Figure 2.6: Un-folded and folded protein. In the compact fold (right), the hydrophobic amino acids (black spheres) are shielded, but not in the unfolded protein (Knorr, Heinz et al. 2006).**

The random coiled stage is related to the losses of functionality of proteins and can lead to fatal consequences for the cell (Heinz and Buckow 2009). Damaged proteins in important functions such as ribosomes, translation apparatus or enzyme activity are especially critical. In combination, these cellular injuries lead to cell inactivation and death (Bowman, Claudio et al. 2007). Pressure may also have an irreversible effect on membrane proteins in two possible ways: either the proteins could be denatured in the membrane or they might be squeezed out of it as a result of closer packing of phospholipids and loss of protein functionality. (Casadei, Mañas et al. 2002, Jofre, Champomier-Verges et al. 2007).

### 2.3.5 **Detection of Damaged Cells**

Experimental staining, such as the method of Løvdal, Hovda et al. (2011), can be used to detect leakage of the cell membrane. This is done by using a fluorescent stain, such as Propidium monoazide (PMA). PMA binds to DNA, but can only enter a leaking cell. This means that after pressurization, one can detect bacteria with membrane damages by their colour. Klotz, Mañas et al. (2010), has examined a possible association between resistance of *E. coli* to high pressure and pressure-induced membrane damage with such staining method. In the experiment, loss of viability coincided with irreversible loss of membrane integrity, as indicated by the uptake of Propidium iodide (PI), another fluorescent stain. The effect of colourization with PI can be seen in Figure 2.7. The results are affected by the time of which the stain has been introduced, showed by a higher detection of stained cells in Figure 2.7A, than in Figure 2.7B.

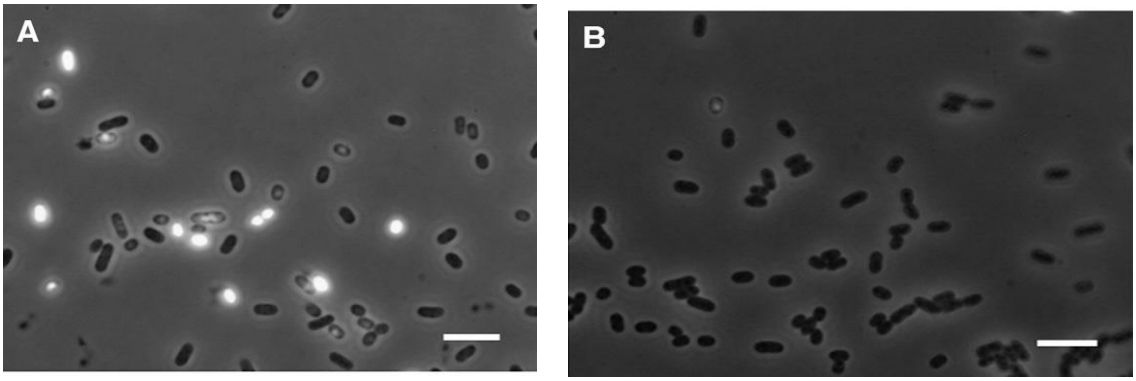



**Figure 2.7: Microscopy of E. coli J1 cells stained with Propidium iodide present during (A), or after (B) pressure treatment at 400 MPa for 10 min. Bar marker 1 μm (Klotz, Mañas et al. 2010).**

Klotz, Mañas et al. (2010), indicated a relationship between uptake of PI during pressure treatment and loss of viability, as shown in Figure 2.8. A correlation was observed between viability and PI uptake. This finding strengthens the theory of an association between loss of envelope integrity during pressure treatment and cell death.

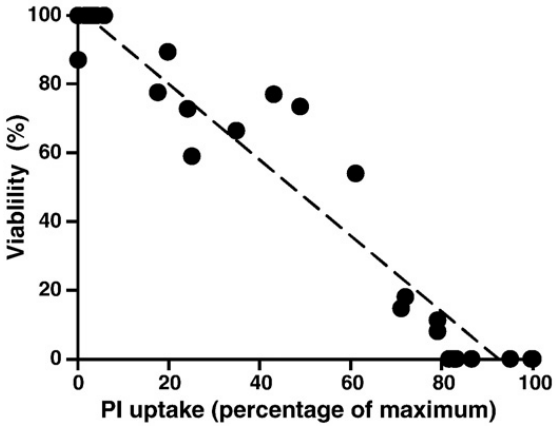



**Figure 2.8: Relationship between PI uptake during pressure treatment and loss of viability in E. coli strains (Klotz, Mañas et al. 2010)**

In the two strains, differences in pressure resistance appeared to be related to the dissimilar ability of their membranes to withstand pressure (Klotz, Mañas et al. 2010). Hence, knowing the state of the bacteria envelope after pressurization can give valuable information on its viability. Since there can be great differences in two strains, the differences between altered species can be quite significant. Knowing the specific effects on *Listeria* could therefore contribute to more insight in this bacterium.

Detection of stained cells is done by using confocal microscopy, an imaging technique that increases the optical resolution and contrast of a micrograph. The micrographs can also give valuable information of the cell state, as abnormalities from healthy cells are visually detected. In Figure 2.9, a difference can be seen between the untreated and pressure treated cells by their appearance. Increasing irregularities with increasing pressure has been reports by the appearance of bud scars, pimples and swelling on the surface of *L. monocytogenes* cells. The extent of these blisters only intensifies with higher pressure (Kaletunç, Lee et al. 2004)

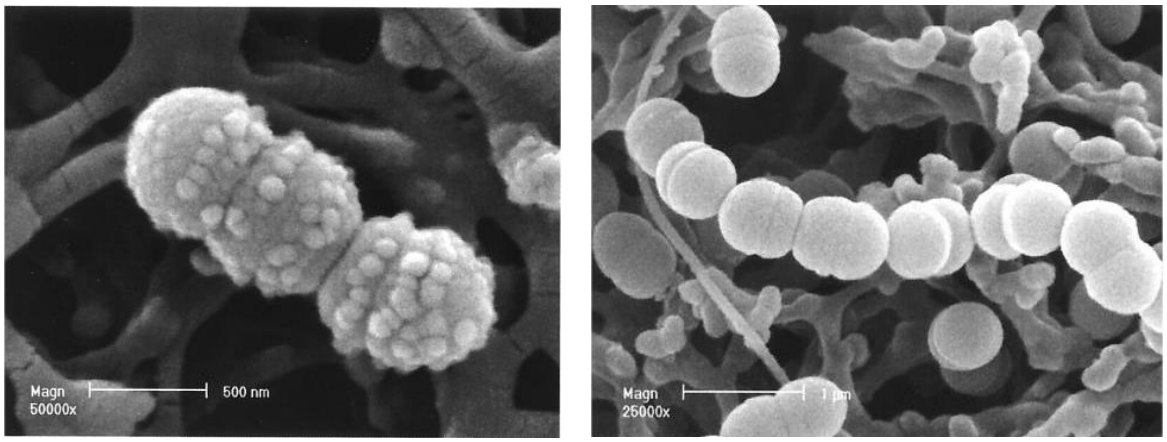



**Figure 2.9: SEM micrographs on gram positive bacteria. Left: One cell treated with 500 MPa at 35 °C for 5 min. Higher magnification (x50 000). Right: Untreated cells. Original magnification (x25 000) (Kaletunç, Lee et al. 2004)**

## 2.4 Bacterial Response to High Pressure

To prevent death from environmental stresses, there are specialized mechanisms in the bacteria cells that help them withstand inhospitable conditions. Currently, stress adaptive responses of bacteria in pressure processed food is not completely understood (Wong 2012). However, the ability of cells to recover is an important concern in food safety. The lack of knowledge in this field may impair the use of HPP treatment as an efficient food preservation method, making it essential to understand these adaption responses (Jofre, Champomier-Verges et al. 2007).

### 2.4.1 Sensing and Signalling of Stress

In many bacteria, the link between variations in environmental conditions and a genetic response has been shown to result from the sensing and regulatory activities of the two component signal transduction systems (TCST) (Hill, Cotter et al. 2001). The two-component signal transduction systems enable bacteria to sense, respond, and adapt to changes in their environment or in their intracellular state These signal transductions originates at the membrane, where the signalling proteins are clustered together (Groves and Kuriyan 2010). A typical two-component system consists of a membrane-associated histidine kinase and a response regulator. The kinase monitors a specific environmental parameter and activates, by phosphorylation, the response regulator when this parameter varies. The activated response regulator can then effect changes in cellular physiology, often by regulating gene expression (Barton 2005, KEGG 2014).

DesRK is an example of a two component system that senses changes in the environment, requiring change of fatty acid composition. By controlling the expression of the des gene that encodes a fatty acid desaturase, the system regulates membrane fluidity. Since high pressure affects membrane fluidity, the desRK system is very likely to be activated under pressure as an adaption response (Mascher, Helmann et al. 2006). DesRK is discovered in *Bacillus subtilis*, but it is assumed that the L.*monocytogenes* equivalent genes of desR and desK are lmo1021 and lmo1022 respectively (STRING 2013).

### 2.4.2 Regulators

A stress response is achieved by changing the patterns of gene expression for those genes who are involved in the bacteria defence. To change the expression of such genes, a transcription factor must be activated. The transcription factor is needed because it interacts with RNA polymerase so they can co-ordinate gene expression together. One family of transcription factors with a role in stress-resistance is the sigma factor. A sigma factor is a subunit of RNA polymerase and it is essential for transcription initiation by recognising the promoter. (Wright and Lewis 2007). Stress response genes often needs interactions of several factors to do transitions and therefore they may be accompanied by co-regulators. These co-regulators can either be multiple alternative sigma factors, or alternative sigma factors with transcriptional regulators (Chaturongakul, Raengpradub et al. 2010).

Sigma B is the central regulator of the stress response in *Listeria*. The activation of SigB is controlled by tight regulation, which limits its expression only to situations where the cell is exposed to stress or when ATP levels are low (Hill, Cotter et al. 2001). This regulation is performed by the Stressosome, which is a type of TCST system. When exposed to stress, the bacteria senses and signals this through a regulatory cascade in the Stressosome, leading to the activation of $\sigma^B$. This is a type of partner-switching cascade, which uses phosphorylation on the proteins that are responsible for the activation of $\sigma^B$. These proteins are the two regulators RbsV and RsbW. In unstressed cells, the sigma factor is held in an inactive state by the anti- sigma factor RsbW. When a type of stress is applied, the anti-anti-sigma factor RsbV is dephosphorylated and connects itself to RsbW. The sigma factor is then freed to bind to RNA polymerase and can activate gene transcription (Wright and Lewis 2007). The pathway from stress-sensing to transcription of the $\sigma^B$ regulon is illustrated in Figure 2.10.

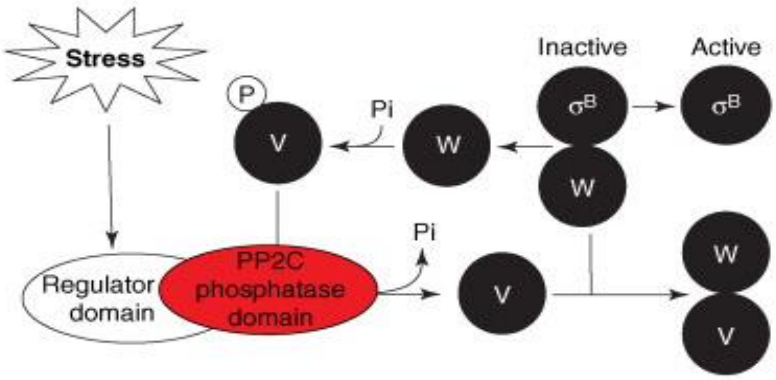



**Figure 2.10: Regulation of $\sigma^B$. Under normal conditions $\sigma^B$ is bound to RsbW (W), but under stress, the anti-anti sigma factor RsbV (V) dephosphorylates and binds to RsbV. Thus releasing $\sigma^B$ to become active (van Schaik and Abee 2005).**

When $\sigma^B$ is activated, the transcription of the $\sigma^B$ regulon starts. The $\sigma^B$ regulon is the set of genes that are regulated by $\sigma^B$, which codes for proteins performing the specific functions that can protect the bacteria cell against stress. (van Schaik and Abee 2005). A typical pathway from stress exposure to cross protection can go through a cascade of events with SigB in a leading role, as shown in Figure 2.11 (Wong 2012). The specific sigma factor used to initiate transcription of a given gene will vary, depending on the genes and environmental signals. SigL, also known as RpoN, is an example of a sigma factor that influences many genes associated with stress response. Together with SigB, RpoN can control and fine-tune the expression of genes that is important for coping with physiological stresses and metabolic requirements in L. *monocytogenes*. Such co-operations can be found for many of the regulators (Chaturongakul, Raengpradub et al. 2008).

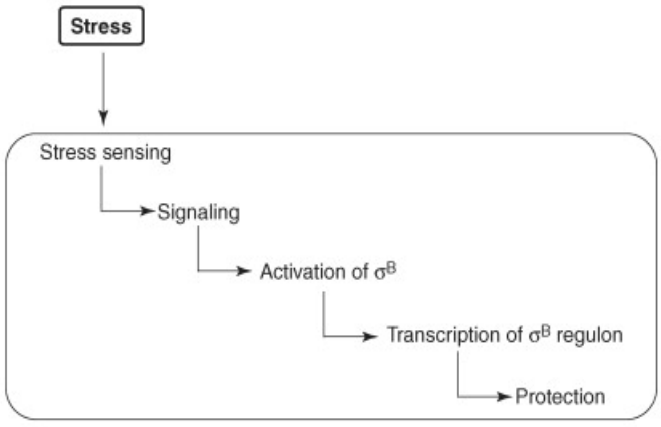



**Figure 2.11: The pathway from exposure of stress to activation of protective functions in the bacteria cell (van Schaik and Abee 2005).**

**Table 2.1: Presentation of relevant genes controlled by the regulators SigB, RpoN, CodY, and HrcA. Adapted from (Bowman, Claudio et al. 2007, Chaturongakul, Raengpradub et al. 2008, Wong 2012, STRING 2013)**

| Category of function | Subcategory | Genes within category |
|---|---|---|
| **SigB regulon** | | |
| See text for specific functions of regulator | | |
| Heat shock proteins | Peptidases | clpEP |
| | Chaperones (folding, stabilization etc) | grpE, dnaKJ, htpG,(groES,groEL - |
| Stress related | General: | lmo1601, lmo0211 |
| | Universal: | lmo0515,lmo1580, lmo2673 |
| Growth | Septal ring | minD, Fts-complex lmo1606 |
| Transport | ABC/ATP | opuCA,CD,CC,CB, lmo1420 |
| | Amino acid, phosphate, carbohydrate | lmo0956, lmo0405, lmo0784 |
| Metabolism | amino acid | lmo1052, lmo1433, lmo2434 |
| | Carbohydrate | lmo0169, lmo0781-4, lmo0956 |
| | Glycerolipid | lmo1539, lmo2695 |
| **RpoN regulon** | | |
| See text for specific functions of regulator | | |
| Stress related | General | lmo1601 |
| Stress protection | autolysins, cell wall hydrolase: | lmo0129,lmo1521 |
| | Protein folding/processing | pflC/lmo1407, clpP |
| Metabolism | Carbohydrate | lmo0096, lmo0097, lmo0098, |
| | Amino acid | lmo1053, lmo1056, lmo1235, |
| | Glycerolipid, glycerophospholipid | lmo1538, lmo1293 |
| Transport | Peptides | lmo0136,lmo0137, lmo0152, |
| Growth | Cell division | ftsZ, mrp, lmo2688 |
| **CodY regulon** | | |
| Transcriptional repressor. DNA- and GTP-binding protein that senses the intracellular GTP concentration as an indicator of nutritional limitations. Represses the expression of many genes that are induced as cells make the transition from rapid exponential growth to stationary phase. | | |
| Cell cycle | Septum | lmo0217, fts complex |
| | Cell shape determining | mreB |
| Transport | Glucose | lmo0169, lmo0768 |
| | Amino acid | lmo2469 |
| Metabolism | Glucose | lmo0022, lmo0183, lmo2566-69 |
| | Amino acid | lmo0458, lmo0561-8, argDBJ |
| **HrcA regulon** | | |
| Transcription repressor regulating chaperone expression. Negatively influences the transcription of class 1 stress response genes | | |
| Stress response | Heat shock proteins | groES, groEL, dnaK, dnaJ and grpE |
| | Peptidases | clpX |

Even though the sigma factors are fundamental, there are other important gene regulators in L. *monocytogene*s. A list over central regulators, together with the most relevant genes they affect, is given in Table 2.1 for overview. It must be emphasized that the bacteria contains many more regulators than enlisted here and the regulators may affect even more genes than given in this table. These proteins and genes are on the other hand given because of their relevance to repair of pressure damaged cell envelopes.

### 2.4.3 **Induced Genes and Proteins**

To be able to recover from stress, specific genes must be activated. Even moderate increases in pressure can result in a myriad of effects on gene and protein expression (Bartlett 2002). Bacteria tend to change many genes slightly rather than certain genes drastically as this could be a safer and less energy demanding procedure. This can result in a broad distribution of regulated genes where the majority of the genes does not seem very affected. L. *monocytogenes* has shown to actively express many genes as a response to high pressure, but some functional categories appears more affected than others. Genes that tends to be expressed at higher levels under high pressure are genes encoding for transport and binding, signal transduction and chemotaxis, cellular processes, transcriptional regulators, metabolism and protein fate (Yanhong and Amy 2008). The stabilization and maintenance of the bacteria cell is at high focus, showed by the significant regulation of ribosomes and proteins, together with components involved in the cell envelope and the septal ring. The activation of genes involved in the lipid and peptidoglycan biosynthetic pathways are assumed to be connected to this function. Up-regulation of genes associated with generalized repair and maintenance has been proved, where the activation of cold- and heat-shock genes is an example of this (Malone, Chung et al. 2006, Scorttia, Monzóa et al. 2007). When high pressure demands more energy to be used on repair, energy production and conversion is supressed. This can be seen by the pressure induced switch from active growth to a cell repair state, the stationary phase. Resulting in a decreased growth rate (Bowman, Claudio et al. 2007)

Several genes associated with cell formation and shape, as well as synthesis or reassembly of peptidoglycan and fatty acids, were observed to have increased expression. Because of this, genes involved in such functions can be considered as very central in the response to high pressure. It is assumed that *L. monocytogenes* may compensate damage by increasing cell division and cell-envelope associated gene expression, as it may lead to replacement of damaged components (Bowman, Claudio et al. 2007).

### 2.4.4 **Recovery Time**

Shelf life studies for detection of possible recovered cells, is a useful method to determine recovery time. Growth experiments is probably the most common way to detect viability of pressurized bacteria cells, but finding the recovery period gives a deeper insight of their viability. By studying the period between low growth appearances, to increases of a colony, the required time for cell reparation can be found. Bacteria have an excellent mechanism of recovering from injury as they have the potential to grow after repairing the site of injury during storage. Shelf life studies are therefore necessary to ascertain the microbiological safety of food products. During recovery time studies, the ratios of injured and non-injured cells can be detected by using selective and non-selective growth mediums. A non – selective medium such as TSAYE (Tryptic Soy Agar supplemented with 0.6 % yeast extract) will allow everything to grow, while a selective medium such as Brilliance is harder to survive in and will therefore be too harsh for injured cells to grow in (scientific 2014). By using both selective and non-selective media in a recovery experiment, viable

cells can be differentiated between injured and non-injured, in the same time as the transition from injured to non-injured cell can be detected and timed (Hansen and Knochel 2001).

The damage caused by high pressure may be repairable, making it possible for bacteria cells to grow during storage. However, the time this takes varies a lot. The pressure level, pressurization time, pressurization temperature, storage temperature, bacteria strain or other inhibiting factors such as salt concentrations or pH, all contribute to the time required for bacteria to repair and start growing again. An experiment done by Bozoglu, Alpas et al. (2004) detected that *L. monocytogenes* strains being pressurized at 450 MPa for 10 minutes needed only 1 day to go from no detected colonies to detection of colonies when stored at 4°C. An increase to 550 MPa on the other hand, required 6 days for the bacteria to recover. Another experiment conducted by Muñoz-Cuevas, Fernández et al. (2012) observed how much time it took from an increase from 1 to 100 bacteria cells. With 350 MPa for 3 minutes, 30 °C storage time and pH 6, this took approximately 16 hours. Increasing the pressure up to 450 MPa required 50 hours to reach 100 cells. The presented data shows that the recovery time is very dependent on the conditions and the recovery for each kind of food matrix should be tested independently as the results varies a lot.

## 2.5 Cellular Processes Potentially Involved in Repair Mechanisms

Many experimental results have given valuable information on how the bacteria is damaged by high pressure, giving a good basis for the existing damage theory we have today. However, despite of all studies on repair of sublethal injury, there appears to be no previous reports comparing the potential repair paths during storage after pressurization. It is clearly important to know more about the mechanism of injury as it will help to understand the properties of pressure damaged cells and hence how to defeat them with high pressure processing (Bozoglu, Alpas et al. 2004).

As there is no existing model for the repair mechanism of the cell envelope, there is no exact description on how bacteria repair the damages either. The mechanisms given below are used as a basis for the repair hypothesis and will therefore be presented in this thesis. The assumptions made from these theories will be discussed in further detail under the Results & Discussion chapter.

### 2.5.1 Peptidoglycan Synthesis and Turnover in the Cell Wall

The cell wall is an important protector of the cell. Hence, a well-developed repair mechanism that makes sure the cell wall is at an optimum state is essential. This state can be maintained by replacing unusable components with functional ones. This section deals with the pathways of the replacement process as well as the components involved in the cell wall maintenance.

### 2.5.1.a *Peptidoglycan Synthesis*

When peptidoglycan is being made, the first step is to transform sugar into aminosugar by the help of the Glm complex. Further on the transformation of the peptidoglycan precursor, LipidII, is synthesized by the help of the Mur complex. Energy and amino acids are added as needed along the pathway. Figure 2.14 shows the stepwise formation from sugar to peptidoglycan with all its associated components (Heijenoort 2007).

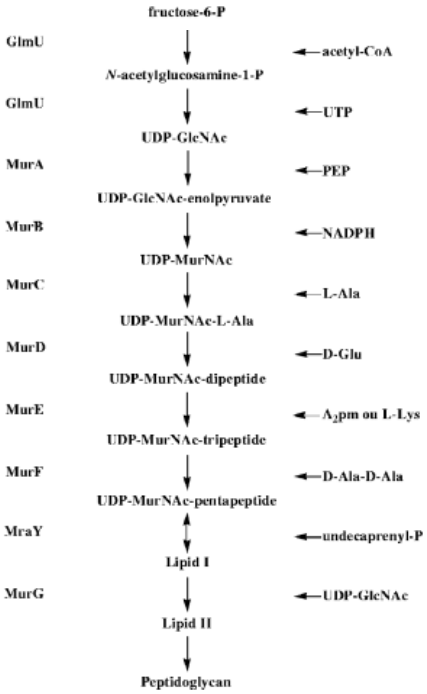



**Figure 2.14: Stepwise assembly of the PG units (Heijenoort 2007).**

The precursor of peptidoglycan must be synthesized within the cytoplasmic cell and not at the cell wall site. Since LipidII is not produced at its functional place, some proteins must make sure this component is brought to its proper location. The DivIVA protein recruits the cell wall synthesis machinery to the cytosolic membrane site, while the transmembrane protein FtsW (RodA) flips LipidII across the membrane. The mechanism of LipidII relocation is illustrated in Figure 2.12 (Vicente, Rico et al. 2006, Sieger, Schubert et al. 2013). When LipidII is located at its proper site, it is incorporated at the outer membrane surface by the Penicillin-binding proteins (Pbp). The Pbp consists if a GT and a PT domain. The GT domain pulls LipidII from the membrane and polymerize it to a peptidyl sugar backbone. The TP domain then cross-links the peptides to construct the peptidoglycan layer. This scheme is illustrated in Figure 2.13 (Borman 2007).

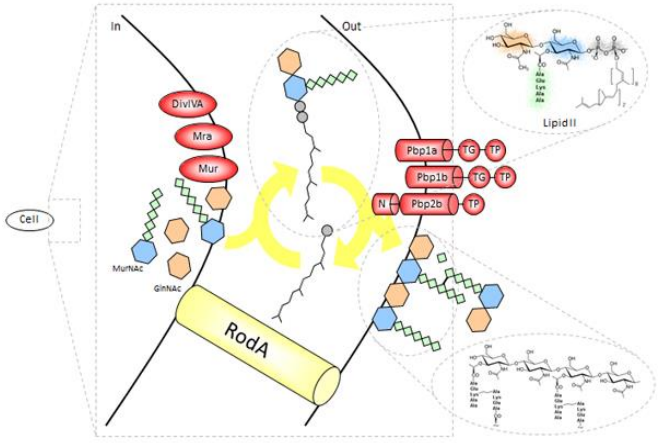



**Figure 2.12: LipidII assembled to the membrane site where FtsW (RodA) uses phospholipids to flip LipidII over the membrane (Sieger, Schubert et al. 2013).**

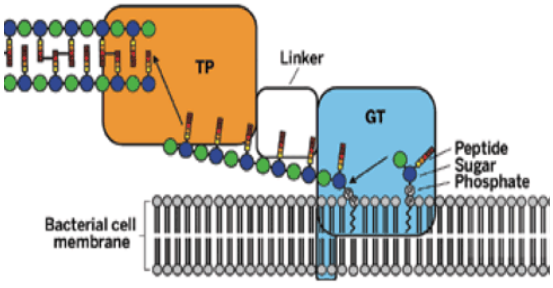



**Figure 2.13: Polymerization mechanism conducted by the penicillin binding proteins to create peptidoglycan (Borman 2007).**

#### 2.5.1.b *Autolytic enzymes and peptidoglycan turnover*

Peptidoglycans can be degraded or recycled when they need to be replaced. The autolytic enzymes, also called muramidases, are responsible for this process. Many of the autolytic enzymes are attached to the expanding regions of the cell wall and contributes to the synthesis of cell wall by a combination of controlled autolysis and biosynthesis. This is the turnover of peptidoglycan. The autolytic enzymes release fragments of the wall into the culture fluid of Gram-positive bacteria, giving an opportunity to reutilize the material for PG synthesis. The lipoteichoic acid that goes through the cell wall can regulate the activity of these enzymes (Barton 2005, STRING 2013).

### 2.5.2 **Phospholipid Synthesis and Turnover in the Cell Membrane**

Membrane integrity is challenged whenever the environment changes, and replacement of membrane proteins or lipids is an ongoing process that makes the membrane able to adapt to these changes. Since the lipids and proteins are not covalently joined, newly synthesized proteins such as transport proteins and cytochromes can easily be inserted into the membrane, without requiring cell division. The stabilization of the membrane by lipophilic and ionic interactions provides greater flexibility for the introduction of proteins than if the membrane structure was secured by covalent bonds, but these interactions also makes it easier to dislocate the proteins from the membrane (Barton 2005). Almost all of the metabolic energy required to produce membrane lipids is used in the formation of fatty acids. The large investment in energy for fatty acid biosynthesis has made the bacteria evolve multiple mechanisms to control pathway activity and precisely match fatty acid production to growth rate. Thus, the regulation of fatty acid production is very tightly controlled so that the wasteful usage of ATP is prevented (Zhang and Rock 2009).

### 2.5.3 ***Phospholipid Synthesis***

Phospholipids are the product of three separate activities: Synthesis of fatty acids, binding of fatty acids onto sn-glycerol-3-phosphate and conversion of polar head groups to produce appropriate phospoholipids. The composition of these three parts is as shown in Figure 2.15. The structure of both fatty acids and phosphate groups can vary, giving rise to many different types of phospholipids. The membrane is assembled by this variety of phospholipids, giving rise to different biophysical properties of the membrane (Barton 2005).

The synthesis of a phospholipid starts with the fatty acid biosynthetic machinery. The FASII pathway is represented in Figure 2.16, with the stepwise formation of fatty acids that are incorporated in the plasma membrane. The synthesis starts with conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC). Important proteins such as the Fab complex that catalyses the formation of the different precursors, as well as the acyl carrier protein (ACP) that carries all of the FASII pathway intermediates, are also presented. The Pls complex are the membrane proteins that transfers the fatty acids into the membrane to become the phospholipid precursor, phosphatidic acid (PA).

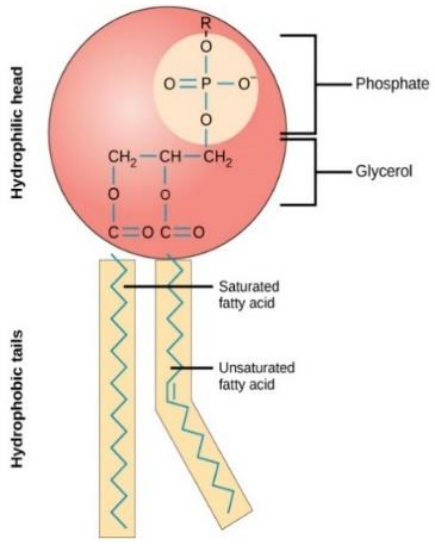



**Figure 2.15: The phospholipid comprising two fatty acids, a glycerol backbone and a phosphate group (Garavito and Ferguson-Miller 2012)**

PA is the key intermediate in the formation of most bacterial phospholipids. From here, PA can be synthesized into various phospholipids such as phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylinositol (PtdIns) or phosphatidylserine (PtdSer) (Berg, Tymoczko et al. 2002).

The last step in Figure 2.16a, where a fatty acid chain is introduced, is the step that determines what kind of fatty acid that will make up the phospholipid. If a bacterium needs to increase the fluidity of the plasma membrane, the introduced fatty acid will most likely be an unsaturated fatty acid. This step is important as it will help the bacteria to adapt to higher pressure. Control at the level of fatty acid biosynthesis is crucial for membrane homeostasis since the biophysical properties of membranes are mainly determined by the composition of the fatty acids that are produced by *de novo* biosynthesis.

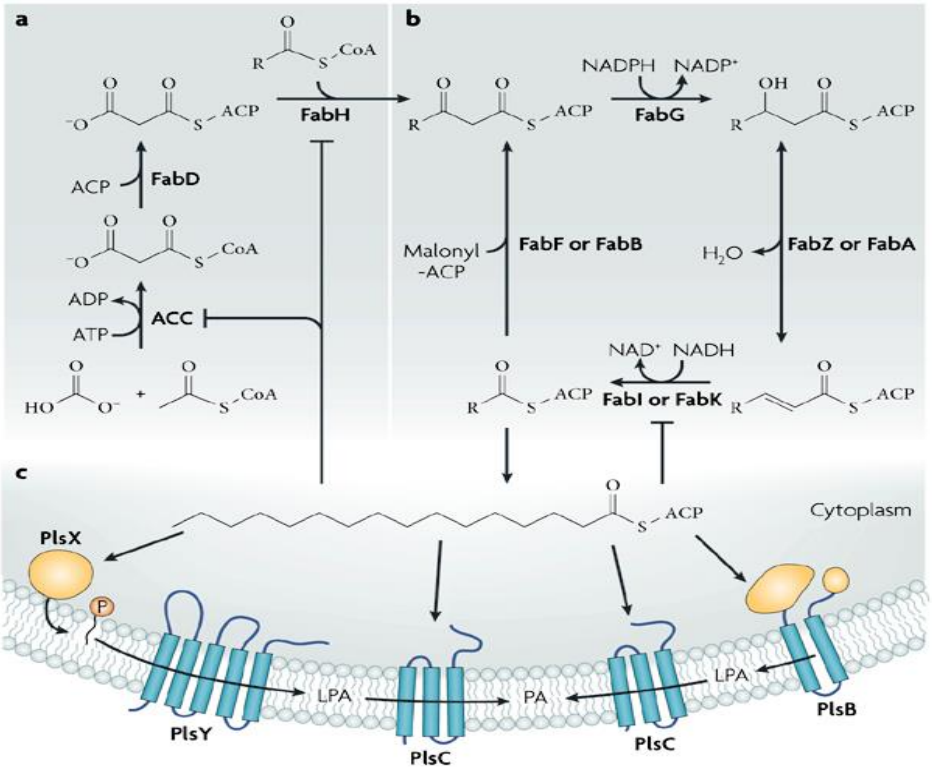



**Figure 2.16abc: The mechanism of phospholipid synthesis. Starting with (a) synthesis of fatty acid (b) to become phosphatidic acid (c) and incorporating it in the membrane where it is transformed to the designated phospholipid (Zhang and Rock 2008).**

Bacteria do not only produce new phospholipids in order to obtain the desired fatty acid composition. They can also adjust the fatty acyl chains that already are attached to the glycerol backbones in the plasma membrane. This regulation often occurs when the bacteria are subjected to abrupt changes that requires an immediate modification of the membrane to optimize growth under the new conditions (Mrozik, Piotrowska-Seget et al. 2004). HPP treatment will cause such an abrupt change. The ability to change existent fatty acids is therefore very relevant for the bacteria's ability to adjust to pressure. Under exposure to high pressure, production of higher amounts of mono- and polyunsaturated fatty acids will help maintaining the appropriate membrane fluidity. (Mangelsdorf, Zink et al. 2005). (Bartlett 2002).

### 2.5.3.a *Phospholipid Turnover*

The high energy consumption from production of phospholipid fatty acyl makes recycling of components necessary, with portions of phospholipids used as precursors in the biosynthesis of other major structural molecules. Phosphatidylglycerol (PtdGro) and phospatidylethanolamine (PtdEtn) are such examples as they both possess recyclable constituents.

PtdGro is a metabolically active phospholipid with a head group that can, with the help of the LtaS enzyme, be used for production of teichoic acid. Teichoic acid is a constituent of lipoteichoic acid (LTA) a common component in the membrane and a regulator of autolytic wall enzymes (muramidases) (Gindsburg 2002). The mechanism of turnover is showed in Figure 2.17. When the headgroup has been used, the remaining part of the phospholipid is a diacylglycerol (DAG). DAG is converted to the phosphatidic acid (PA) intermediate, by the soluble DAG kinase, DgkB.

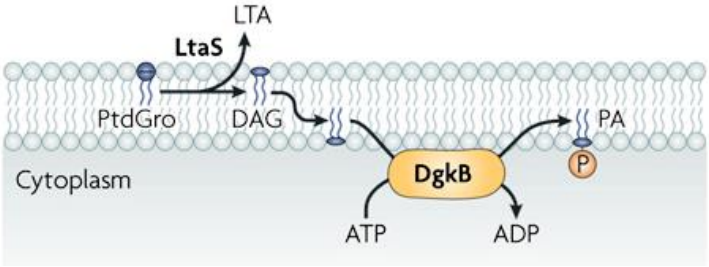



**Figure 2.17: Recycling of PtdGro with the help of LtaS and DgkB (Zhang and Rock 2008).**

PtdEtn can be reused by transferring the fatty acids to a membrane lipoprotein (Lpp). This process is catalysed by the phospholipid-protein acyltransferase (Lnt). The remains of PtdEtn, is transferred to the cytosolic side by the lysophospholipid flippase (LplT) and will either be recycled or degraded with the help of the acyl carriers. The mechanism of this turnover is illustrated in Figure 2.18.

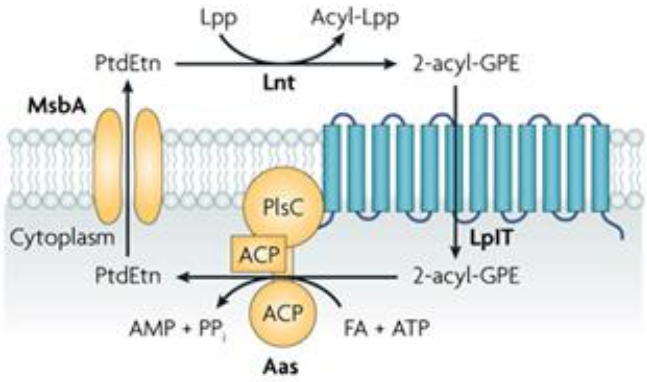



**Figure 2.18: Turnover of PtdEtn with the help of Lpp, Lnt, LplT, Acp and Ass proteins (Zhang and Rock 2008)**

### 2.5.4 **Protein Repair and Turnover**

Keeping the functionality of proteins in the cell is of great importance as nearly all reactions will halt without the enzymatic activity of proteins. Because of this, the bacteria has developed a highly sophisticated mechanism that makes sure that the proteins are functional. The proteins especially developed to repair faults in a stressed cell are designated cold - and heat-shock proteins. Despite their names, they operate during most kind of stresses. The heat-shock proteins (HCPs) are especially relevant for the cell envelope as they work to repair damaged proteins, which includes the membrane associated proteins. This section will only focus on the membrane-associated proteins, though the heat shock proteins do work on repairing all proteins inhabiting the bacteria cell. The heat-shock proteins can roughly be divided into two groups: The repair chaperones and the peptidases. The repair chaperones works on the misfolded proteins by trying to refold them back to their functional states. The peptidases however, will target the damaged proteins that are beyond repair and degrade them so they can be recycled into new functional proteins again.

#### 2.5.4.a ***Repair Chaperones***

The heat-shock proteins protect other proteins by chaperone activities. The DnaK chaperone is especially important for these mechanisms as it contributes to modulation of polypeptide folding, repair, degradation of protein aggregates, translocation across membranes and protein-protein interactions. The reparation is done through cycles of binding and release, which is possible because DnaK undergoes a conformational change to reach states with high or low affinity for the substrate proteins. Figure 2.20 gives an overview of how the reparation cycle may look like. This illustration focuses on the function of Hsp70, the eukaryotic version of DnaK, but the same mechanism can be applied to the function of a variety of DnaK homologs. (Wilbank 2014). The specific features of the repair process, can be seen in Figure 2.19. Developed from the bacteria specific system in *E. coli*.

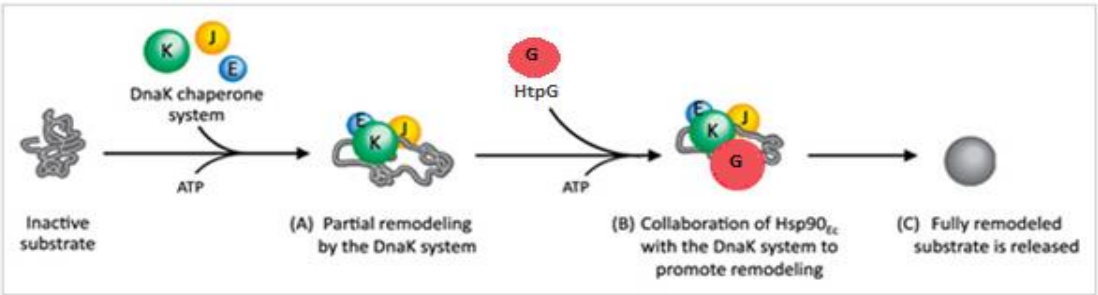



**Figure 2.19: Reactivation of proteins by the activities of HtpG and DnaK system in *E. coli*. (A) The chaperones in the DnaK system (DnaK, DnaJ and GrpE) interact with a denatured protein and initiate the remodelling process. (B) HtpG interacts with the complex. (C) When remodelling is completed, the functional protein is released (Kerrigan 2011)**

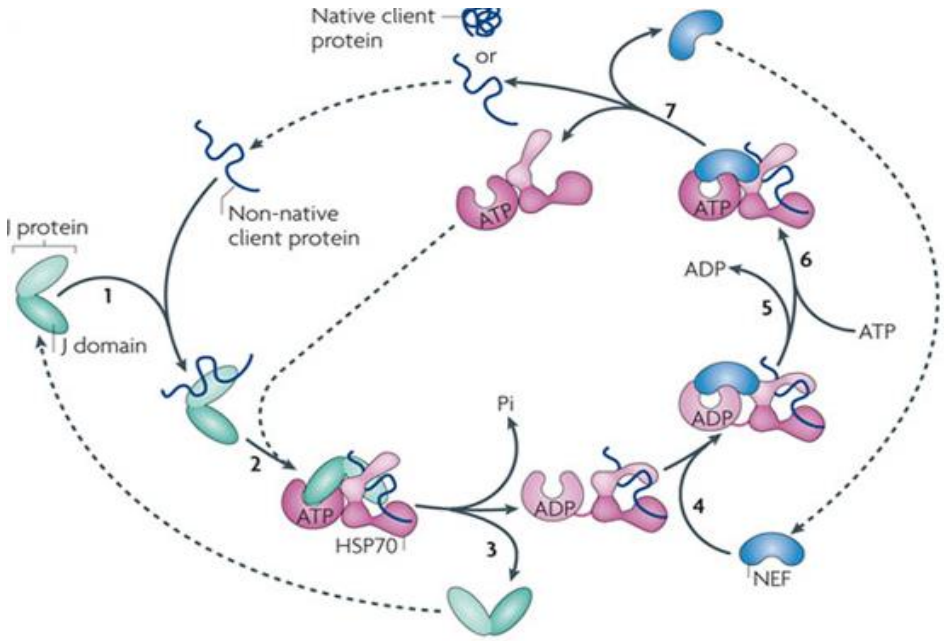



**Figure 2.20: The reparation cycle of damaged proteins. (1) The DnaJ protein (J) binds to the unfolded protein (2) Interaction with DnaK (3) DnaK stabilizes the interaction with the unfolded protein and DnaJ leaves the complex. (4,5,6) Attempt to refold protein (7) Dissolving of complex, leaving either a folded and functional protein or an unfolded protein that can undergo a new repair cycle (Kampinga and Craig 2010).**

### 2.5.4.b ***Chaperone regulators***

SigB and HrcA are two of the regulators of the repair chaperones in *L.monocytogenes*. At un-stressful conditions, the SigB level is kept low by rapid turnover, but upon exposure to stress stimuli, the concentration of SigB is greatly increased, resulting in transcription of SigB dependent heat shock genes. The transcription of SigB is controlled by a negative feedback system to prevent accumulation of heat shock proteins. As an example, accumulated DnaK-DnaJ-GrpE chaperones can bind to SigB, which inhibits its activity or promote its degradation. Because of this, the availability of DnaK-DnaJ is a direct sensor of cellular stress and a regulator of heat shock transcription. The HrcA repressor regulates major heat shock proteins by binding to negatively acting CIRCE elements (Controlling Inverted Repeat of Chaperone Expression). The GroE chaperonin system facilitates folding of HrcA, but in response to increased protein damage, the GroE folding machinery is occupied. Leading to a stall of HrcA folding. Thereby this regulatory system relies on the availability of heat shock proteins to provide a direct sensing mechanism for protein misfoldings (Gahan, O'Mahony et al. 2001). The regulating mechanisms of SigB and HrcA are illustrated in Figure 2.21.

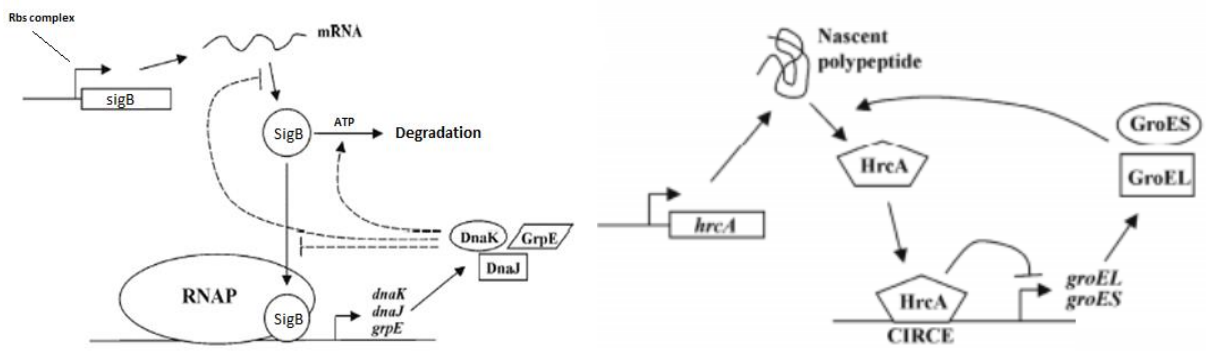



**Figure 2.21: Activation and deactivation of the chaperone regulators SigB (left) and HrcA (right) (Gahan, O'Mahony et al. 2001).**

### 2.5.4.c *Protein degradation*

Although chaperones can facilitate folding or refolding, such rejuvenation is often not possible. In such cases, chaperones can assist degradation, either by simply preventing aggregation and thus keeping misfolded proteins susceptible to proteolysis or by actively facilitating their transfer to proteolytic systems (Kampinga and Craig 2010). Peptidases and proteases (such as the Clp protease family) contribute to degradation of peptides and proteins by running the proteolytic mechanisms. In the degradation process, the proteins are cleaved to peptides which are further degraded into amino acid sequences. These sequences can be used to synthesize new proteins, allowing the proteins to be recycled (Jofre, Champomier-Verges et al. 2007).

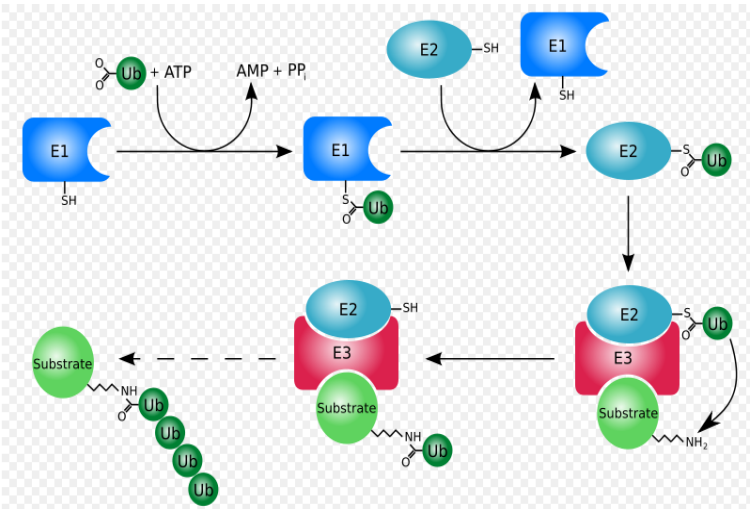



**Figure 2.22: The ubiquitination process that signals proteases to degrade a protein (Qian 2009)**

A damaged protein that is to be removed is tagged for degradation with a small protein called ubiquitin and the tagging reaction is catalysed by the ubiquitin ligases. Once the protein is tagged with one ubiquitin molecule, additional ubiquitin molecules will attach, as shown in Figure 2.22. This makes the proteasome degrade the tagged protein. Transmembrane proteins are first dislocated from the membrane and then lead to destruction in lysosymes (Qian 2009).

### 2.5.4.d ***Protein insertion***

If a protein is missing from the membrane, either by being squeezed out because of its changed structure or because of relocation from degradation tagging, it must be replaced by a new one. The bacteria has a well-developed system for this: The Sec translocation complex. The Sec translocon is an essential system for protein translocation across and into the membrane. The core channel is the SecYE complex with a peripheral SecA ATPase that provides the motor for the translocation. This channel is located in the membrane and most of the polypeptide chains to be inserted use this pathway. The signal recognition particle (SRP) (or the chaperone SecB) is also necessary as it mediates the targeting of the protein substrate to the translocon.

The polypeptide to be inserted contains a leader peptide which gives correct interaction with the translocon. The channel opens and accommodates the leader peptide by moving away the blocking plug domain. When the polypeptide is connected to the translocon, it is threaded through the channel as an unfolded string of amino acids. This is done by creating an opening of the translocation pore which allows it to embrace the polypeptide chain. Once translocation is finished, a signal peptidase cleaves off the leader peptide while the nascent protein is leaded to its proper location in the membrane (Dowhan and Bogdanov 2009, Maillard, Chan et al. 2013). The process of protein insertion is illustrated in Figure 2.23.

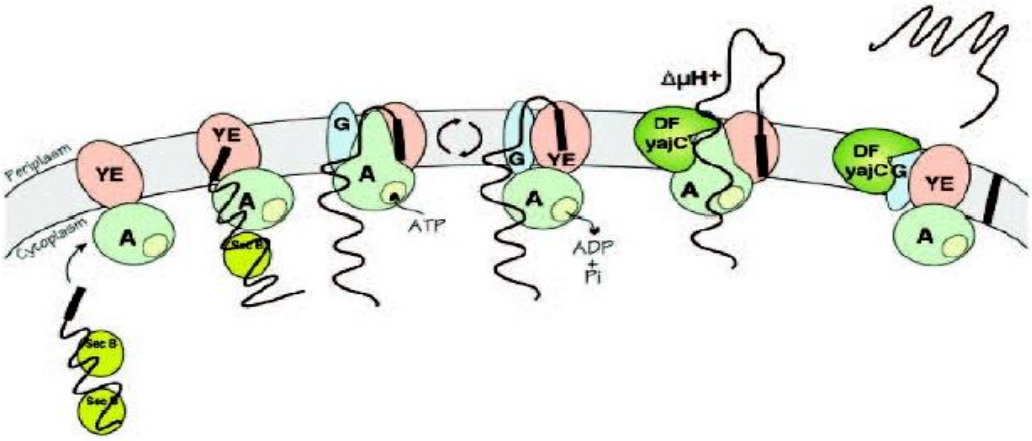



**Figure 2.23: Insertion of a new protein in the cytoplasmic membrane (Maillard, Chan et al. 2013)**

## 2.6 Modelling of Biological Systems

The ability to model and predict the behaviour of genetic circuits has an increasing importance as molecular genetic techniques have made it possible to construct *de novo* genetic circuits. This is called synthetic biology and it comprises the design and construction of biological devices and systems. This technology, which combines biology and engineering, approach to create new biological systems that can help us on finding out how life works or how to use it for beneficial purposes (Keasling 2012). Synthetic biology aims to apply the engineering principles into the design of biological systems. By considering each DNA-encoded component in isolation, these individual parts can be combined to produce new pathways and devices that give a predictable response (Ellis, Adie et al. 2011).

A very common application of genetic modelling is to manipulate bacteria to synthesize new products, but as this method reveals the genetic circuits in a bacteria, it may also help to discover unknown pathways and mechanisms. By combining mathematical modelling programs with a pathway model, the behaviour of a biological pathway can be simulated. It is then possible to simulate the exposure of a system to different conditions, such as altered pressure levels, and see how it responds (Almaas 2013) When a system is investigated through a computational program instead of laboratory experiments, a lot of time and expenses can be saved as this represents a more efficient analytical method. Mathematical models complement experimental procedures that are done to identify the molecular components and interactions in a system of interest. Of course, laboratory experiments are the foundation for all models as this is the only way to find out what actually happens. This is where one obtains all parameter values such as reaction kinetics or genetic expressions. Still, a computational program that can simulate what has been found through experiments, can make the further work easier.

# 3 Materials and Methods

The method of developing a cell envelope repair model was divided into three parts. The following chapter is therefore divided into three sections, where the methods of each step has been explained. The first part, chapter 3.1, introduces the methods used to develop a *de novo* repair pathway. The second part, chapter 3.2, explains experimental procedures that were done to give supporting data for the repair model development. The last part, chapter 3.3, explains the methodology of making mathematical expressions of the created models and simulations of the repair process.

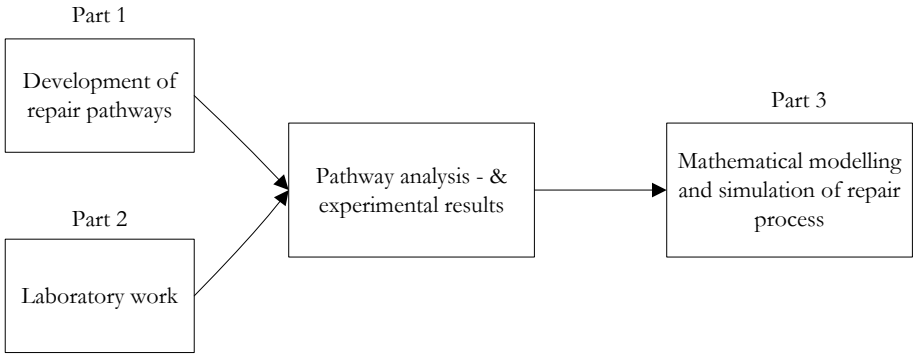



**Figure 3.1: Flowchart of methodology. First, repair pathways are created. Second, experiments are conducted. Third, results are used for modelling purposes.**

## 3.1 Pathway Analysis

As there is no existing pathways for pressure induced repair at today's date, a strategy of gathering information from different sources and putting them together in a reasonable matter was used. These were sources such as published articles, protein databases and experimental results. Different articles described different mechanisms that possibly can be involved in the reparation process. STRING is a database of known and predicted protein interactions that uses sources from genomic context, high-experiment throughputs, conserved co-expressions and previous knowledge to provide protein connections (STRING 2013). Therefore, STRING provided proofs that the specific repair proteins existed in *L. monocytogenes* as well as showing if they were connected. Information for each protein was provided in this database, but there was little information on how the proteins affected each other. This means that connection systems could be obtained, but not interaction systems.

Gene regulation data from Bowman, Claudio et al. (2007), gave additional proofs that the specific repair proteins were active in *L. monocytogenes*. In addition, these data provided values of gene regulations at 400 and 600 MPa immediately after pressurization (no recovery time). This information is very valuable when choosing proteins to be used in the repair pathways. Gene regulation values indicates if a gene has become more or less active as a response to high pressure.

This makes it possible to sort pressure relevant genes from the pressure irrelevant ones. A fold change value between -1 and 1 can be regarded upon as unchanged while everything above or below this range will represent upregulation or downregulation of genes respectively. Supplementary regulation values of the relevant proteins could also be found from other experiments, though there is not much specifically for pressurized *L. monocytogenes* cells. Bowman, Claudio et al. (2007), provided information on each gene encoding the specific proteins, but not any information about possible network formations. For this, the KEGG pathway database could be used. This database works as an encyclopaedia for genes and genomes and could therefore give some general information on pathway formation and interaction systems. By putting together the information given about possible repair mechanisms, which of these proteins are existing in *L. monocytogenes* together with their regulation values made it possible to create a hypothetic protein interaction system for the repair mechanisms.

### 3.1.1 Fold Change and P-value for Gene Expression Changes

The fold change is a method used in the analysis of microarray experiments as it can identify genes with changed expression at two different conditions. The fold change is simply calculated as the ratio between two conditions, dividing the initial value by the final value. If the value has increased, the result is a positive number and if the level of expression has decreased, the result will often be presented as a negative number. In this way, one can easily see if a gene has been expressed at a higher or lower level and at which grade it has changed (Witten and Tibshirani 2007).

Additional information is needed in order to determine the significance of the fold change result. For this purpose, the statistical p-value method is used as a complimentary evaluation scheme to the fold-change method. When using the p-value, a null hypothesis is evaluated. In this case the null hypothesis represents no fold change occurring in the experiment. The P-value is a number between 0 and 1. If the P-value is very high (reaching 1), the chance for no fold change is very high. The evaluated gene can then be discarded as the probability for expression change is very small. On the other hand, the smaller the P-value, the more strongly the test rejects the hypothesis being tested, meaning that there has occurred a change. Shortly said, a small P-value is desired because it means that the result should be true (Statsdirect 2014).

### 3.1.2 Hypothesises and Assumptions

There is little existing documentation on the actual repair pathway of a bacteria cell after HPP. However, documented theory on bacteria repair can give indications of how this process may be conducted. With this in mind, a possible scheme has been created for the outfall when the bacteria senses damage on the cell envelope as a result of exposure to high pressure. Loss of integrity is only one of many ways that the bacteria is affected by high pressure, but as this probably is one of the main causes for why bacteria are inhibited by high pressure, it is important to know more about. In this thesis, it has been assumed that the loss of cell integrity is a result of holes created in the cell

wall and membrane, since reports has verified this event. When the bacteria cell senses these leakages, it will activate three main mechanisms in order to reseal the holes. The assumptions are as follows:

1. Cell Wall Repair
   High pressure will create holes in the otherwise rigid cell wall as a result of breaks in the pressure sensitive hydrogen bonds between peptides binding the wall vertically. The horizontal covalent bonds between the glucans are assumed not affected, but the cell wall will regardless be affected when the vertical connections are broken. The repair mechanism of the induced holes is assumed to be by removal and degradation of the broken peptidoglycan components and replacement with new peptidoglycans which are produced through regular biosynthesis. Recycling of damaged components will also occur. As the cell wall is the most rigid component of the three parts comprising the envelope, it will be the first to repair.

2. Membrane Repair
   The hypothesis for the membrane is that high pressure will disrupt the membrane integrity by causing holes in the phospholipid bilayer. Among others, because of water penetrating the lipids so that bonds of hydrophobic parts are disrupted. It has been showed that the bacteria can repair this undesired permeability and it has therefore been assumed that the membrane repair is done by producing new phospholipids that can fill in the introduced holes. The damaged and non-functional phospholipids must also be removed from the membrane as they will not contribute to membrane homeostasis. This is assumed to be done in the same manner as the natural phospholipid turnover, where these components are either recycled or decomposed. Recycling is however preferred because of energy conservation.

3. Envelope Associated Protein repair
   The assumed ways of damage to the cell envelope associated proteins are that high pressure induces misfoldings of proteins and hence making them dysfunctional. Some of the misfolded transmembrane proteins may also be squeezed out of the membrane because of their changed form. This leaves a gap in the membrane where the missing protein must be replaced. The repair mechanisms of these events are assumed to be by the induction of heat shock proteins, comprising the repair chaperones and peptidases. The chaperones will try to repair the damaged proteins by refolding and if they do not succeed, the damaged proteins will be removed and either degraded by the peptidases or recycled. Insertion of new proteins to replace the either removed or dislocated protein is done in order to bring the cell membrane back to its desired protein composition.

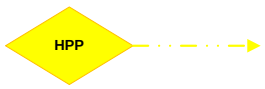
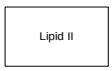
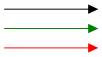
The possible repair pathways were created from a hypothetical scenario where one bacteria being exposed to high pressure is studied. The result of the pressurization is an activated repair mechanism that repairs the damaged cell envelope. Not all bacteria responds equally: Some bacteria dies straight away as the loss of components from the permeable cell envelope has been too fatal to survive, even

if they manage to reseal their walls and membranes. Other bacteria are rough enough to maintain their integrity even during pressurization and appear unaffected. These are not the bacteria that are under focus in this thesis. The relevant bacteria are those who are so affected by the pressure that they become permeable, but manage to reseal and start to grow again before the loss of components within the cell becomes fatal.

A repair model has been developed for the described components and for each of the three models, a protein interaction system has been developed. The signal of protein activation will go through the two component signal transduction system (TCST), which is the main sensor of changes in both environmental conditions and cell state. The developed repair systems may be presented with only the nearest documented regulator (such as RpoN, CodY, SigB or HrcA), or directly from the TCST system if a near regulator is unknown. In reality, it is assumed that a regulation will always go through a TCST system with several links in between that determines the regulation of a gene. These links are not included in the network for simplicity, as they are not directly relevant. A diamond shaped node named HPP has been used to shorten the whole pathway, though it symbolizes the TCST system that senses high pressure exposure and sends signals to regulate enzyme activity after necessity of the cell.

The created repair pathways are presented with different types of nodes and arrows in the models. A register of their functions is given below for better overview:

**Table 3.1: Function of nodes and arrows in the repair models**

| | |
|---|---|
|  | HPP inducing the activation of proteins. A simplification of the TCST system |
|  | Precursors or products in the system |
|  | Source of building blocks |
|  | Active enzyme – Inactive enzyme |
|  | Active regulator – Inactive regulator |
|  | Activation signal – Deactivation signal |
|  | Component transition |

## 3.2 Staining and Recovery Experiments with High Pressure

Knowledge about the specific features on how bacteria react to high pressure is limited. However, by doing microbiological experiments, more information that supports the theories of both high pressure induced damage and repair can be obtained. This section explains the methods conducted for the two experiments done in combination with high pressure. It comprises the methodology of staining pressurized cells for injury detection and the method of storing cells to detect recovery after pressure exposure.

Processing conditions of 400 MPa and 20 °C during 5 minutes, with subsequent storage at 4 °C, was used. This is similar to common settings for food processing and therefore comparable to a real life situation. Fish soup was used as a model food product in the experiments. Preservation of fish soup is common with the high pressure processing technique and can therefore provide a situation that is more similar to real life. *Listeria innocua* has been used as a substitute for L. *monocytogenes* during the laboratory work. There are limitations with the usage of pathogen bacteria in laboratories, but such substitution made the experiments feasible.

### 3.2.1 Fish Soup, *Listeria* and Plate Preparations

Fish soup was prepared with a common recipe. Ingredients were approximately 2 % fish bouillon, 8 % fat (from butter, milk and cream), 0.66 % salt, and the rest being mainly water. The soup was cooked, packed and sterilized. Samples were afterwards kept at 1 °C until the day of experiments.
*L. innocua* ATCC 33090 (Oxoid, Hampshire, U.K) was stored in Microbank (Pro-Lab Diagnostics, Canada) at -80 °C. *L. innocua* was initially grown in Tryptic Soy Broth (Oxoid) supplemented with 0.6% w/w Yeast Extract (Merck, Darmstadt, Germany) (TSB-YE) at 37 °C for 20 h at 150 rpm. The overnight culture was then sub-cultured in TSBYE with 20 h incubation at 30 °C, 150 rpm. Resulting cells in TSBYE at a concentration of $10^9$ cells/ml was used for staining experiment. For cell recovery experiment, bacteria were added to 20 ml fish soup samples at a calculated ratio to obtain an initial concentration of $10^5$ cells/ml. Then, 20 ml inoculated fish soup (for cell recovery experiment) and 20 ml TSBYE with bacteria (for staining experiment) were transferred into separate sterile plastic bags and heat-sealed under vacuum. For heat treatment, bacteria were added to sterile tubes with TSBYE at a concentration $10^9$ cells/ml. All samples were stored on ice until they were pressurized.

Counting of cells on conventional medium was used for comparison. Along with positive and negative controls, triplicate pressurized samples in Tryptic Soy Broth (Oxoid) supplemented with 0.6 % yeast extract (TSBYE) were prepared with ten-fold serial dilutions for subsequent surface plating on Tryptic Soya Agar (Oxoid) supplemented with 0.6 % yeast extract (TSAYE). Additionally, dilutions were plated on Brilliance *Listeria* Agar added with selective and differential supplements (Oxoid). The latter medium, that is selective for *Listeria*, can give information about cell injury, as injured cells have difficulties to grow on this medium. Eddy Jet spiral plater instrument (IUL Instruments, Barcelona, Spain) was used for surface plating procedure unless manual plating was necessary to count lower amounts of bacteria. All plates were incubated at 30 °C for 2-5 days.

### 3.2.2 **High Pressure and Heat Treatment**

Pressurization of samples for cell recovery and staining experiments was carried out using high hydrostatic pressure machine QFP 2L-700 (Avure Technologies, Västerås, Sweden/Columbus, USA) at 400 MPa for 5 min at 20 °C. The pressure come-up rate was approximately 250 MPa/min and the pressure release was < 1 sec. The duration of treatment did not include the come-up time. Samples were immediately placed on ice-water following pressure treatments.

### 3.2.3 **PMA Staining Protocol**

PMA is a fluorescent stain, used to demonstrate the appearance of holes in a cell, as it can only enter and bind to a leaking cell. The protocol from Løvdal, Hovda et al. (2011) was followed in the staining experiment, as it provides a successful procedure for this technique.

Step 1 : 1mg PMA (phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride (Biotum, Hayward, USA) is dissolved in 980 µL 20% dimethyl sulfoxide (DMSO; Sigma, St. Louis, USA) to create a stock solution of 2 mM and stored at −20 °C in the dark.

Step 2 : A concentration of 50 µM PMA is added to 500 µl aliquots of pressurized cells in Eppendorf tubes. Then, they are incubated on ice in the dark for 5 min prior to light exposure.

Step 3 : Following the incubation period, samples are laid horizontally on ice (to prevent heating) with the more transparent side facing upwards and light exposed using a Tempo f650 lamp (Luci della Ribalta, Castel Goffredo, Italy) equipped with a Showbiz Quartzline Halogen 650-W bulb (GE Lightning, Northampton, UK). Samples are then exposed to light for 10 min, placed about 30 cm from the light source. The temperature of samples is checked at the end of the incubation time. After light exposure, samples are washed twice with 1.5% NaCl and resuspended in 500 µL MilliQ autoclaved water.

Step 4 : After PMA staining the bacteria are double labeled in solution with fluorescent DNA dye Hoescht 33342 (1 µg $mL^{-1}$), washed and fixed in 4% paraformaldehyde for 30 min at room temperature and mounted onto glass slides in Mowiol mounting media containing 2.5% (w/v) DABCO anti-fading reagent. Confocal fluorescence microscopy (CFM) images are then taken using an inverted Nikon A1R confocal laser scanning microscope using a 60× oil objective and 6× zoom. Fluorescence images of Hoescht 33342 and PMA stained bacteria are acquired at 425/475 nm and 570/620 nm, respectively. Differential interference contrast (DIC) images are recorded in parallel. Images have been quantified using ImageJ software (National Institutes of Health, USA) and verified manually.

Cells can be divided into three categories by CFM; 1) cells stained only with PMA (presumed to be dead by cell membrane damage), 2) cells stained only with Hoechst 33342 (presumed to represent

viable cells), and 3) cells stained both with PMA and Hoechst 33342 (presumed to be dead or heavily injured).

### 3.2.4 **Statistical Analysis of Data**

Bacterial colonies were calculated as $\log_{10}(N)$, where N was the final bacteria concentration (cells/ml) on TSAYE and Brilliance plates during the storage test. The lower limit for accurate detection was 100 cells/ml. Mean values and standard errors were calculated from five sample replicates for recovery experiments and three for staining experiments.

Sublethal injury of *L. monocytogenes* exposed to pressure treatment was assessed by the difference between the counts on the nonselective agar medium (TSAYE) and the selective agar medium (Brilliance).
Percentages of viability and injury ratios was calculated for the average of the 10 samples using the following equations:

Percentage left from total colony (Equation 3.1):

$$\frac{Inoculum\ level\ TSAYE}{Initial\ inoculum\ level} * 100 = \%\ viable\ cells, injured\ and\ noninjured \tag{3.1a}$$

$$\frac{Inoculum\ level\ Brilliance}{Initial\ inoculum\ level} * 100 = \%\ viable\ cells, noninjured \tag{3.1b}$$

$$Viable\ cells\ (TSAYE) - viable, noninjured\ cells\ (Brilliance) = viable\ \ cells, injured \tag{3.1c}$$

Injury ratios of all viable cells (Equation. 3.2):

$$\frac{Injured\ cells}{Viable\ cells} * 100 = \%\ injured\ cells \tag{3.2a}$$

$$\frac{Noninjured\ cells}{Viable\ cells} * 100 = \%\ noninjured\ cells \tag{3.2b}$$

Daily average growth of cell concentration (Equation 3.3).

$$\frac{C_{increase}/_{week}}{days/_{week}} = C_{increase}/_{day} \tag{3.3}$$

Where $C_{increase}$ is the concentration increase of a bacteria colony

## 3.3 Mathematical Modelling of Cell Recovery

The procedure to mathematically express the repair pathways goes by the help of certain tools. Ordinary differential equations (ODEs) are essential to express the biological functions. ODEs are based on the assumption that the variables in a system can be considered as continuous and that it is possible to define the rates of change of these variables. The ODE representation of a genetic circuit is represented by accounting for the rates of creation and destruction for each variable (Almaas 2013).

The ODEs are necessary for mathematical expressions, but the programming language Matlab is the main tool for simulations. Matlab is a program used for numerical computation, visualisation and programming. It is possible to analyse data and create models with this program, which makes it ideal to use for simulations of biological pathways. The Ordinary differential equation solver is an algorithm developed in Matlab, which evaluates the right side of a differential equation. Depending on the type of system (stiff or non-stiff), solvers with different accuracy can be chosen. A stiff system cannot require as much accuracy as a non-stiff system, since this may be too demanding for an algorithm that takes very small steps for each iteration. The Ode15s and ODE23s solvers have mainly been used for the simulations in this thesis, as they can solve stiff differential equations. Over a given time-range, these solvers will give the tendency of a system from the given differential equations, showing potential steady states and giving opportunities to test model stability (Mathworks 2014).

The created models have been implemented in Matlab, but some definitions are needed for their simulations to give meaning. The program is based on providing an input that describes a change in a condition, leading to an output that shows the response to this change. The input in the cell envelope model is the pressure level being changed, while the state of the cell envelope is the output. This procedure is illustrated and explained in Figure 3.2.

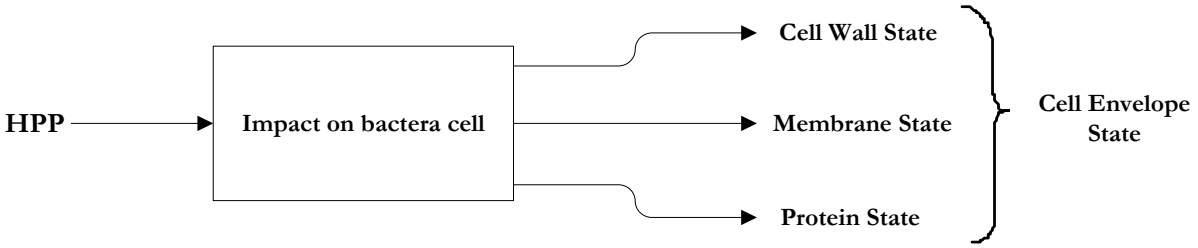



**Figure 3.2: Input and output of the Matlab model. HPP is the input applied on the cell and the box receiving HPP is where the ODEs are solved. The ODE solutions are the outputs, giving the state of the cell wall, membrane and proteins, comprising the cell envelope**

The state of the cell envelope, with its associated cell wall, membrane and proteins, is the main measurement. It is a way of giving an index to which extent the components are affected by pressure and this index is described by the Hill function. The Hill function is often used in biochemistry

where it can describe the dynamics of regulated gene circuits. This is done by taking the concentration of a product as a function of either an activator or a repressor (Burril and Silver 2010). The Hill function can also be used to understand how a pressure input will give a cell state output. The specific way that the Hill function describes the state of a cell is given in Figure 3.3. The cell envelope state will be a function of pressure, where the value of the index varies after which level is applied. The cell envelope state index has a maximum level of 1 and a minimum level of 0. The higher the pressure, the lower is the index, following the Hill repressor equation (Equation 3.3):

$$Index = \frac{P^n}{P^n + Km} \tag{3.3}$$

There are three key parameters; P, n and Km. P is the pressure level and Km is the repressor coefficient, defining the value of P needed to reach the middle state of the cell (Index = 0.5). The most important parameter is n, the Hill coefficient. This value determines how the function responds to the input. A smaller n results in a more graded response whereas a larger n produces a switch-like response (Burril and Silver 2010).

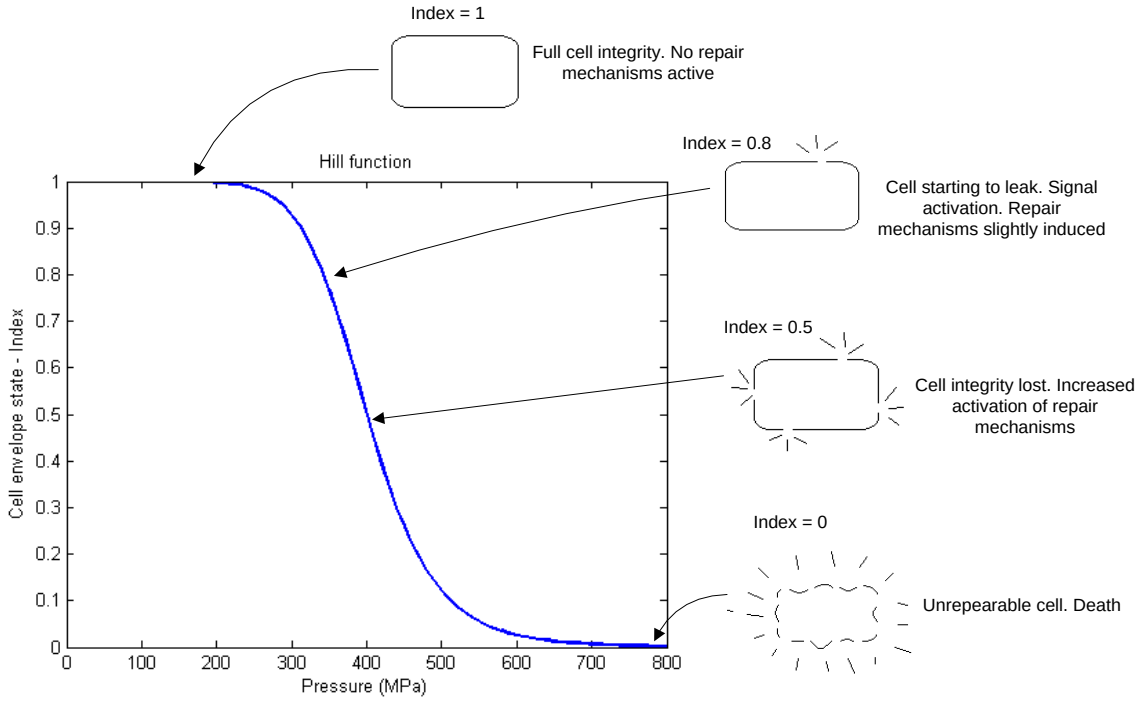



**Figure 3.3: Representation of the cell envelope by the Hill function. Different pressure levels will reduce the cell envelope by different magnitudes.**

Different indexes represents different extents of leakages appearing in the envelope structure where 1 is the optimal state and 0 is the worst case. An index equal to 1 represents a normal cell with full integrity. This is how it normally would appear at atmospheric pressure. An index below 0. 1

represents a cell that is so heavily damaged that it cannot repair itself and can be regarded upon as dead. This has been observed to happen at pressures above 600 MPa. In this model, a pressure level of 400 MPa represents a threshold value where the bacteria is starting to be seriously affected, represented by an index of the middle value 0.5. This index has been used in all the repair simulations where the states are presented as percentages going from 0-100 %. The cell wall, membrane and proteins that build up the cell envelope, have their states measured. The proteins and intermediates that are responsible for their repair are not measured with such an index. The value of these components represents the activity or usage that is needed from them, in order to get the cell envelope back to its optimum state.

### 3.3.1 **Sensitivity Analysis**

The sensitivity analysis is very useful in mathematical modelling, as it describes dependencies between different elements of a model. The sensitivity analysis investigates the relations between uncertain parameters in a model and a property of the observable outcome. The observable outcome represent a phenotypic feature of the modelled system, such as the state of a cell envelope. This analysis has been used for various parameterization tasks in models of biological systems, such as finding essential parameters to use in research or identifying insignificant parameters for model reduction. The sensitivity analysis can also be applied on empirical experiments as it can verify a theoretical model by checking its authenticity to experimental results. Errors in the model can then be recognized, whether they are conceptual or in implementation (Charzynska, Nalecz et al. 2012). The sensitivity analysis is therefore a useful tool to test out how well the created model reflects the real system. By going through the cycle of creation, testing and refining as shown in Figure 3.4, it can be possible to create a practical mathematical model that correctly describes a biological phenomenon.

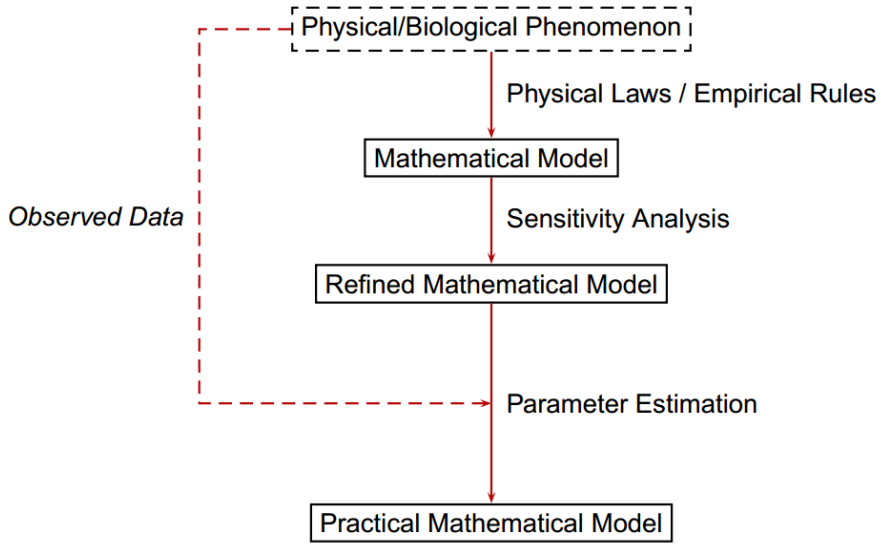



**Figure 3.4: Creation cycle of a mathematical model (ZivariPiran 2008).**

The analysis determines how different values of an independent variable will impact the dependent variables in a system by introducing a perturbation and detecting the response. In the sensitivity equations, a perturbation of 1% was individually applied on each rate constant to investigate the response of the cell wall, membrane and protein repair model. Equation 3.4 has been used to determine the sensitivity for perturbations in the cell envelope repair system:

$$|S_i| = \frac{State_{(k_i)} - State_{(k_i + k_i * \Delta k_i)}}{k_i - k_i + k_i * \Delta k_i} = \frac{State_{(k_i)} - State_{(k_i + k_i * \Delta k_i)}}{k_i * \Delta k_i} \quad (3.4)$$

Where $k_i$ is the independent parameter, State is the dependent variable of the model, and $S_i$ is the i-th sensitivity coefficient of the model. This is a one-factor-at-a-time method as the net effect of one parameter is taken while assuming that all other factors are fixed (Charzynska, Nalecz et al. 2012).

The sensitivity coefficients are normalized relative to each other by dividing them with the highest coefficient value and then multiplying by 1000 to obtain a normalization per thousands. This gives a range of significance going from 0 to 1000, where 1000 represents the highest coefficient in each system and 0 represents no impact.

# 4 Results & Discussions

In this chapter, the results from the pathway analysis, the laboratory experiments and the simulations of the models are presented and discussed. First, the developed repair models of the cell envelope are given with all proteins and substrates involved in this. Secondly, the experimental results from the staining and recovery experiment are presented. Lastly, mathematical simulations of the created repair models are shown. The models have been evaluated for their quality and improvements have been done where they showed to be needed and feasible.

## 4.1 Possible Mechanisms of Repair

The following section provides the findings from the investigation on biological reactions in pressurized bacteria cells. The results are hypothetical interaction systems, developed from known pathways and experimental data. They have been combined in a way that altogether will represent possible outcomes of cell envelope reparation after high pressure exposure. The cell envelope consists of a cell wall, a cytoplasmic membrane and the cell envelope associated proteins. Each individual model is presented first, giving a better overview of their specific setup, and then combined together to become one complex network of the cell envelope.

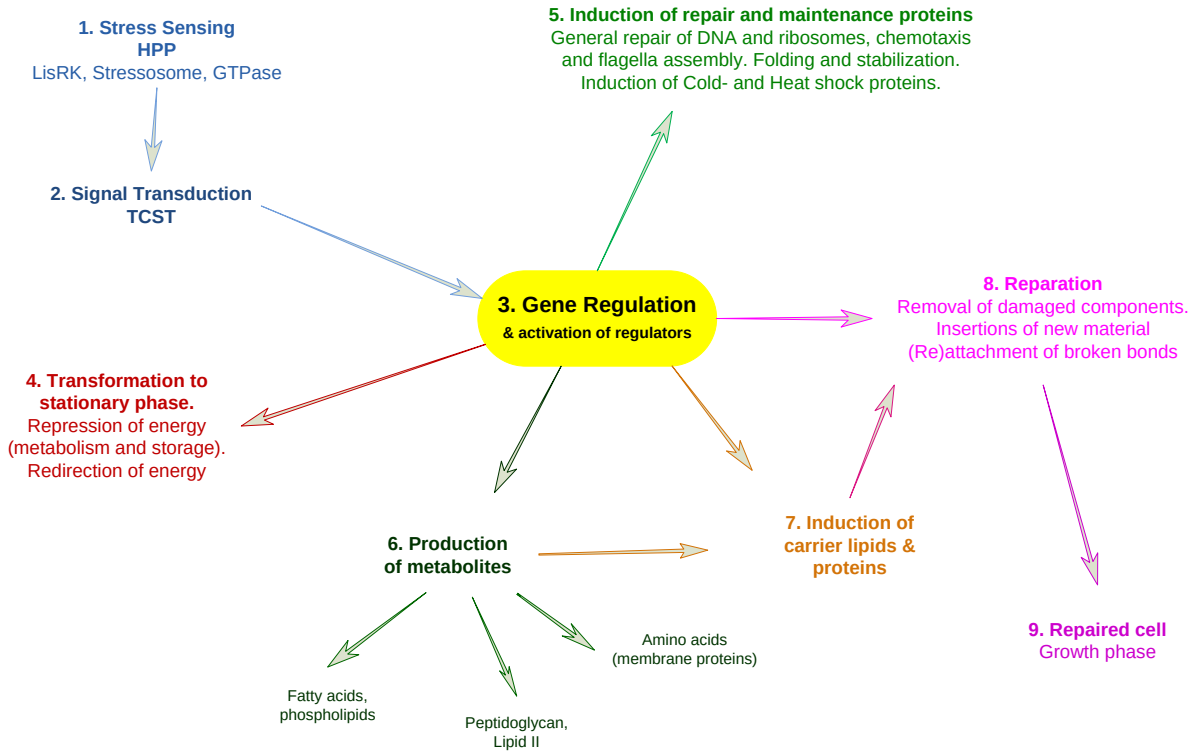



**Figure 4.1: The pathway from sensing of high pressure to reparation of pressure induced injuries. (1) The cell senses high pressure exposure and (2) the signal transduction system signalizes that (3) genes must be regulated in order to respond to this exposure. (4) Before reparation begins, the cell**

**turns into a stationary phase where it is not using energy on growth. (5, 6, 7) Then it can start using energy on general repair and maintenance, production of metabolites and induction of repair processes and carriers. (8) After this, reparation of the cell can be done. (8) Lastly, the cell will be fully repaired and return to growth phase.**

**Feil! Fant ikke referansekilden.** provides a repair scheme that shows all mechanisms involved when a cell is pressurized. The scheme goes from sensing of high pressure to repair of the cell, where the role of gene regulation is emphasized. When the cell senses damage from high pressure, it will send signals that activates genes involved in the repair mechanism. Transformation to stationary phase is a necessary step before repair can begin. It is assumed that the repair of the cell envelope will comprise these mechanisms, though the focus in this thesis is on part 8 in **Feil! Fant ikke referansekilden.**, reparation.

### 4.1.1 Hypothetical Cell Wall Repair Model

This section provides the developed model of cell wall repair. All components either being repaired or doing the reparation are presented first. After, a hypothetical repair pathway for the cell wall is presented along with explanation around the pathway setup.

#### 4.1.1.a *Proteins and substrates involved in cell wall repair*

All proteins potentially involved in repair of the cell wall are enlisted in Table 4.2 and all substrates involved in the system are listed in Table 4.1. These proteins can be involved in three different parts. Biosynthesis of new peptidoglycan layers through synthesis of various intermediates, transportation of the intermediates and products or cleavage of bonds to components that needs to be removed. The *L. monocytogenes* specific genes are enlisted for each protein. The gene regulation values detected from pressurization with 400 MPa and no recovery time, along with their associated P-values, are obtained from Bowman, Claudio et al. (2007). They are presented to show the significance of each protein involved in the repair network. Regulators are given if they have been proved. The DivIVA protein is mentioned as a regulator in parenthesis as it does not function as a regulator in practice, but still required for the Mur complex to function properly. Most of the enlisted proteins shows to have an up-regulated gene activity, giving a higher probability for these proteins to be involved in the bacteria's pressure defence mechanism.

**Table 4.1: Substrates involved in the cell wall repair pathway. Adapted from (Bowman, Claudio et al. 2007, STRING 2013).**

| Intermediates and products | |
|---|---|
| **Name** | **Function** |
| Aminosugars | Building blocks for Lipid II |
| Lipid II | Peptidoglycan precursor |
| MurNAC | Constituent of peptidoglycan |
| GlcNAC | Constituent of peptidoglycan |
| Lipoteichoic Acid | Membrane connector |
| Peptidoglycan | Main component in cell wall |

**Table 4.2: Proteins and regulators involved in reparation of the cell wall. Proteins are divided in functional groups. Gene regulation values (fold change) are given for each protein along with the P-value determining the probability of the fold change result. Down-regulation and high P-values are marked in red, significant up-regulations and low P-values are marked in green. Adapted from (Bowman, Claudio et al. 2007, STRING 2013)**

| Cell Wall System - Interactive proteins | | | | | | |
|---|---|---|---|---|---|---|
| **Group** | **Protein name** | **Gene name** | **Gene reg** | **P-value** | **Regulator** | **Function** |
| Mur complex: Mediates Lipid II synthesis | MurA | lmo2691 | -1.23 | 0.38 | | UDP-N-acetylglucosamine 1-carboxyvinyltransferase |
| | GlmS | lmo1999 | 3.06 | 0.01 | CodY | glucosamine--fructose-6-phosphate aminotransferase |
| | MurC | lmo1605 | 3.56 | 0.02 | | UDP-N-acetyl muramate-alanine ligase |
| | MurD | lmo2036 | 1.42 | -0.08 | (DivIVA) | UDP-N-acetylmuramoylalanine D-glutamate ligase |
| | GcaD/ Tms | lmo0198 | 3.55 | 0.00 | | Glucosamine-1-phosphate N-acetyltransferase |
| | MurF | lmo0856 | -1.6 | 0.03 | | Catalyzes the final step in the synthesis of UDP-N-acetylmuramoyl-pentapeptide |
| | MurG | lmo2035 | 1.99 | 0.05 | (DivIVA) | Phospho-N-acetylmuramoyl-transferase |
| | DdlA | lmo0855 | 1.62 | 0.06 | | D-alanine-D-alanine ligase |
| | MraY | lmo2037 | 1.90 | 0.01 | (DivIVA) | Phospho-N-acetylmuramoyl-pentapeptide transferase |
| Cell division initiation protein | DivIVA | lmo2020 | 5.23 | 0.00 | CodY | Crucial role in recruiting the cell wall synthesis machinery to the cell poles |
| Lipid II flippase | FtsW | lmo1071 | 1.23 | 0.09 | | Flips the peptidoglycan precursor across the membrane, cell division protein |
| Penicillin Binding Proteins | PbpA | lmo1892 | 1.69 | 0.04 | | Involved in the final stages of peptidoglycan synthesis. Receives the flipped precursors and |
| | PbpB | lmo2039 | 8.90 | 0.00 | (DivIVA) | Penicillin-binding protein 2B |
| Autolysins: Cell wall hydrolase | N/A | lmo0717 | 6.20 | 0.00 | | Putative lytic murein transglycosylase |
| | N/A | lmo1521 | 4.09 | 0.01 | RpoN- | N-acetylmuramoyl-L-alanine amidase |
| Teichoic acid Synthesis | TagA | lmo2521 | 3.67 | 0.04 | | Undecaprenyl-phosphate alpha-N-acetylglucosaminyltransferase |
| | TagO | lmo2519 | 4.16 | 0.08 | | Teichoic acid biosynthesis protein TagA |

#### 4.1.1.b *Repair Pathway for the Cell Wall*

The resulting pathway for cell wall repair is shown in Figure 4.2, but a thorough model that includes references for each reaction is provided in Appendix 1. The network shows that high pressure will cause damages in the form of holes in the cell wall. This is presented by high pressure inducing the autolysins (represented as the enzyme Al in the illustration) which cleaves out components in the cell wall that has to be replaced. The activity of the autolysins can be controlled by both RpoN and the lipoteichoic acid. Damages can either be broken bonds in the peptidoglycan layer or loss of functionality of peptidoglycan constituents. These components are degraded to either be used as new building blocks for the cell wall or for other parts in the cell. Synthesis of new cell wall components that are to replace the lost ones will go through the regular pathway of peptidoglycan biosynthesis. This is illustrates by the Mur enzyme which comprises the whole Mur-complex that is involved in Lipid II synthesis, the precursor of peptidoglycan. The Mur enzyme-complex will indirectly be induced by high pressure since the Mur regulators, RpoN and CodY, will be affected by high pressure. CodY regulates the Mur complex through the DivIVA enzyme, which is needed to properly assemble the Mur complex. Further on the enzymes FtsW and the Pbp-complex will be responsible for transporting Lipid II to its proper location and then polymerize it to become peptidoglycan layers that can fill in the missing components.

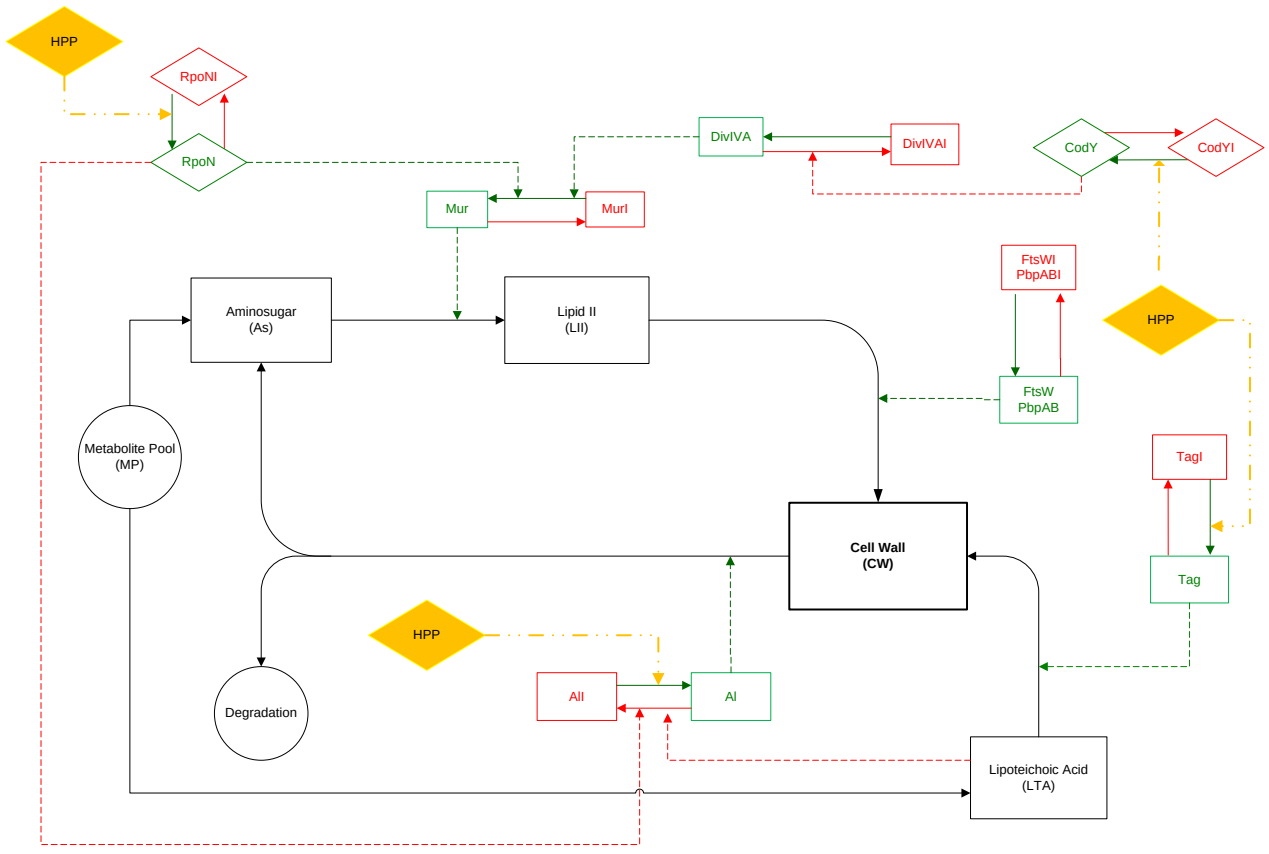



**Figure 4.2: Hypothetical pathway for cell wall repair.**

The reactions and differential equations associated with the interactive system are as follows:

Reactions:

$$As + Mur \rightarrow LII + Mur \tag{4.1a}$$

$$LII + FtsW + PbpAB \rightarrow CW + FtsW + PbpAB \tag{4.1b}$$

$$LTA + Tag \rightarrow CW + Tag \tag{4.1c}$$

$$CW + Al \rightarrow Deg + Al \tag{4.1d}$$

$$CW + Al \rightarrow As + Al \tag{4.1e}$$

Ordinary differential equations (ODEs):

Substrates:

$$\dot{As} = k_1 * MP + k_5 * PG * Al - k_2 * Mur * As \tag{4.2a}$$

$$\dot{LII} = k_2 * Mur - k_3 * LII * FtsW * PbpAB \tag{4.2b}$$

$$\dot{PG} = k_3 * LII * FtsW * PbpAB - k_4 * PG * Al - k_5 * PG * Al \tag{4.2c}$$

Proteins:

$$\dot{Mur} = \frac{kM_1 * (MurT - Mur) * RpoN * DivIVA}{k_{Mm1} + (MurT - Mur)} - \frac{kM_2 * Mur}{k_{Mm2} + Mur} \tag{4.3a}$$

$$\dot{FtsW} = \frac{kF_1 * (FtsWT - FtsW) * HPP}{k_{Fm1} + (FtsWT - FtsW)} - \frac{kF_2 * FtsW}{k_{Fm2} + FtsW} \tag{4.4b}$$

$$\dot{Al} = \frac{kA_1 * (AlT - Al) * HPP}{k_{Am1} + (AlT - Al)} - \frac{kA_2 * Al * RpoN * LTA}{k_{Am2} + Al} \tag{4.4c}$$

$$\dot{DivIVA} = \frac{kD_1 * (DivIVAT - DivIVA) * RpoN * CodY}{k_{Dm1} + (DivIVAT - DivIVA)} - \frac{kD_2 * DivIVA}{k_{Dm2} + DivIVA} \tag{4.4d}$$

$$\dot{CodY} = \frac{kC_1 * (CodYT - CodY) * HPP}{k_{Cm1} + (CodYT - CodY)} - \frac{kC_2 * CodY}{k_{Cm2} + CodY} \tag{4.4e}$$

$$\dot{RpoN} = \frac{kR_1 * (RpoNT - RpoN) * HPP}{k_{Rm1} + (RpoNT - RpoN)} - \frac{kR_2 * RpoN}{k_{Rm2} + RpoN} \tag{4.4f}$$

All the enzymes in this model will follow the enzymatic mass equation: $E + EI = ET$. Where "E" is the active form of the enzyme, "EI" is the inactive form of the enzyme and "ET" is the total amount of enzyme.

### 4.1.2 Hypothetical Membrane Repair Model

The following section provides the developed model for membrane repair. All components either being repaired or doing the reparation are presented first. After, a hypothetical repair pathway for the membrane is presented along with explanation around the pathway setup.

#### 4.1.2.a *Proteins and Substrates Involved in Membrane Repair*

All the proteins potentially involved in repair of the cytoplasmic membrane are enlisted in Table 4.4 and all substrates involved in the system are listed in Table 4.3. These proteins can be involved in three different mechanisms. Biosynthesis of phospholipids through synthesis of different precursors and constituents, transportation of the components or cleavage of parts in the phospholipid layer that needs to be removed or recycled. The *L. monocytogenes* specific genes are enlisted for each protein. The gene regulation values detected from pressurization with 400 MPa and no recovery time, along with their associated P-values, are obtained from Bowman, Claudio et al. (2007). They are presented in the table to show the significance of each protein involved in the repair network. If the *Listeria* specific gene is not found, possible genes that might have the same function in this bacterium is provided. Regulators are given if they have been proved. Most of the enlisted proteins shows to have an up-regulated gene activity, giving a higher probability that they are involved in the bacteria's pressure defence mechanism. The Pls- and Dlt-complex however, does not show to be very much activated by their fold change values. There are several possibilities for this outcome. These proteins can have a delayed activation as they might not be immediately needed, there may be other proteins that in reality are responsible for this function or this mechanism might in reality not be that important for the cell repair.

**Table 4.3: Components involved in the repair pathway of the membrane. Adapted from (Bowman, Claudio et al. 2007, STRING 2013).**

| Intermediates and products | |
|---|---|
| **Name** | **Function** |
| Acetyl CoA | Initial precursor fatty acid synthesis |
| Fatty Acid | Constituent in phospholipids |
| Lipoteichoic acid | Connects membrane and cell wall |
| Phosphatidic acid | Simplest form of phospholipids |
| PtdGro | Type of phospholipid |
| PtdEtn | Type of phospholipid |
| PtdChl | Type of phospholipid |
| PtdSer | Type of phospholipid |

**Table 4.4: Proteins and regulators involved in reparation of the membrane. Proteins are divided in functional groups. Gene regulation values (fold change) are given for each protein along with the P-value determining the probability of the fold change result. Down-regulation and high P-values are marked in red, significant up-regulations and low P-values are marked in green. Adapted from (Bowman, Claudio et al. 2007, STRING 2013).**

| Membrane System - Interactive proteins | | | | | | |
|---|---|---|---|---|---|---|
| Group | Protein name | Gene name | Gene reg | P-value | Regulator | Function |
| Fab complex: Mediates fatty acid biosynthesis. Influences acyl carriers | FabD | lmo1808 | 2.10 | 0.00 | FapR | Acyl-carrier-protein S-malonyltransferase |
| | FabH | lmo2202 | 11.20 | 0.00 | RpoN | 3-oxoacyl-(acyl carrier protein) synthase III |
| | FabG | lmo2201 | 1.60 | 0.03 | FapR | 3-oxoacyl-(acyl-carrier-protein) reductase |
| | FabZ | lmo2524 | 1.80 | 0.01 | | (3R)-hydroxymyristoyl-ACP dehydratase |
| Transcriptional regulator | FapR | lmo1810 | 4.49 | 0.00 | CodY, CtsR | Fatty acid biosynthesis transcriptional regulator; Transcriptional factor involved in regulation of membrane lipid biosynthesis by repressing genes involved in fatty acid and phospholipid metabolism |
| Acyl carriers: carries acyl intermediates | AccD | lmo1573 | 3.79 | 0.00 | | Acetyl-CoA carboxylase biotin carrier protein |
| | AcpP | lmo1414 | 6.25 | 0.00 | FapR | acetyl-CoA acetyltransferase Branched chain amino acids-, Tryptophan- and Fatty acid metabolism |
| | AcpA | lmo1806 | 9.97 | 0.00 | | Acyl carrier protein, carrier of the growing fatty acid chain in fatty acid biosynthesis |
| Pls complex: Phospholipid synthesis | PlsX | N/A | N/A | N/A | FapR | Putative glycerol-3-phosphate acyltransferase; Involved in phospholipid synthesis |
| | PlsY | lmo1284 | -1.75 | 0.05 | FapR | Phospholipid biosynthesis enzyme, putative glycerol-3-phosphate acyltransferase |
| | PlsC | lmo1647 | -1.00 | 0.98 | FapR | Glycerophospholipid metabolism, putative 1-acylglycerol-3-phosphate O-acyltransferase. |
| Dlt-complex (similar to LtaS). Teichoic acid biosynthesis | DltA | lmo0974 | -1.03 | 0.87 | | D-alanine-D-alanyl carrier protein ligase |
| | DltB | lmo0973 | 1.60 | 0.09 | | D-alanine export protein |
| | DltC | lmo0972 | 2.18 | 0.06 | | D-alanyl carrier protein |
| | DltD | lmo0971 | 1.50 | 0.07 | | Lipoteichoic/teichoic acid transfer protein |
| | TagB | lmo1088 | -1.4 | 0.11 | | Glycosyl/glycerophosphate transferase involved in teichoic acid biosynthesis |
| DAG kinase | DgkD (similar) | lmo1753 | 2.10 | 0.03 | | Similar to sphingosine kinase and enzymes related to eukaryotic diacylglycerol kinases. Formation of diacylglycerol to phosphatidic acid |
| Recycling of fatty acids | Lnt (similar) | lmo2262 | 2.50 | 0.00 | | similar to predicted hydrolases or acyltransferases of the alpha/beta hydrolase superfamily |
| | Lpp (similar) | lmo2751 | 6.97 | 0.00 | | Membrane lipoprotein, ABC transporter, ATP-binding/permease protein |
| | Ass (similar) | lmo0354 | 1.66 | 0.06 | | Similar to acyl-coenzyme A synthetases/AMP-(fatty) acid ligases |
| | N/A | lmo2074 (similar) | 2.30 | 0.01 | | Similar to predicted hydrolases or acyltransferases of the alpha/beta hydrolase superfamily |
| Transmembrane proteins | Lplt (similar) | lmo0981 | 2.70 | 0.00 | | Lysophospholipid transporter/flippase; Catalyzes the facilitated diffusion of 2-acyl-glycero-3-phosphoethanolamine |
| | TcsA (similar) | lmo1388 | 5.90 | 0.00 | RpoN | Putative ABC transporter, permease component/surface lipoprotein |
| | MsbA | LMHCC_1792 | N/A | N/A | | Lipid A export/permease protein MsbA |

### 4.1.2.b *Repair Pathway for the Cytoplasmic Membrane*

The resulting pathway for cytoplasmic membrane repair is shown in Figure 4.3, but a thorough model that includes references for each reaction is provided in Appendix 1.

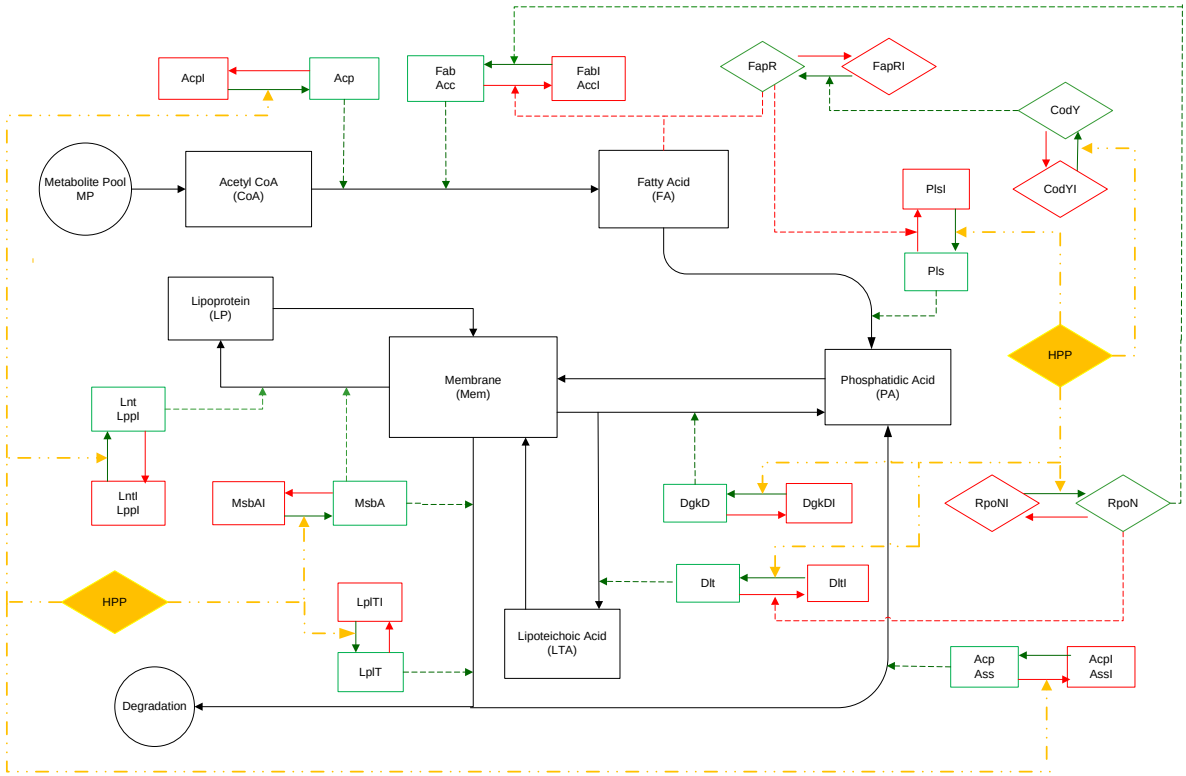



**Figure 4.3: Hypothetical pathway for membrane repair.**

The network shows that high pressure will cause damages in the form of holes in the membrane. This is presented by high pressure inducing the enzymes responsible for degradation and turnover of the phospholipids. Which proteins that are active will be decided by which phospholipid that has been damaged. Degradation or recycling of phospahtidylethanolamine (PtdEtn) goes through a cycle where the MsbA protein will split it up. The fatty acid is used in lipoprotein, another constituent in the membrane, by the activity of the Lpp and Lnt proteins. The remaining part of the phospholipid is either degraded by the LplT protein or recycled by the help of Acp and Ass proteins. The route depends on the state of this constituent. Phospahtidylglycerol (PtdGro) will go through a recycling route. The phosphate group will be taken by the Dlt enzyme-complex which uses it in the formation of lipoteichoic acid. The remaining part is converted to phosphatidic acid by the kinase DgkD. PhosphatidylSerine (PtdSer) and phosphatidylcholine (PtdChl) will also go through similar routes, but for simplicity, only the routes of PtdGro and PtdEtn are described. Synthesis of new membrane components that are to replace the lost ones will go through the regular pathway of phospholipid biosynthesis. This is illustrated by the initial biosynthesis of fatty acids, mediated by the Fab enzyme complex along with Acc and Acp. When the fatty acids has been synthesized, they are transferred to glycerol-3-phosphate so they can be formed into phospholipids. Phosphatidic acid is the simplest form of the phospholipid and a precursor of the different types of phospholipids. Because of this,

formation of phophatidic acid is a separate step and this happens by the help of the PlsX complex. Conversion of phosphatidic acid into another type of phospholipid and the transfer of this component to its proper position has for simplicity been assumed spontaneous (no protein involved in the conversion). These components will be transported where the holes in the membrane are, to fill in the leakages. RpoN induce production of fatty acids used in repair, which itself is induced by high pressure. FapR is a central regulator in the fatty acid biosynthesis that hinder overproduction of the energy demanding fatty acids. Together they finely adjust synthesis activity.

The reactions and differential equations associated with the interactive system are as follows:

Reactions:

$$CoA + Acp + Fab + Acc \rightarrow FA + Acp + Fab + Acc \quad (4.5a)$$

$$\mathrm{FA} + \mathrm{Pls} \rightarrow PG + PA + Pls \quad (4.5b)$$

$$\mathrm{PA} \rightarrow Mem \quad (4.5c)$$

$$\mathrm{M}em + DgkD \rightarrow PA + DgkD \quad (4.5d)$$

$$Mem + Dlt \rightarrow LA + Dlt \quad (4.5e)$$

$$\mathrm{LA} \rightarrow Mem \quad (4.5f)$$

$$\mathrm{Mem} + \mathrm{MsbA} + \mathrm{Lnt} + \mathrm{Lpp} \rightarrow LP + MsbA + Lnt + Lpp \quad (4.5g)$$

$$\mathrm{LP} \rightarrow Mem \quad (4.5h)$$

$$\mathrm{Mem} + \mathrm{MsbA} + \mathrm{LplT} \rightarrow Deg + MsbA + LplT \quad (4.5i)$$

$$\mathrm{Mem} + \mathrm{MsbA} + \mathrm{LplT} + \mathrm{Acp} + \mathrm{Ass} \rightarrow PA + MsbA + LplT + Acp + Ass \quad (4.5j)$$

Ordinary differential equations (ODEs):

Substrates:

$$\dot{CoA} = k_1 * MP - k_2 * Acp * Fab * Acc * CoA \quad (4.6a)$$

$$\dot{FA} = k_2 * Acp * Fab * Acc * CoA - k_3 * Pls * FA \quad (4.6b)$$

$$\dot{PA} = k_3 * Pls * FA + k_4 * DgkD * Mem + k_6 * MsbA * LplT * Acp * Ass * Mem - k_5 * PA \quad (4.6c)$$

$$\dot{Mem} = k_5 * PA - k_4 * DgkD * Mem - k_6 * MsbA * LplT * Acp * Ass * Mem - k_7 * Dlt * Mem + k_8 * LA - k_9 * MsbA * Lnt * Mem - k_{10} * MsbA * LplT * Mem \quad (4.6d)$$

$$\dot{LA} = k_7 * Dlt * Mem - k_8 * LA \quad (4.6e)$$

Proteins:

$$\dot{Acp} = \frac{kA_1 * (AcpT - Acp) * HPP}{k_{Am1} + (AcpT - Acp)} - \frac{kA_2 * Acp}{k_{Am2} + Acp} \quad (4.7a)$$

$$\dot{Fab} = \frac{kF_1 * (FabT - Fab) * RpoN}{k_{Fm1} + (FabT - Fab)} - \frac{kF_2 * Fab * FapR * FA}{k_{Fm2} + Fab} \quad (4.7b)$$

$$\dot{Acc} = \frac{kAc_1 * (AccT - Acc) * RpoN}{k_{Acm1} + (AccT - Acc)} - \frac{kAc_2 * Acc * FapR * FA}{k_{Am2} + Acc} \quad (4.7c)$$

$$\dot{Pls} = \frac{kP_1 * (PlsT - Pls) * HPP}{k_{Pm1} + (PlsT - Pls)} - \frac{kP_2 * Pls * FapR}{k_{Pm2} + Pls} \quad (4.7d)$$

$$\dot{DgkD} = \frac{kD_1 * (DgkDT - DgkD) * HPP}{k_{Dm1} + (DgkD - DgkD)} - \frac{kD_2 * DgkD}{k_{Dm2} + DgkD} \quad (4.7e)$$

$$\dot{Dlt} = \frac{kDl_1 * (DltT - Dlt) * HPP}{k_{Dlm1} + (DltT - Dlt)} - \frac{kDl_2 * Dlt * RpoN}{k_{Dlm2} + Dlt} \quad (4.7f)$$

$$\dot{MsbA} = \frac{kM_1 * (MsbAT - MsbA) * HPP}{k_{Mm1} + (MsbAT - MsbA)} - \frac{kM_2 * MsbA}{k_{Mm2} + MsbA} \quad (4.7g)$$

$$\dot{LplT} = \frac{kL_1 * (LplTT - LplT) * HPP}{k_{Lm1} + (LplTT - LplT)} - \frac{kL_2 * LplT}{k_{Am2} + LplT} \quad (4.7h)$$

$$\dot{LntLpp} = \frac{kLL_1 * (LntLppT - LntLpp) * HPP}{k_{LLm1} + (LntLppT - LntLpp)} - \frac{kLL_2 * LntLpp}{k_{LLm2} + LntLpp} \quad (4.7i)$$

$$\dot{FapR} = \frac{kFR_1 * (FapRT - FapR) * CodY}{k_{FRm1} + (FapRT - FapR)} - \frac{kFR_2 * FapR}{k_{FRm2} + FapR} \quad (4.7j)$$

$$\dot{CodY} = \frac{kC_1 * (CodYT - CodY) * HPP}{k_{Cm1} + (CodYT - CodY)} - \frac{kC_2 * CodY}{k_{Cm2} + CodY} \quad (4.7k)$$

$$\dot{RpoN} = \frac{kR_1 * (RpoNT - RpoN) * HPP}{k_{Rm1} + (RpoNT - RpoN)} - \frac{kR_2 * RpoN}{k_{Rm2} + RpoN} \quad (4.7l)$$

All the enzymes in this model will follow the enzymatic mass equation: $E + EI = ET$. Where "E" is the active form of the enzyme, "EI" is the inactive form of the enzyme and "ET" is the total amount of enzyme.

### 4.1.3 **Hypothetical Protein Repair Model**

The following section contains the last developed model and comprises a repair model of the cell envelope associated proteins. The section has a setup where all components doing the reparation are presented first and then a hypothetical repair pathway for the proteins is presented along with explanation around the pathway setup.

#### 4.1.3.a ***Proteins and Substrates Involved in Protein Repair***

All the proteins potentially involved in repair of the envelope associated proteins are enlisted in Table 4.5. These proteins can either be repair chaperones that will try to repair unfolded or damaged proteins, they may be involved in the lytic pathway that will degrade unrepairable proteins or they can be involved in the synthesis of new proteins that will replace removed or dislocated proteins. The *L. monocytogenes* specific genes are enlisted for each protein. The gene regulation values detected from pressurization with 400 MPa and no recovery time, along with their associated P-values, are obtained from Bowman, Claudio et al. (2007). They are presented in the table to show the significance of each protein involved in the repair network. If the *Listeria* specific gene is not found, possible genes that might have the same function in this bacterium is provided. Regulators are given if they have been proved. Few of the enlisted proteins shows to have an up-regulated gene activity, making the probability that they are involved in the bacteria's pressure defence mechanism quite low. The reasons why they still are used is explained under section 4.1.5, Discussions of Repair Models.

#### 4.1.3.b ***Repair Pathway for Envelope Proteins***

The resulting pathway for envelope protein repair is shown in Figure 4.4, but a thorough model that includes references for each reaction is provided in Appendix 1. The network shows that high pressure will turn a functional protein un-functional, which is assumed to happen by unfolding of the protein or it being squeezed out of its proper position in the cell envelope. Reparation of the un-functional protein will go through a repair cycle where the heat shock chaperones GroES, GroEL, DnaK, DnaJ, GrpE and HtpG are involved. These are induced by either SigB, HcrA or indirectly by high pressure. During the repair cycle, the chaperones will either manage to refold the damaged protein so that it turns into a functional protein again, or they will not succeed and the protein stays un-functional. If so, the chaperones will send it to the proteolysis pathway. Proteases are signalled to degrade a protein by the ubiquitination process which consists of tagging it with an ubiquitin protein. This is illustrated by the E-complex/Ubiquitin node that leads the damaged protein (DP) to the tagged protein node (TP). When the protein is tagged, the Clp complex along with MecA will degrade the protein where the resulting amino acids are available to be re-used. The activation of the peptidases are done by CodY, RpoN and SigB. The Sec complex along with Srp, will use the amino acids and build them up to proteins that can replace unrepairable or dislocated envelope proteins. These are indirectly induced by high pressure.

**Table 4.5: Proteins and regulators involved in reparation of proteins in the cell envelope. Repair proteins are divided in functional groups. Gene regulation values (fold change) are given for each protein along with the P-value determining the probability of the fold change result. Down-regulation and high P-values are marked in red, significant up-regulations and low P-values are marked in green. Adapted from (Bowman, Claudio et al. 2007, STRING 2013)**

| Protein Repair System - Interactive proteins | | | | | | |
|---|---|---|---|---|---|---|
| Group | Protein name | Gene name | Gene reg | P-value | Regulator | Function |
| Heat shock proteins: Chaperones | GroES | lmo2069 | -1.00 | 0.75 | (SigB) | Class I heat-shock protein (chaperonin) GroES |
| | GroEL | lmo2068 | 2.10 | 0.00 | HcrA, (SigB) | Class I heat-shock protein (chaperonin) GroEL |
| | DnaJ | lmo1472 | -1.30 | -0.19 | SigB | Molecular chaperone (heat shock protein) |
| | DnaK | lmo1473 | -2.60 | 0.00 | HrcA | Class I heat-shock protein (molecular chaperone) |
| | GrpE | lmo1474 | 1.02 | -0.88 | HrcA | Molecular chaperone (heat shock protein) |
| | HtpG | lmo0942 | 1.60 | -0.10 | HrcA | Putative chaperonin (heat shock protein htpG) |
| Ubiquitin ligases: Tags proteins for degradation | MecA | lmo2190 | -1.16 | 0.46 | | Adaptor protein; Enables the recognition and targeting of unfolded and aggregated proteins to the clpC protease or to other proteolyses |
| | AceF | lmo1374 | -2.50 | 0.00 | | Similar to branched-chain alpha-keto acid dehydrogenase E2 subunit ( acyltransferase) |
| | N/A | lmo1371 | 1.16 | -0.51 | RpoN | Similar to branched-chain alpha-keto acid dehydrogenase E3 subunit |
| | PdhA | lmo1052 | -2.80 | 0.00 | SigB | Pyruvate dehydrogenase E1 component, α subunit |
| | PdhB | lmo1053 | -9.40 | 0.00 | RpoN | Pyruvate dehydrogenase E1 component, β subunit |
| | PdhC | lmo1054 | -17.70 | 0.00 | | Pyruvate dehydrogenase E2 component |
| | PdhD | lmo1055 | -12.20 | 0.00 | RpoN | Dihydrolipoamide dehydrogenase, E3 subunit of pyruvate dehydrogenase complex |
| Heat shock proteins: Peptidases | ClpC | lmo0232 | -5.20 | 0.00 | SigB | Clp endopeptidase ATP-binding subunit |
| | ClpE | lmo0997 | -15.20 | 0.00 | | Clp protease ATP-binding subunit |
| | ClpP | lmo1138 | 1.20 | 0.16 | RpoN, SigB | ATP-dependent Clp protease proteolytic subunit |
| | ClpX | lmo1268 | 1.90 | 0.03 | HrcA | Clp protease ATP-binding subunit |
| | ClpQ/ HclV | lmo1278 | 1.00 | -0.77 | CodY, RpoN | Similar to ATP-dependent HslUV protease, peptidase subunit HslV |
| | ClpY/ HclU | lmo1279 | -1.20 | -0.21 | CodY, RpoN | Similar to HslUV protease ATP-binding subunit HslU |
| | ClpB | lmo2206 | 1.60 | -0.16 | HrcA | Clp endopeptidase ATP-binding subunit |
| | PepT | lmo1780 | -1.50 | 0.06 | | Peptidase T; Cleaves the N-terminal amino acid of tripeptides |
| Sec-complex: Protein insertion | SecA | lmo0583 | -1.30 | -0.22 | | Protein export, preprotein translocase SecA subunit |
| | SecE | lmo0245 | 23.08 | 0.01 | | Protein export, preprotein translocase SecY subunit |
| | SecY | lmo2612 | 1.40 | 0.05 | | Protein export, preprotein translocase SecE subunit |
| | SecDF | lmo1527 | -1.80 | 0.02 | | Protein export, preprotein translocase SecDF |
| | Ffh (SRP) | lmo1801 | 4.30 | 0.00 | | Protein export, signal recognition particle, subunit SRP54 |
| | N/A | lmo1802 | 4.01 | 0.00 | CodY | Putative regulator of the SRP pathway |

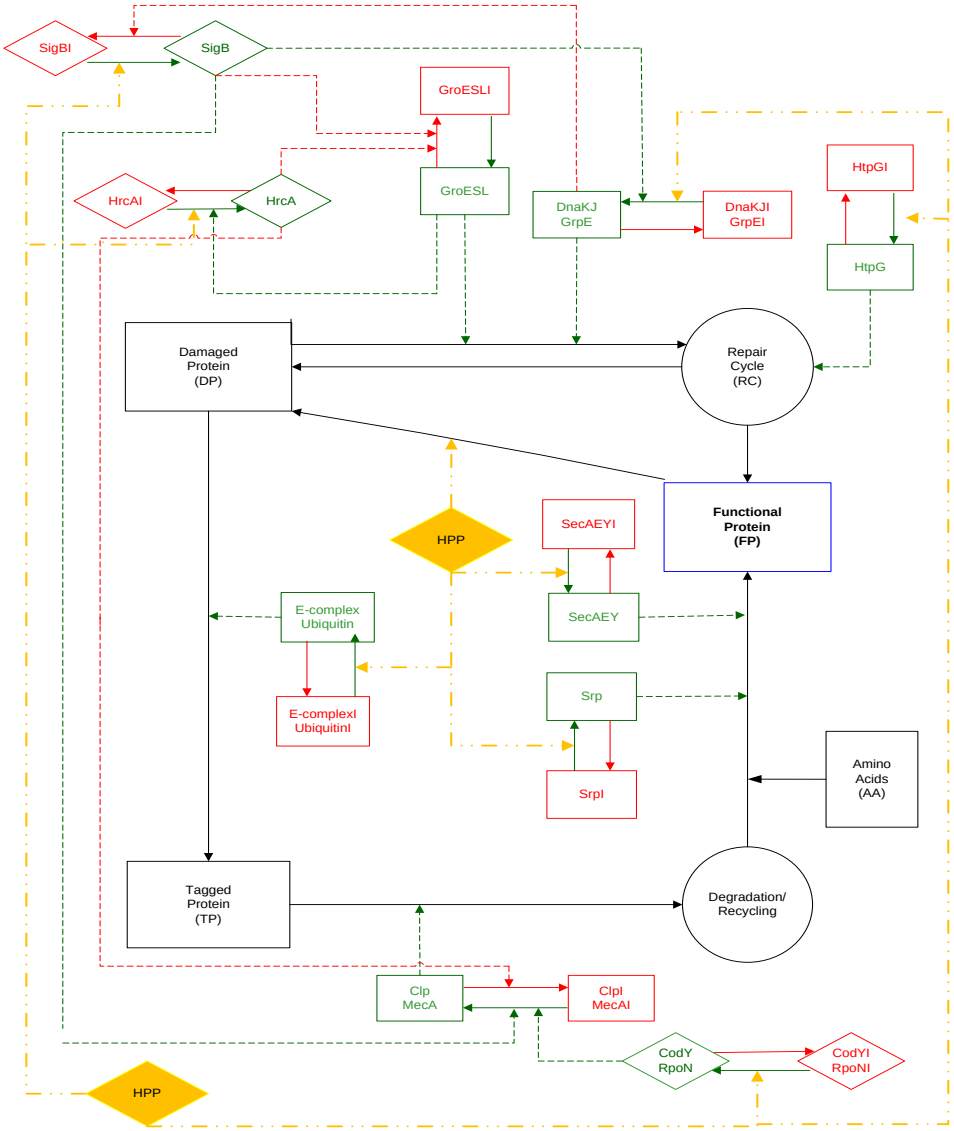



**Figure 4.4: Hypothetical pathway for protein repair.**

The reactions and differential equations associated with the interactive system are as follows:

Reactions: $FP + HPP \rightarrow DP + HPP$ (4.8a)

$DP + GroESL + DnaKJ + GrpE + Htp \leftrightarrow \mathrm{RC} + GroESL + DnaKJ + GrpE + HtpG$ (4.8b)

$\mathrm{RC} \rightarrow FP$ (4.8c)

$DP + Ecomplex + Ubiquitin \rightarrow TP + Ecomplex + Ubiquitin$ (4.8d)

$\mathrm{TP} + \mathrm{Clp} + \mathrm{MecA} \rightarrow DegRec + Clp + MecA$ (4.8e)

$$DegRec + AA + Srp + SecAEY \rightarrow FP + Srp + SecAEY \tag{4.8f}$$

Ordinary differential equations (ODEs):

Substrates:

$$\dot{FP} = k_1 * RC - k_2 * HPP * FP + k_8 * DegRec * AA * Srp * SecAEY \tag{4.9a}$$

$$\dot{DP} = k_2 * HPP - k_3 * GroESL * DnaKJ * DP + k_4 * RC - k_5 * DP \tag{4.9b}$$

$$\dot{UP} = k_5 * DP - k_6 * Ecomplex * Ubiquitin * UP \tag{4.9c}$$

$$\dot{TP} = k_6 * Ecomplex * Ubiquitin * UP - k_7 * Clp * MecA * TP \tag{4.9d}$$

$$\dot{DegRec} = k_7 * Clp * MecA * TP - k_8 * DegRec * AA * Srp * SecAEY \tag{4.9e}$$

Proteins:

$$\dot{GroESL} = \frac{kG_1 * (GroESLT - GroESL) * HPP}{k_{Gm1} + (GroESLT - GroESL)} - \frac{kG_2 * GroESL * SigB * HcrA}{k_{Gm2} + GroESL} \tag{4.10a}$$

$$\dot{DnaKJ} = \frac{kD_1 * (DnaKJT - DnaKJ) * SigB}{k_{Dm1} + (DnaKJT - DnaKJ)} - \frac{kD_2 * DnaKJ}{k_{Dm2} + DnaKJ} \tag{4.10b}$$

$$\dot{GrpE} = \frac{kGr_1 * (GrpET - GrpE) * SigB}{k_{Grm1} + (GrpET - GrpE)} - \frac{kGr_2 * GrpE}{k_{Grm2} + GrpE} \tag{4.10c}$$

$$\dot{HtpG} = \frac{kH_1 * (HtpGT - HtpG) * HPP}{k_{Hm1} + (HtpGT - HtpG)} - \frac{kH_2 * HtpG}{k_{Hm2} + HtpG} \tag{4.10d}$$

$$\dot{Ecomplex} = \frac{kE_1 * (EcomplexT - Ecomplex) * HPP}{k_{Em1} + (EcomplexT - Ecomplex)} - \frac{kE_2 * Ecomplex}{k_{Em2} + Ecomplex} \tag{4.10e}$$

$$\dot{Ubiquitin} = \frac{kU_1 * (UbiquitinT - Ubiquitin) * HPP}{k_{Um1} + (UbiquitinT - Ubiquitin)} - \frac{kU_2 * Ubiquitin}{k_{Um2} + Ubiquitin} \tag{4.10f}$$

$$\dot{Clp} = \frac{kCl_1 * (ClpT - Clp) * SigB * CodY * RpoN}{k_{Clm1} + (ClpT - Clp)} - \frac{kCl_2 * Clp}{k_{Clm2} + Clp} \tag{4.10g}$$

$$\dot{MecA} = \frac{kM_1 * (MecAT - MecA) * SigB * CodY * RpoN}{k_{Mm1} + (MecAT - MecA)} - \frac{kM_2 * MevA}{k_{Mm2} + MecA} \tag{4.10h}$$

$$\dot{Srp} = \frac{kSr_1 * (SrpT - SrpT) * HPP}{k_{Srm1} + (SrpT - SrpT)} - \frac{kS_2 * Srp}{k_{Sm2} + Srp} \tag{4.10i}$$

$$\dot{SecAEY} = \frac{kSe_1 * (SecAEYT - SecAEY) * HPP}{k_{Sem1} + (SecAEYT - SecAEY)} - \frac{kSe_2 * SecAEY}{k_{Sem2} + SecAEY} \tag{4.10j}$$

$$\dot{SigB} = \frac{kS_1 * (SigBT - SigB) * HPP}{k_{Sm1} + (SigBT - SigB)} - \frac{kS_2 * SigB * DnaKJ * GrpE}{k_{Sm2} + SigB} \quad (4.10k)$$

$$\dot{HrcA} = \frac{kHr_1 * (HrcAT - HrcA) * HPP * GroESL}{k_{Hem1} + (HrcAT - HrcA)} - \frac{kHr_2 * HrcA}{k_{Sm2} + HrcA} \quad (4.10l)$$

$$\dot{CodY} = \frac{kC_1 * (CodYT - CodY) * HPP}{k_{Cm1} + (CodYT - CodY)} - \frac{kC_2 * CodY}{k_{Cm2} + CodY} \quad (4.10m)$$

$$\dot{RpoN} = \frac{kR_1 * (RpoNT - RpoN) * HPP}{k_{Rm1} + (RpoNT - RpoN)} - \frac{kR_2 * RpoN}{k_{Rm2} + RpoN} \quad (4.10n)$$

All the enzymes in this model will follow the enzymatic mass equation: $E + EI = ET$. Where "E" is the active form of the enzyme, "EI" is the inactive form of the enzyme and "ET" is the total amount of enzyme.

### 4.1.4 **Cell Envelope Model**

The cell envelope pathway, shown in Figure 4.5, is an assemblage of the hypothetical cell wall, membrane and protein repair models. In the illustrated cell envelope model, the high pressure node has been left out and only the active form of the repair proteins are shown. This has been done to reduce the complexity of the network so that it can be more viewable. Regulation signals to proteins are shown by either a striped green arrow for induction or a striped red stopper for repression. This model shows how the three systems are linked together. The functional proteins will be inserted in the membrane and connected to the cell wall. The membrane and cell wall is connected through lipoteichoic acid, which also regulates the activity of the autolysins (Al). The Cody and RpoN regulators will affect all the three networks while SigB and HrcA will only affect the protein repair system. The metabolite pool (MP) is a source for any component that is needed for the pathway to work.

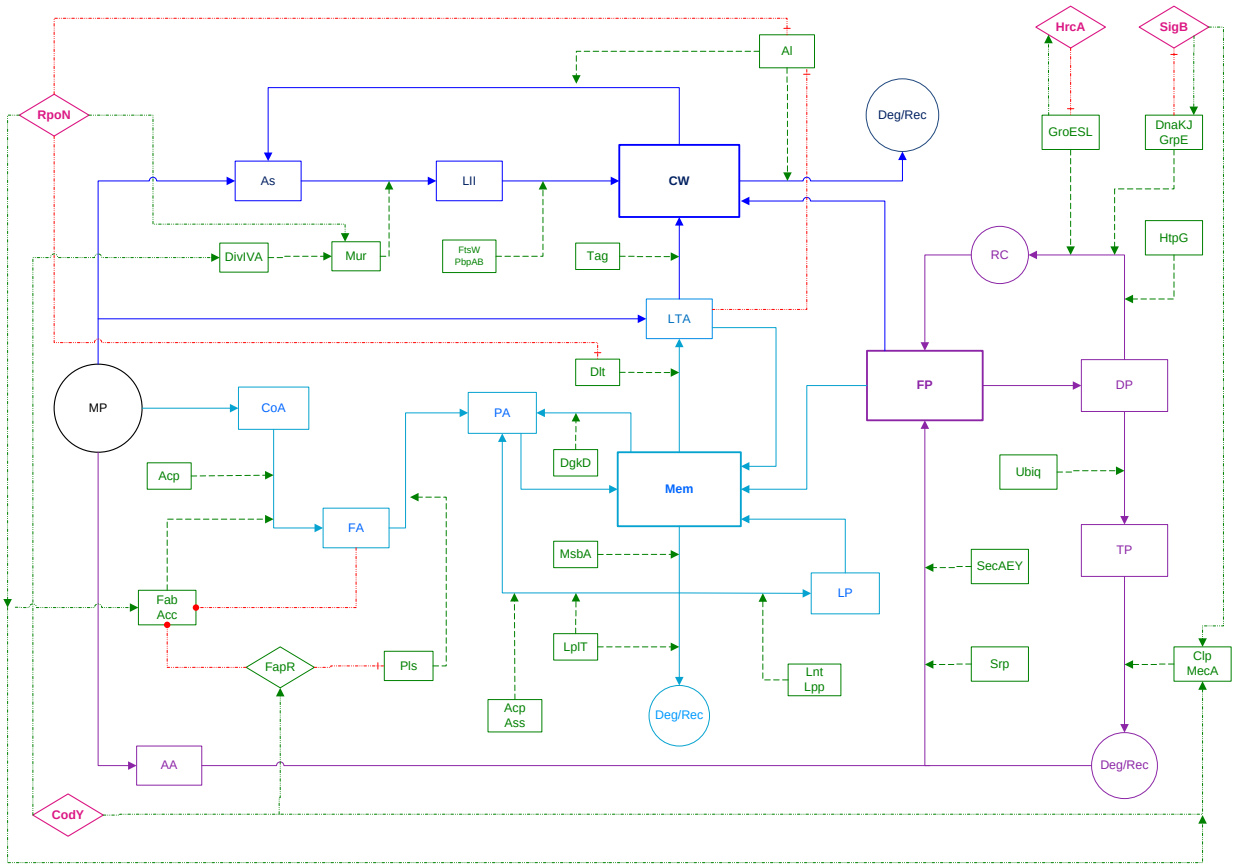



**Figure 4.5: Hypothetical pathway for cell envelope repair. Dark blue elements derives from the cell wall repair model, light blue elements comes from the membrane repair model and purple elements are from the protein repair model. Pink nodes are regulators.**

### 4.1.5 Discussions of Repair Models

The objective of the pathway analysis was to create models that could show and explain the mechanism of cell envelope repair after exposure to high pressure. This sections discusses the many challenges involved in setting up a novel model for repair. Especially challenging is the creation of a correct model and the choice of correct repair genes.

#### 4.1.5.a *Model Setup*

The trail that leads the bacteria cell from sensing stress and damage to reparation and adaption, is a complex and comprehensive pathway. Even though the bacteria response of these situations are considerably faster than for most other organisms, numerous of genes and proteins are involved for this to happen. Most often there are several different pathways leading to the same mechanisms. Because of this, setting up a complete system where all the involved components are linked together can be a hard task. Some important components or alternative routes may mistakenly not be included or a misunderstanding of how one gene effects another may occur. Many experiments has been done where the recovery of sub lethally injured cells that can survive HPP has been observed, but the

status of bacterial cells during this recovery period as well as the exact mechanism of repair is still unknown. The setup of the repair model therefore represents many possible pitfalls on the search for the correct system and with the lack of enough relevant experimental data, many incorrect assumptions may have been done. Most of the mechanisms are taken from general explanations on gram-positive bacteria. Also, much of the existing theory that is found for *L. monocytogenes* today, is based on knowledge on the gram positive bacteria, *Bacillus subtilis*. There is a lack of knowledge on some of the genetic systems in *L. monocytogenes*, but *B. subtilis* is a well-studied bacteria with many similarities to *Listeria*. That makes this replacement possible. However, only genes taken from *L. monocytogenes* has been used in the described pathways as these are the only ones that can be considered valid.

The created models are very simplified versions of the real systems and should not be regarded upon as completely reliable models that can be used for practical purposes yet. The models could have been created in other ways and this is not necessarily the most correct version. There may be more than one way or one protein to do a specific function. This means that these repair networks may not be enough as a basis for determining key elements in the repair network. Some proteins may even overlap in function, most likely because genetic redundancy makes the bacteria less susceptible to mutations or other influences that can knock out genes. By having a "backup-gene", the bacteria will not be too greatly affected if it loses the function of one gene. The ideal model should be bigger, showing all the details of the whole pathway. From sensing of high pressure to reparation and adaption.

There are many other functions in the bacteria cell that can be damaged than the cell envelope and they are all connected with each other in some way. The models have been narrowed down to show cell envelope repair, which means that influences from other events in the cell are left out. If some parts in the cell are affected, other parts will also be affected, resulting in a myriad of inactivated functions or damages in the bacteria. The cell envelope is not an independent system, meaning that other damages and functions in the cell will affect the envelope too. As the created repair pathways are simplified models, they were never intended to include these effects, but the simplifications represents limitations in the model. By allowing more complexity to the system, the models could become more authentic and hence, more representative for the actual repair mechanisms.

#### 4.1.5.b ***Gene Regulation Values***

Repair proteins were chosen by a combination of finding which proteins that were involved in the different mechanisms and which proteins that were significantly up-regulated by high pressure. The various mechanisms could be taken from other bacteria species than the *Listeria* genus and therefore the corresponding *Listeria* genes had to be found. If a specific protein was uncertain for *L. monocytogenes*, similar proteins that may have the same function was listed. This could result in less authentic models if some of the chosen proteins were not correct for some given reactions. Nevertheless, since proteins also were chosen by their fold change value after pressurization, they might still be very relevant for pressure stress response, regardless of their specific function.

Using the value of gene expression (fold change) to determine pressure stress adaption genes is not a straightforward procedure. A good example of how experimental conditions can affect the outcomes of experimental results is the detection of gene or protein regulations. Pressure, exposure time and temperature are processing variables that have different impact on the bacteria, all depending on their given values. Different pressure levels will induce genes at different scales. A pressure level of 400 MPa will for example give a much higher effect on the gene regulations than a pressure level of 600 MPa, as bacteria has less specific stress response to higher pressure levels. This can give different values of expression change which makes the significance of the protein appear different, but they rarely give opposite signals. The recovery time however, has a great influence on the detected changes in gene expression. All depending on how much time the bacteria cells get to recover, the measured gene expression values can show different results. Some genes have a tendency to be down-regulated during pressurization or immediately after pressurization, but after some time they are up-regulated. Other genes can show opposite tendencies by initially being up-regulated and then be down-regulated after some recovery time. This means that the detected gene expression values can turn out differently, depending on the allowed recovery time of the bacteria cells before measurement. A comparison made from three different high pressure experiments show how different the gene regulation results can be between no recovery (immediate analysis) and 2 hours of recovery.

Table 4.**6** presents some chosen genes that illustrate both differences and similarities between gene regulations. Especially noticeable is the different impact on heat shock proteins (also comprising peptidases), showing that some proteins involved in repair and adaption may have a delayed activation. Ribosomal proteins on the other hand, shows consistency by being immediately up-regulated and staying so during recovery.

**Table 4.6: Comparison between some regulated genes. Data obtained from 1: Bowman, Claudio et al. (2007) 2: Jofre, Champomier-Verges et al. (2007) 3: Kamlesh, Ramakrishna et al. (2011)**

| | Protein name | Gene name | No recovery (1) | 2h recovery (2) | 2 h recovery (3) | Comparison |
|---|---|---|---|---|---|---|
| **Ribosomal proteins** | RplJ | lmo0250 | Up | Up | Up | Correlation |
| | RplL | lmo0251 | Up | Up | Up | Correlation |
| | RpsF | lmo0044 | Up | Up | Up | Correlation |
| | RpsB | lmo1658 | Up | Up | Up | Correlation |
| **Metabolism** | Pta | lmo2103 | Down | N/A | Up | Mismatch |
| **Stress protein Detoxification Adaption** | OsmC | lmo0903 | Down | Up | N/A | Mismatch |
| | GshR | lmo0906 | No change | Up | N/A | Mismatch |
| | Flp | lmo0943 | Down | Up | Up | Mismatch |
| **Peptidase, degradation** | PepF | lmo2188 | Down | Up | Up | Mismatch |
| | Amp | lmo1603 | No change | Up | N/A | Mismatch |
| | PepT | lmo1780 | Down | Up | Up | Mismatch |
| | PfpI | lmo2256 | Down | Up | Up | Mismatch |
| **Heat shock chaperone** | GroES | lmo2069 | Down | Up | Up | Mismatch |
| | DnaK | lmo1473 | Down | Up | N/A | Mismatch |

## 4.2 Experimental Results

This section consists of the results from the two experiments conducted with high pressure. The first experiment is a staining method on pressurized cells, differentiating injured from non-injured cells by colorization. The second experiment is a recovery time detection of pressurized cells, differentiating growth between injured and non-injured cells.

### 4.2.1 PMA Staining Results

There were made 9 samples for the staining experiment: 3 replicates of pressurized-stained cells, one untreated-stained sample, one untreated-unstained sample, 3 replicates of pressurized-unstained cells and one sample of heat treated-stained cells.

The results from the pressurized-stained replicates were evaluated, while the other samples were used for comparison or control. In total, 28 pictures were taken of the pressurized (3 replicates) and the heated (1 sample) cells that were stained. All representative images are provided in Appendix 2. The pictures were taken at different angles and with different magnifications. Figure 4.7 gives a selection of the stained cells. Only good quality pictures comprising few cells were used for counting, such as those given in Figure 4.6.

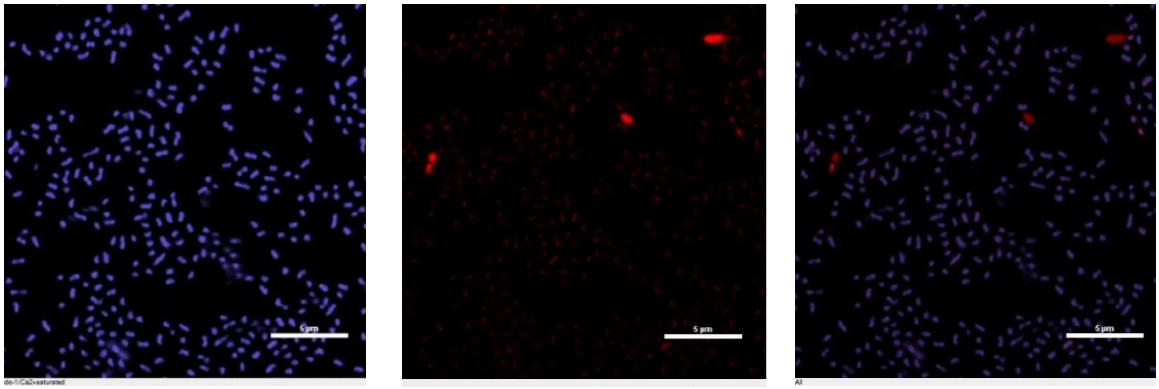


**Figure 4.6: Microscopy images used for stained cell counting. Left: Cells stained with Hoechst 33342 (purple). Middle: Cells stained with PMA (red). Right: Both Hoechst 33342 (purple) and PMA stained cells (red).**

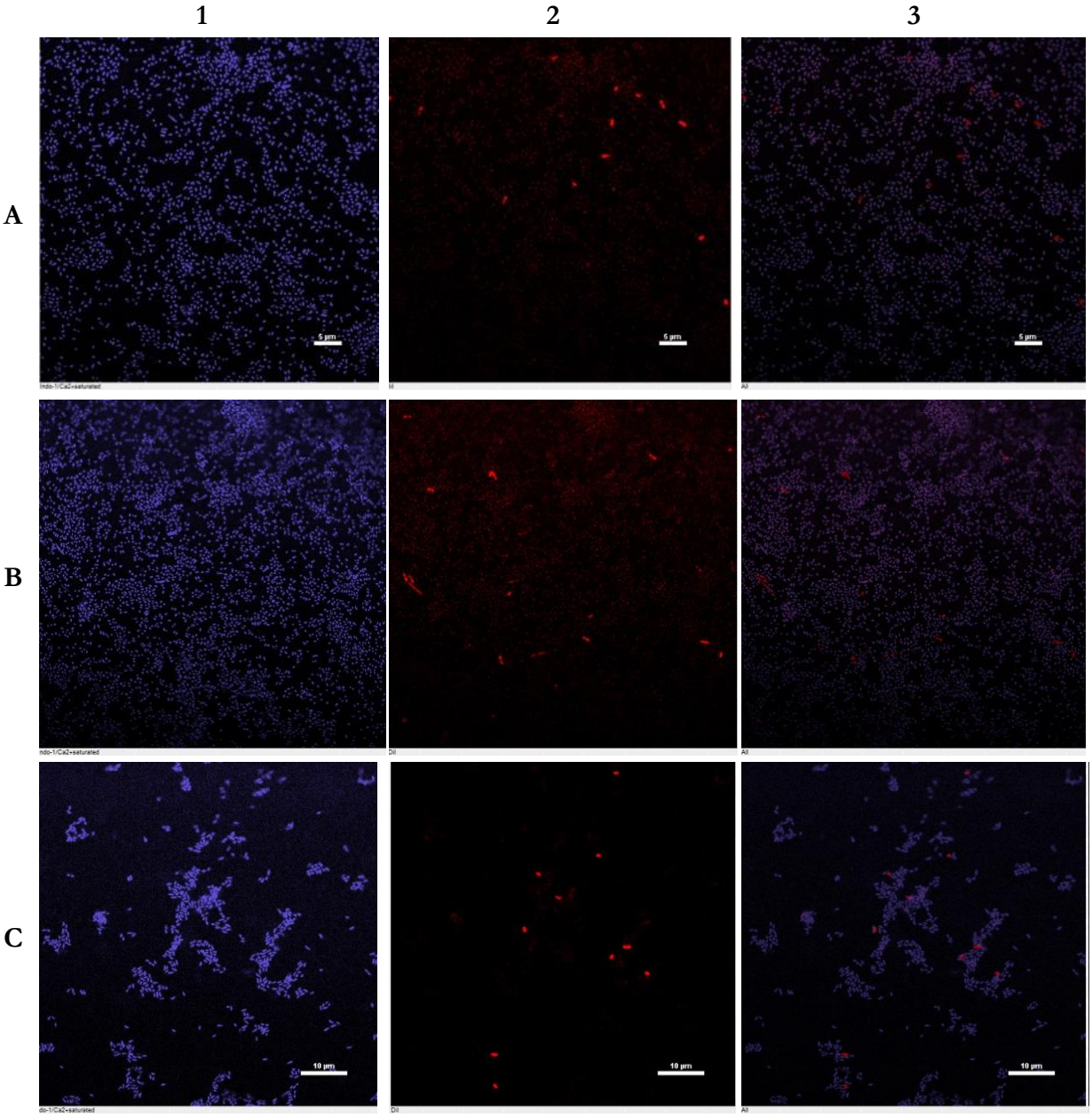



**Figure 4.7: Microscopy images of staining results. Row A and B displays different replicated of cells exposed to 400 MPa for 5 minutes, row C is used as a comparison, showing heated cells exposed to 60 °C for 6 minutes.**
**Column 1: Cells stained only with Hoechst 33342 (purple). These cells have intact membranes and presumed to represent viable cells.**
**Column 2: Cells stained only with PMA (red). These cells have lost their integrity and are assumed to be dead or injured.**
**Column 3: Column 1 and 2 merged together. Showing both Hoechst 33342 stained cells (purple) and PMA stained cells (red).**

10 pictures were counted, giving quotas of cells with full integrity (viable) and cells with lost integrity (dead/injured). The counting results are given in Table 4.7.

**Table 4.7: Counting results from pressurized and heated cells. Two pictures were counted for each of the three replicates of pressurized cells. Four pictures were counted for the one sample of heated cells.**

| Pressurized cells | | | |
|---|---|---|---|
| Replicate | Viable cells (purple) | Leaking cells (red) | Percentage leaking cells |
| 1 | 177 | 4 | 2.2 |
| 1 | 110 | 5 | 4.5 |
| 2 | 350 | 2 | 0.5 |
| 2 | 780 | 6 | 0.7 |
| 3 | 484 | 2 | 0.4 |
| 3 | 500 | 2 | 0.4 |
| Average leaking cell percentage: | | | 1.45 |

| Heated cells | | | |
|---|---|---|---|
| Picture no | Viable cells (purple) | Leaking cells (red) | Percentage leaking cells |
| 1 | 100 | 1 | 1 |
| 2 | 107 | 0 | 0 |
| 3 | 190 | 5 | 2.6 |
| 4 | 193 | 3 | 1.5 |
| Average leaking cell percentage: | | | 1.28 |

### 4.2.2 Analysis of Staining Results

The objective of the staining experiment was to demonstrate that the cell envelope is affected by high pressure. The experiment did show influence on the cell envelope, but not at a particularly high degree. The highest percentage of leaking cells was 4. 5 % after pressurization and 2. 6 % after heating, while the average percentage of leaking cells were 1. 45% and 1. 28% for pressurization and heating respectively. These numbers are considerably low, as such percentages do not contribute to any significant part of the population. By looking at these results, it could be interpreted that neither heat nor pressure has a noteworthy effect on the cell envelope, despite that the opposite has been proven in other studies. Such a low outcome of the results can probably best be explained by the time of applying the stain. As in the experiment of Klotz, Mañas et al. (2010), their results were significantly different from when stain was added to *E. coli* cells before, rather than after pressurization. For the samples in which the stain was present during pressurization, a high number of cells were stained. However, for the samples where the stain was added after pressurization, a low amount of cells were stained. As shown in Figure 2.7 from chapter 2.2, no *E. coli* cells were coloured after pressure exposure. The reason for this is most likely because the bacteria cells have resealed their leakages already during pressurization so that when the stain was added after processing, most of the cells did already have full integrity. As this experiment with *Listeria* only tested for stain uptake after pressure processing where the PMA uptake was low, it cannot prove that most *Listeria* in fact loses integrity by pressure. However it can be assumed that they react similar to the *E. coli* cells and do lose their integrity, but manage to reseal very quickly. The greater part of a population probably manage to reseal during a couple of minutes, but as shown from the resulting pictures, not all cells reseal that quickly.

Heating did not seem to give very different impact on cell integrity. There was only one sample to count of the heated cells, but the tendency of heating seemed to be rather similar to that of pressurization. A treatment of 60 °C for 6 minutes represents an amount of stress exposure that can be similar to 400 MPa for 5 min, which are both mild conditions. Since none of these process conditions gave a significant impact on cell integrity, it may seem like a higher stress exposure would

be needed to get more efficient results. A variety of pressure levels should be used in conjunction with the staining method. Especially a higher level than 400 MPa should be used to see if intensified conditions gives a more efficient outcome. Therefore, a pressure level of 600 MPa would be beneficial to test out, where processing with 80 °C for 15 minutes could be used for comparison.

The experimental conduction could be a source of error for the results. If the PMA stain was not properly added in the bacteria population, there might not be any stain available for the bacteria to take up and hence a low staining result. Other possible errors coming from the experimental procedure must also be taken into account when evaluating the results. Cell counting is a possible source of error as areas chosen for counting may be selective. However, as long as a minimum of 100 cells are included for each counting, the results can be regarded as reliable enough as it satisfies statistic criteria.

Flow cytometry can be used for this kind of research as it would give valuable insight on how a single cell reacts and repair itself. Flow cytometry is a laser based, biophysical technique that suspends cells in a stream of fluid and pass them by an electronic detection apparatus. In this way, analysis of the physical and chemical characteristics can be done for of up to thousands of cells per second (Martz 2000). Flow cytometry would therefore be the ideal measurement, but a too comprehensive experiment for this thesis.

### 4.2.3 **Recovery Results**

Fish soup added *Listeria* was used as a model food product. The fish soup had pH 6, which does not have any inhibitory effect on bacteria. After pressure processing, the bacteria were stored at 4 °C during the recovery period. The bacteria colony sizes throughout the weeks of recovery are given in Table 4.8.
By using Equation 3.1, viable cells left from colony was found immediately after pressurization (Day 0) was found to be:

0.00077 % viable cells, both injured and non-injured
0.00052 % viable, non-injured cells
0.00025 % viable, injured cells

From these results, injury ratios of all viable cells (0, 00077% of initial inoculum level) immediately after pressurization was calculated by the use of Equation 3.2. The ratio were 33 % injured cells and 67 % non-injured cells.

**Table 4.8: Table of injury extent throughout the 6 weeks of recovery. Applied for all viable cells (0, 0007 % of initial inoculum level)**

| Injury detection of pressurized cells | | | | | | | |
|---|---|---|---|---|---|---|---|
| Medium | Day 0 | Day 7 | Day 14 | Day 21 | Day 28 | Day 35 | Day 42 |
| TSAYE ($log_{10}N$) | 0,77 | 1,04 | 1,8 | 2,35 | 2,91 | 3,52 | 4,69 |
| Brilliance ($log_{10}N$) | 0,52 | 0,52 | 1,3 | 1,74 | 2,38 | 3,03 | 4,02 |

The process gave a 4 log reduction of the population as the inoculum size was reduced to below 1 log for both TSAYE and Brilliance. The population in TSAYE had grown during the first week. On Brilliance, growth was first detected after 2 weeks. After 6 weeks, the bacteria populations in both growth mediums had almost reached back to initial inoculum level.

**Table 4.9: Table of injury extent throughout the 6 weeks of recovery. Applied for all viable cells (0, 0007% of initial inoculum level)**

| Injury extent of pressurized cells | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day 0 | Day 7 | Day 14 | Day 21 | Day 28 | Day 35 | Day 42 |
| Injured (%) | 33 | 50 | 28 | 26 | 18 | 14 | 14 |
| Non-injured (%) | 67 | 50 | 72 | 74 | 82 | 86 | 86 |

During the 6 weeks of storage, the ratio of injured bacteria cells decreased while the ratio of non-injured cells increased.

### 4.2.4 Analysis of Recovery Results

The objectives of the recovery experiment were to detect the time consumption for recovery of both injured and non-injured bacteria cells. A high reduction of viable cells was obtained, as only 0. 00077 % of the initial colony was left. However, the population reached back to a hazardous level. After 6 weeks, the bacteria populations in both growth mediums had almost reached back to original inoculum level. Still, this was during a long time period and it can probably not be expected that a bacteria population stays at minimum for a long time under such conditions.

More interesting is the comparison between the selective and the non-selective growth mediums. The inoculum level in Brilliance was slightly lower than the level in TSAYE from day 0. Since Brilliance does not allow for injured cells to grow, the difference between the two mediums represented injured cells. This gave an injury/non-injury ratio of 33/67 immediately after pressurization. During the first week, cells managed to grow in TSAYE, but not in Brilliance. This can be because Brilliance is a tougher growing medium, making bacteria require more time to grow, but it may also be due to cell reparation. Even though the Brilliance culture needed more time to grow than the TSAYE culture, it managed to reach a high inoculum level throughout the storage period, though lower than that of TSAYE. Table 4.9 shows that the injured cell ratio is decreasing

during time, which is a result of the Brilliance inoculum level reaching the TSAYE inoculum level. Since the ratio of non-injured cells increases with time, it could seem like bacteria are able to repair and start growing again. This could however be a sought solution. It is difficult to differentiate between regular cell division and actual repair. The longer the cells are allowed to recover, the harder it is to know if a colony has grown because non-injured cells has started to divide or because injured cells are repaired and able to grow again. Because of this, one cannot make any specific assumptions on injury repair throughout a period of 6 weeks. The first week would be much more useful for such analysis as the populations are still small and the recovery period is still short enough to do a differentiation between normal cell growth and cell repair.

During the first week, the TSAYE colony went from inoculum level 0. 77 to 1. 04 in log scale, meaning that the population managed to increase 13. 5 times during 7 days. As mentioned, the shorter the time period, the less errors are involved in analysis and the same applies for population size measurements. Even though the Brilliance population did not grow during the first week, the population increase in TSAYE is too high to make an accurate conclusion on recovery time. The whole recovery experiment should in fact be conducted during a period of two weeks where the measurement frequency could by daily instead of weekly. The time steps were too long to obtain useful information and therefore not sufficient to obtain the desired data. The time usage for doubling of the bacteria concentration could be suitable to find as it would give an indication of how much time one bacteria cell would need to divide. Such answer is not possible with these data, as it only detects growth after the concentration has increased 13. 5 times. However, by calculating the daily average growth during this period, an approximation of the time needed for cells to double can be found with Equation 3.3:

$$\frac{13{,}5\ {}^{C_{increase}}/_{week}}{7\ {}^{days}/_{week}} = 1.92\ {}^{C_{increase}}/_{day} \tag{3.3}$$

To say that a pressurized bacteria cell, under the given conditions, in average needs almost 2 days to recover and start growing is a very rough estimation. There are too many sources of errors included. The calculation may not be applicable, as cell growth follows a logarithmic curve which this calculation does not take into account. This assumption is very weak if it is only taken with the experimental results as a basis, but by combining it with previous published experiments and comparing the results, the assumptions may be more feasible. As shown in chapter 2.4, Muñoz-Cuevas, Fernández et al. (2012) and Bozoglu, Alpas et al. (2004) detected growth after 1 and 2 days respectively. These examples has relatively similar process conditions as the recovery experiment conducted with Nofima. The experiment of Bozoglu, Alpas et al. (2004) would probably be most comparable as the storage temperature was the same and since milk can have similarities to fish soup. By comparing these results with the recovery experiment, the hypothesis that pressurized bacteria cells needs around two days to repair and start growing again may be assumed, but cannot completely

be defended. All depending on the conditions, recovery can take from hours to weeks to happen. Because of this, it is very difficult to give a generalized recovery period for a bacteria population and can probably only be used on independent cases. For each model there are still limitations as it is difficult to know what actually is happening in a population. Predicting an average recovery time for a single cell is especially difficult as there is too much variation in how each cell responds.

Even though specific calculations on recovery time could not be done with this experiment, it still showed that bacteria are most likely able to repair after pressurization. This was also revealed from the staining experiment, which showed that cells are able to reseal and they do so very quickly. Because of this, the lack of integrity might not be the main reason for cell death. There may be two possibilities; Bacteria cells can reseal the envelope very quickly and become viable shortly after, or bacteria cells do not manage to reseal before they have lost too much components and therefore dies. With this assumption, the actual resealing is not directly crucial, but the timing of it is. Cells might survive due to envelope repair as long as it is done quickly enough, making the efficiency of repair very important.

## 4.3 Mathematical Modelling and Simulations

Simulations provided in this section are based on the findings from the pathway analysis and the high pressure experiments. The models used in Matlab are simplified versions of the created repair pathways with their associated differential equations. These equations were used to simulate the behaviour of each constituent when exposed to high pressure. The behaviour of the cell wall, membrane and proteins were based upon the findings that the cell wall is immediately being resealed, with the membrane needing more time for repair because of its complexity. Protein repair is assumed to have a delayed activity after what was found from the pathway analysis. The simulations are showing how pressure exposure is affecting the time and capability of the components to repair themselves.

Since the simulations are based on the pathway analysis and high pressure experiments, the settings and assumptions will be similar to these. This means that the simulations have been based on a hypothetical scenario where one high pressure exposed bacteria is being investigated. This bacteria represents the average behaviour for how a bacteria would respond when the envelope is damaged because of preddure. The simulations show what genes it activates and how much time it uses to repair the damages on the cell wall, membrane and the associated proteins. The specific conditions involved with these simulations are as in the experiments, bacteria contaminated fish soup, processed for 5 minutes at 20 °C and subsequently stored at 4 °C. Pressure levels will vary.

It has been assumed that the cell has full integrity at a state above 90 %, though this is not an optimal state. A state over 90 % means that major damages are repaired and all holes are patched, but 100 % is the optimal level and therefore also the steady state. Any disturbance that knocks the system out of its steady state will make the system seek back to it. This behaviour is representing the effort

the bacteria cell does to repair damages after being exposed to high pressure. The repair mechanisms will be active as long as they receive a signal saying that the envelope state is not being optimal.

### 4.3.1 Cell Wall Repair Model

The mathematical model of the cell wall system being used in Matlab is a simplified version of the original cell wall model. In this model, RpoN is the only regulator and it up-regulates the biosynthesis of peptidoglycan through the Mur-complex. The state of the cell wall will decide the activity of RpoN. The extra Lipid II link has been removed as it does not have a big impact on the system. Lipoteichoic acid is also removed for simplicity, as it is only a minor constituent in the cell wall. Loss of peptidoglycan is represented by two different pathways where one of them shows the natural turnover of peptidoglycan components and the other one represents loss of peptidoglycan specifically by high pressure. These are in reality in the same path, but presented as two different routes in Matlab. The recycling path is not included, as the source of aminosugar is assumed constant in the simulations and will therefore not be affected by changes in the system. The Al protein is stated as continuously active and available. The simplified model is illustrated in Figure 4.8.

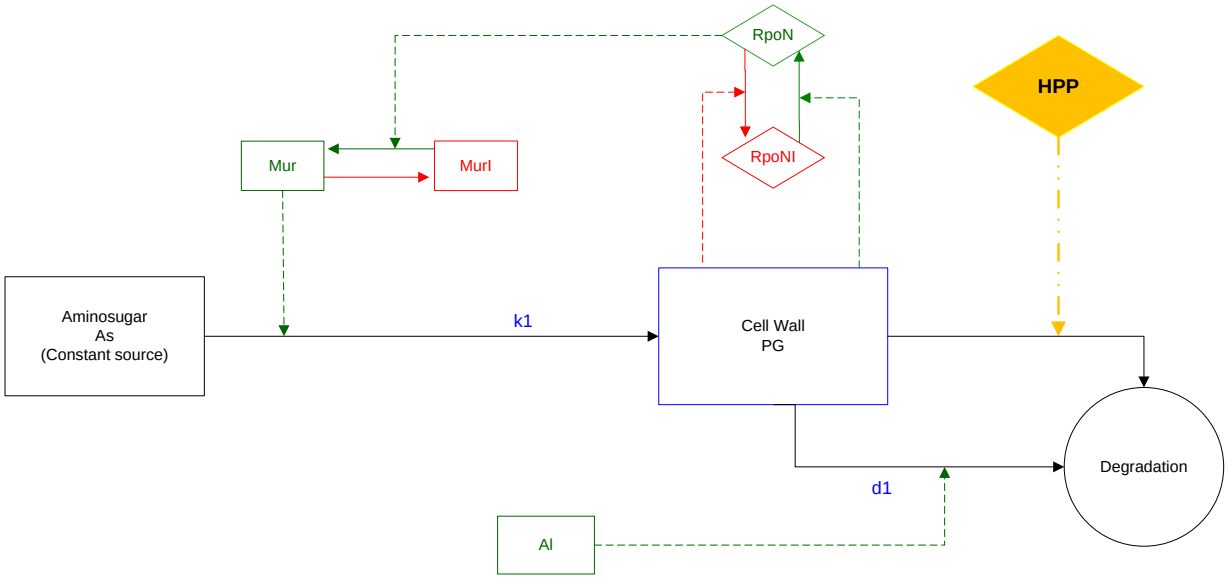



**Figure 4.8: Simplified version of the developed Cell wall model. Used in simulations.**

The simulations for the Cell wall model are developed to show how the state of the cell wall and activity of the repair proteins are affected by pressurization. Figure 4.9 shows how all the components are affected by a pressure of 400 MPa. The simulation starts when the cell is under normal conditions at atmospheric pressure. After 100 minutes, 400 MPa is applied to the bacteria for 5 minutes. Once pressure is relieved, the bacteria will attempt to get back to its optimum state by activating the RpoN regulator and the Mur complex, which allows synthesis of new peptidoglycan.

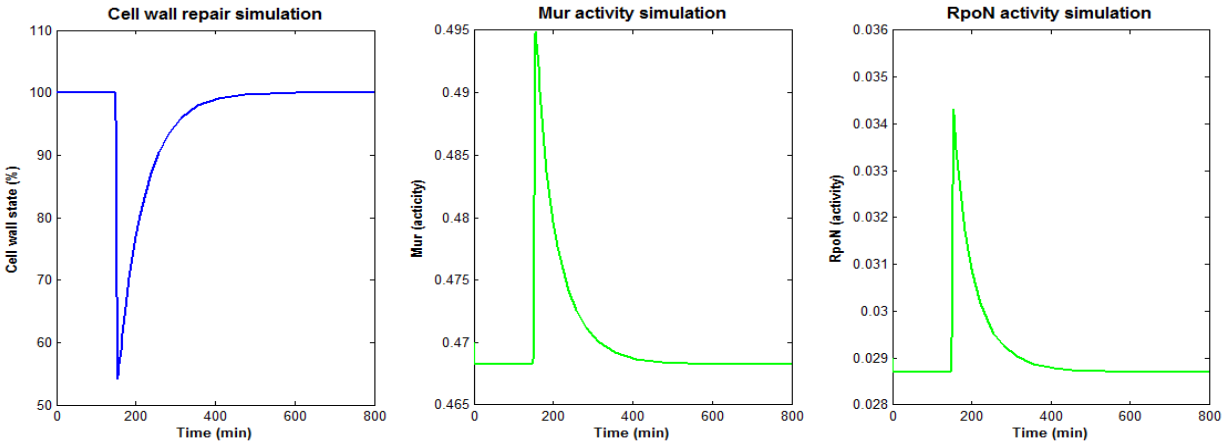



**Figure 4.9: Simulation of cell wall state, activity of RpoN regulator and Mur complex, being affected by a pressure of 400 MPa. Pressure applied after 150 minutes and relieved after 155 minutes.**

The state of the cell wall is reduced to 55 % during the 5 minutes of pressure exposure, while the activity of Mur and RpoN increases during these 5 minutes. The increased activities are direct responses to the reduced state. It takes 5-6 hours before the cell wall is fully recovered (100 % state), but full integrity where all holes are patched, is assumed to be reached at an earlier state (before 90 % state). Details on cell wall state at different times are given in Figure 4.10 and simulations with different pressures are given in Figure 4.11.

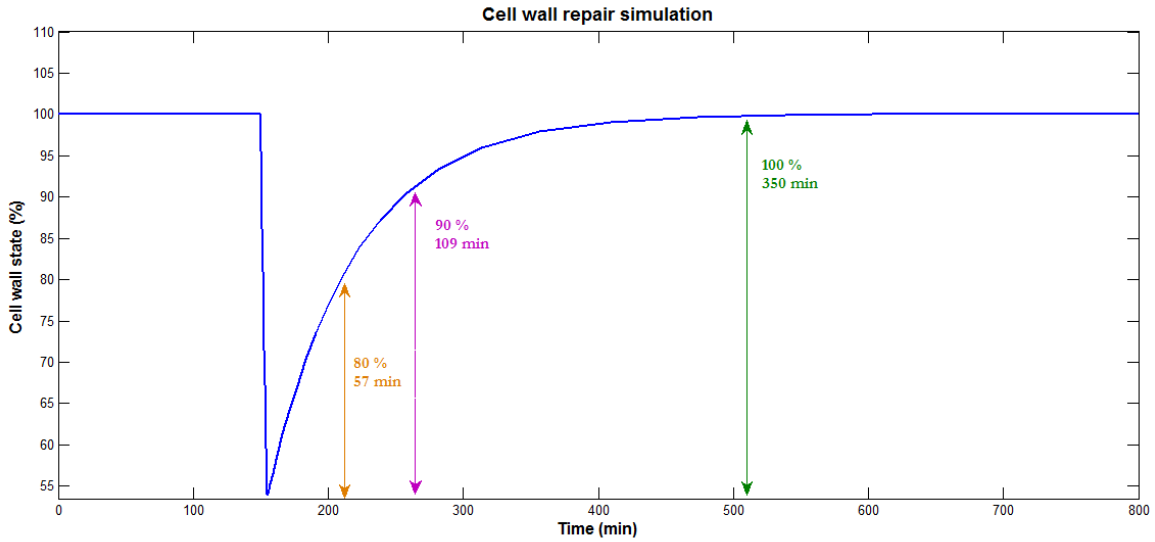



**Figure 4.10: Time consumption to reach different states in the cell wall system. Pressure applied after 150 minutes and relieved after 155 minutes. It takes approximately 57, 109 and 360 minutes to reach back to a state of 80, 90 and 100 % respectively.**

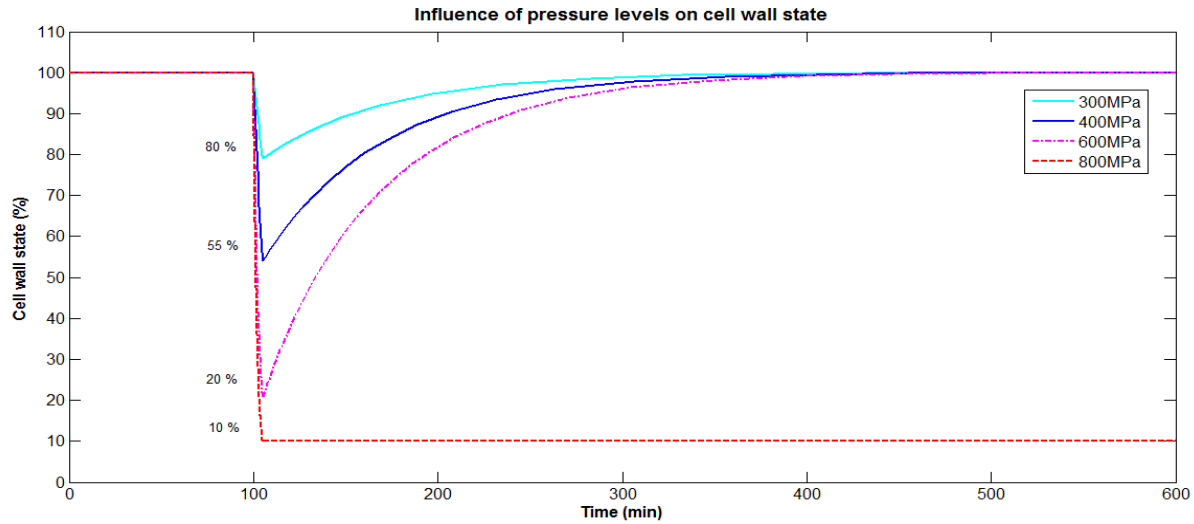



**Figure 4.11: Pressure levels affecting the cell wall state. Pressure applied after 100 minutes and relieved after 105 minutes. Pressures of 300, 400, 600 and 800 MPa will reduce the cell wall state to 80, 55, 20 and 10 % respectively.**

Simulations with different pressure levels illustrate the diverse impact on the cell wall state. Figure 4.11 shows how the state of the cell wall is affected by pressures of 300, 400, 600 and 800 MPa. A pressure of 300MPa will have low impact on the cell wall state as it is only reduced to 80 %. Pressures of 400 and 600 MPa has bigger influence by reducing the state down to 55 and 20 % respectively. A pressure level over 600 MPa is needed to completely destroy the cell wall, where 800 MPa is such a stress. 800 MPa reduces the state below 10 %, which in the simulation is a threshold value where the cell wall cannot be repaired back to its optimum state.

### 4.3.2 **Membrane Repair Model**

The mathematical model of the membrane system that has been used in Matlab is a simplified version of the original membrane model. In this model, RpoN is the only regulator in the simplified pathway for phosphatidic acid synthesis and it is regulated by the membrane state. CodY and FapR are not included as they are not crucial for synthesis to happen. Recycling of membrane components is simplified by only going through phosphatidic acid where DgkD induces this. Loss of membrane is represented by two different pathways. One of them shows the natural turnover of membrane components and the other one represents loss of membrane specifically by high pressure. These are in reality assumed to go through the same way, but presented as two different paths in Matlab. DgkD and MsbA are stated as continuously active and available during simulations. The simplifications are illustrated in Figure 4.12.

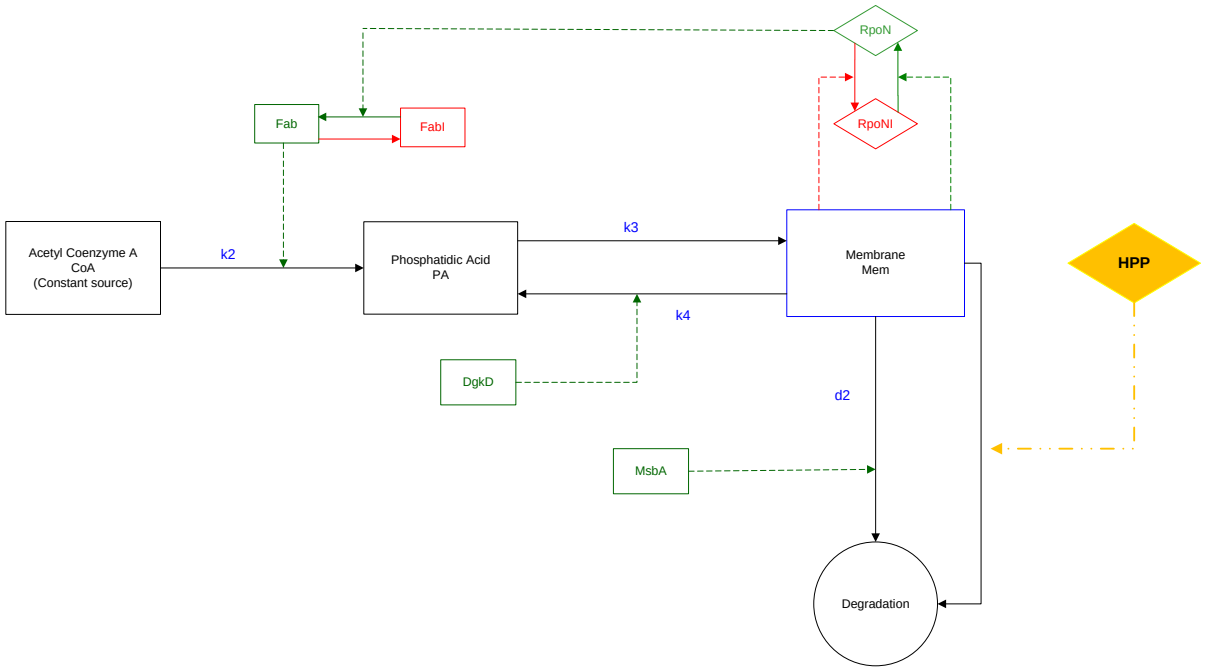



**Figure 4.12: Simplified version of the developed membrane model. Used in simulations.**

The simulations for the membrane model are developed to show how the state of the membrane, usage of phosphatidic acid and activity of repair proteins are affected by pressurization. Figure 4.13 shows how all the components are affected by 400 MPa, while the specific time points for reaching back to the upper membrane states are showed in Figure 4.14. Simulations with different pressure levels are given in Figure 4.15 to illustrate their diverse impact on the membrane state.

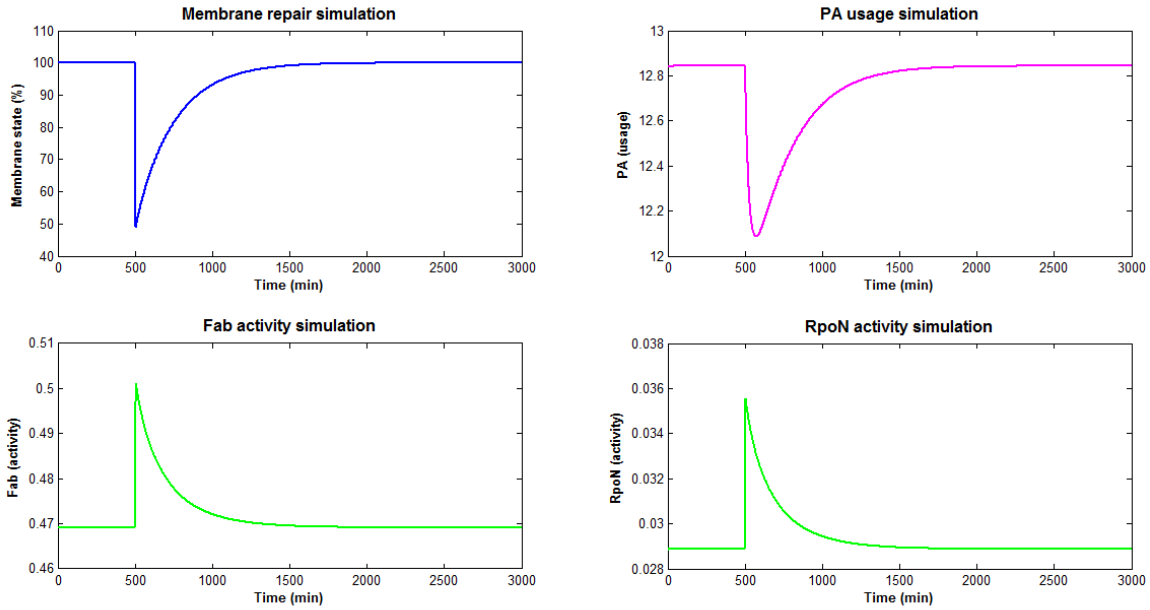



**Figure 4.13: Simulation of the membrane state, usage of phosphatidic acid and activity of RpoN regulator and Fab complex, affected by a pressure of 400 MPa. Pressure applied after 500 minutes and relieved after 505 minutes.**

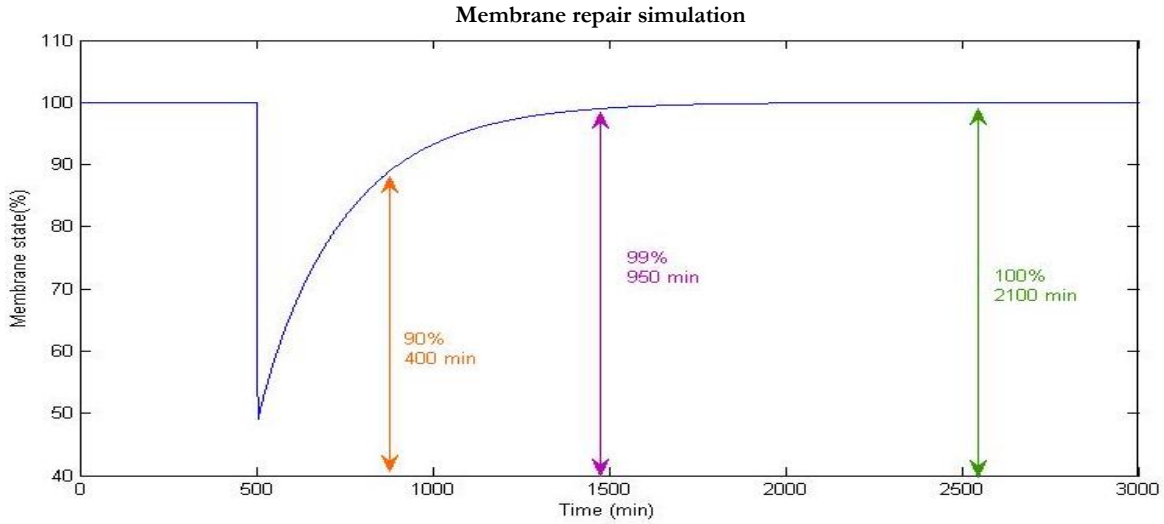



**Figure 4.14: Time usage for reaching different states in the membrane system. Pressure applied after 500 minutes and relieved after 505 minutes. It takes approximately 400, 950 and 2100 minutes to reach back to a state of 90, 99 and 100 % respectively.**

The beginning of the simulation shows normal conditions under atmospheric pressure, but after 500 minutes, 400 MPa is applied to the bacteria for 5 minutes. After pressure relief, the bacteria will strive to get back to its optimum state. This is done by using the source of new phosphatidic acid (PA) to be inserted in the membrane. The source of phosphatidic acid goes down when used, but activation of the RpoN regulator and Fab protein complex allows synthesis of more PA. This will increase the membrane state. It takes around 35 hours before the membrane is fully recovered (100 % state).

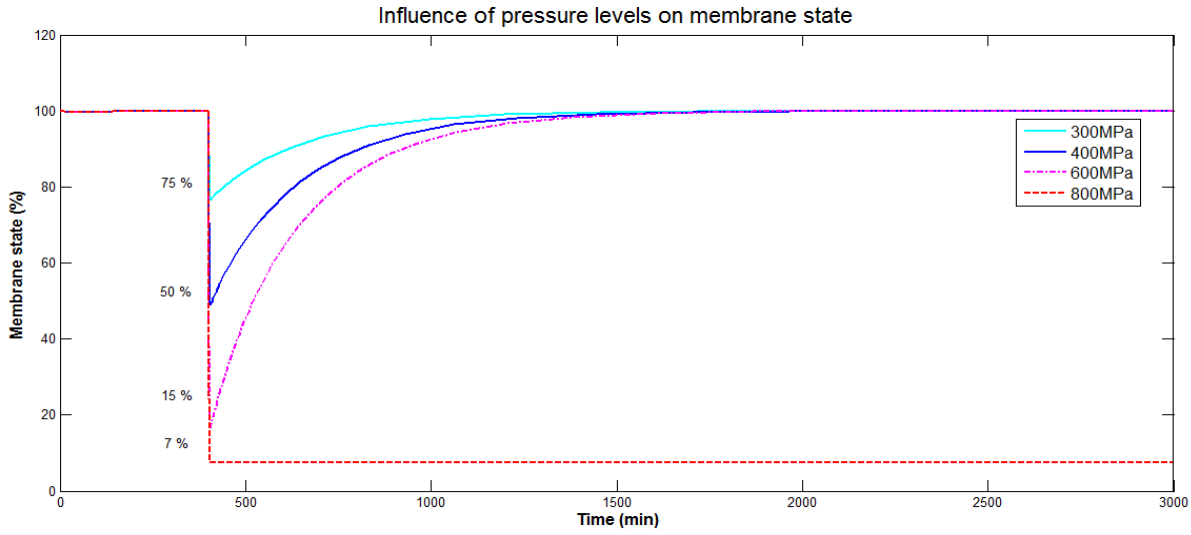



**Figure 4.15: Pressure levels affecting the membrane state. Pressure applied after 500 minutes and relieved after 505 minutes. Pressures of 300, 400, 600 and 600 MPa will reduce the membrane state to 75, 50, 15 and 7 % respectively.**

Pressures of 300, 400 and 600 MPa will reduce the state of the membrane down to 75, 50 and 15 % respectively. Pressures over this level will reduce the membrane state to such a low level that it cannot repair itself anymore. 800 MPa will reduce the state below 10 % and the state cannot increase from here.

### 4.3.3 **Protein Repair Model**

The mathematical model of the Protein repair system being used in Matlab, is a simplified version of the original protein repair model. In the simplified version, SigB is the only regulator in the system and all the repair chaperones are combined as the Chap complex. The repair cycle is not included in this model. Recycling of components is not included as the source of amino acids is assumed constant and will therefore not be affected by changes in the model. The Ubiq, Clp and Sec proteins are stated as continuously active and available during simulations. Repair Activation is an extra differential equation included in the Matlab model. It is meant to simulate the delay of activation in the repair proteins, making the repair dynamics a little slower for this system. The simplifications are showed in Figure 4.16.

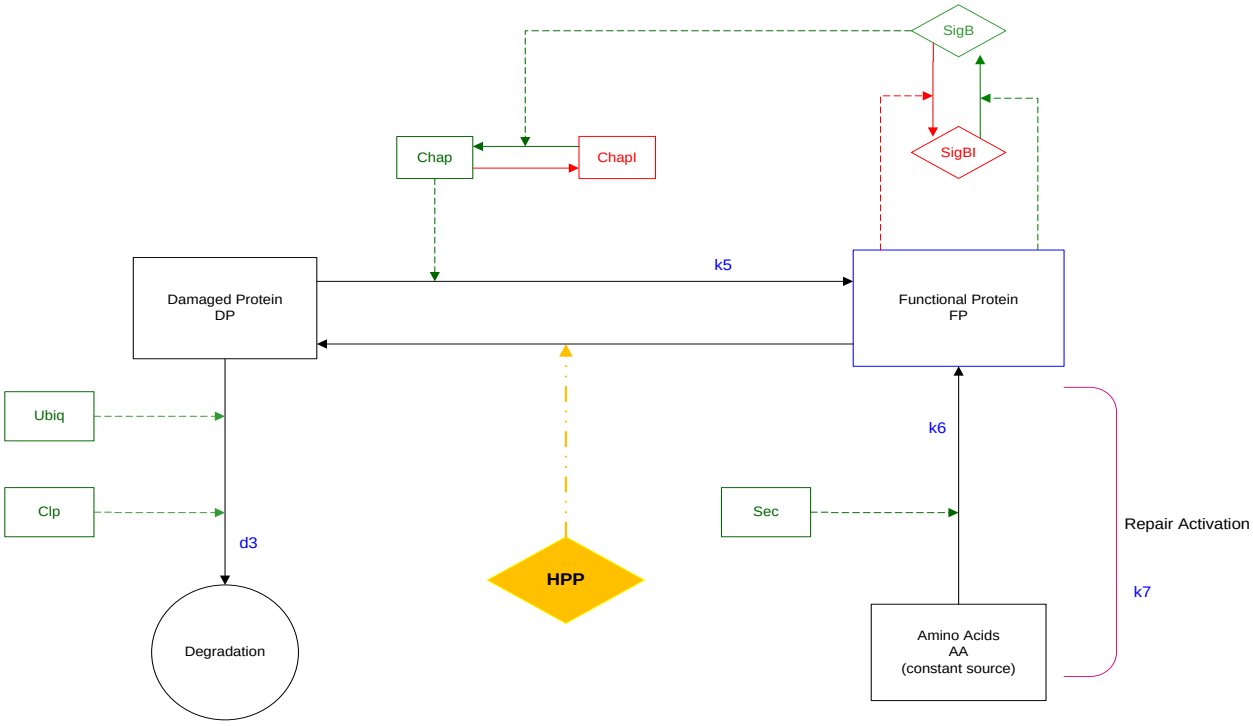



**Figure 4.16: Simplified version of the developed Protein repair model.**

The simulations for the protein repair model is developed to shows how the state of the functional protein and activity of the repair proteins are affected by pressurization The beginning of the simulation shows normal conditions under atmospheric pressure, but after 200 minutes, 400 MPa is applied to the bacteria for 5 minutes. After pressure relief, the bacteria will try to get back to its optimal state. This is done by repairing the damaged proteins or insert new proteins to replace

unrepairable ones. The Chap protein is responsible for reparation and SigB is responsible for its activation. The responses to pressurization are illustrated in Figure 4.17, while the specific time points for reaching back to the upper protein states are showed in Figure 4.18. Simulations with different pressure levels are given in Figure 4.19, to illustrate their diverse impact on the protein state.

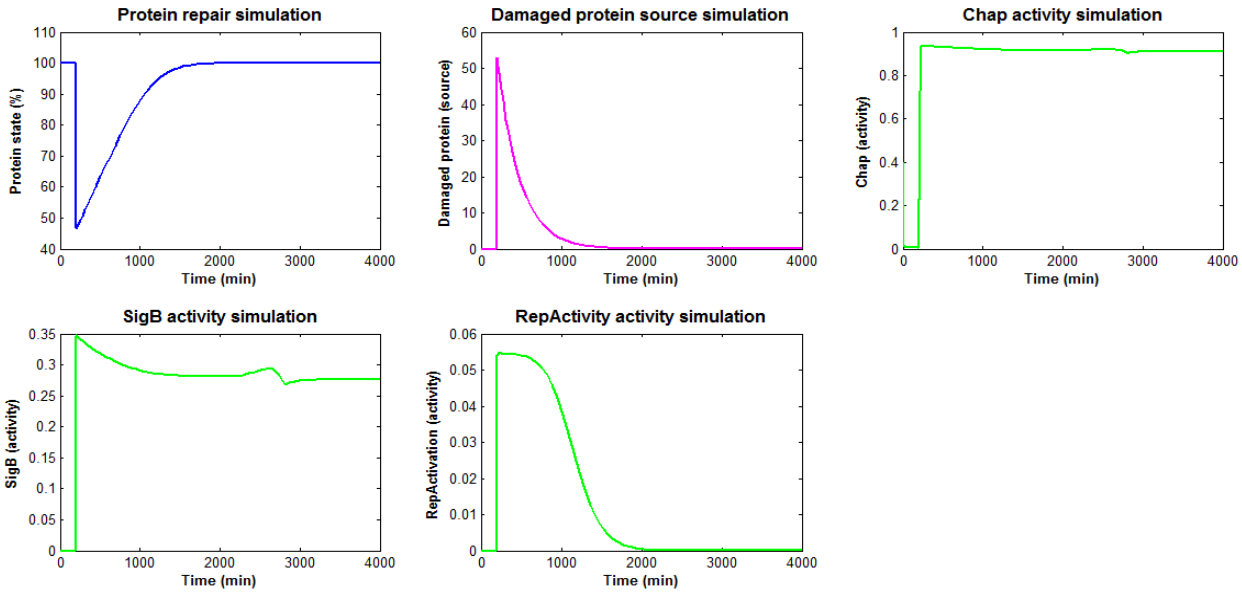



**Figure 4.17: Simulation of the functional protein state, transformation to damaged proteins and activity of the repair proteins when being affected by a pressure of 400 MPa. Pressure applied after 200 minutes and relieved after 205 minutes. The repair activation is also included to show the dynamics of activation delay.**

The state of the protein is reduced to 45 % during the 5 minutes of pressure exposure, while the activity of Chap and SigB increases during these 5 minutes. The increased activities are direct responses to the reduced state, but they do not reduce activity after the optimum state has been reached. The amount of damaged proteins is however a reversed simulation of the functional protein state as the proteins will either be in the functional or damaged protein pool. The preferred amount of damaged proteins is 0, meaning that this is the level it will reach at steady state. It takes around 38 hours before the envelope proteins are back at optimal state (100 %).

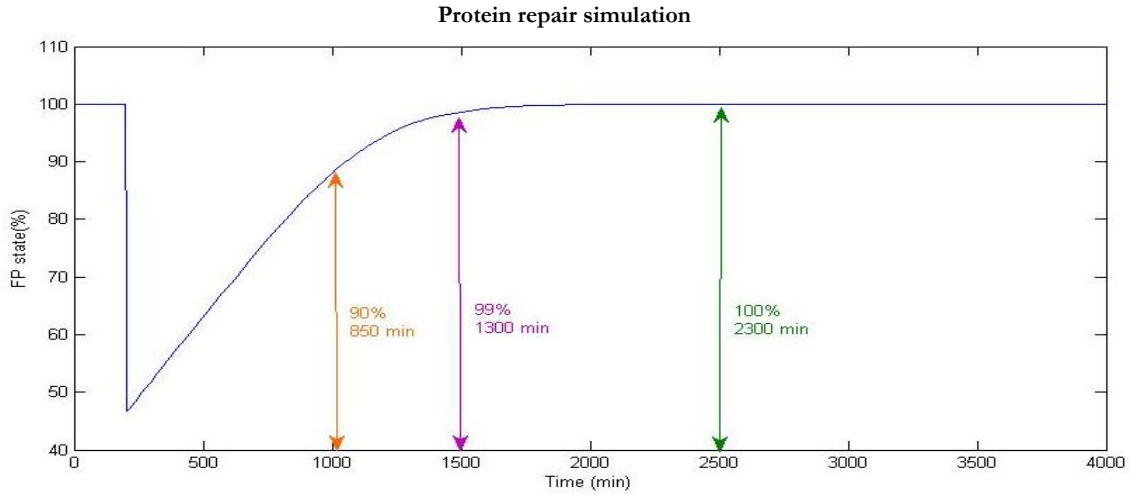



**Figure 4.18: Time used to regain different states in the protein system. Recovery starts after 205 min, when pressure is relieved. It takes approximately 850, 1300 and 2300 minutes to reach back to a state of 90, 99 and 100% respectively.**

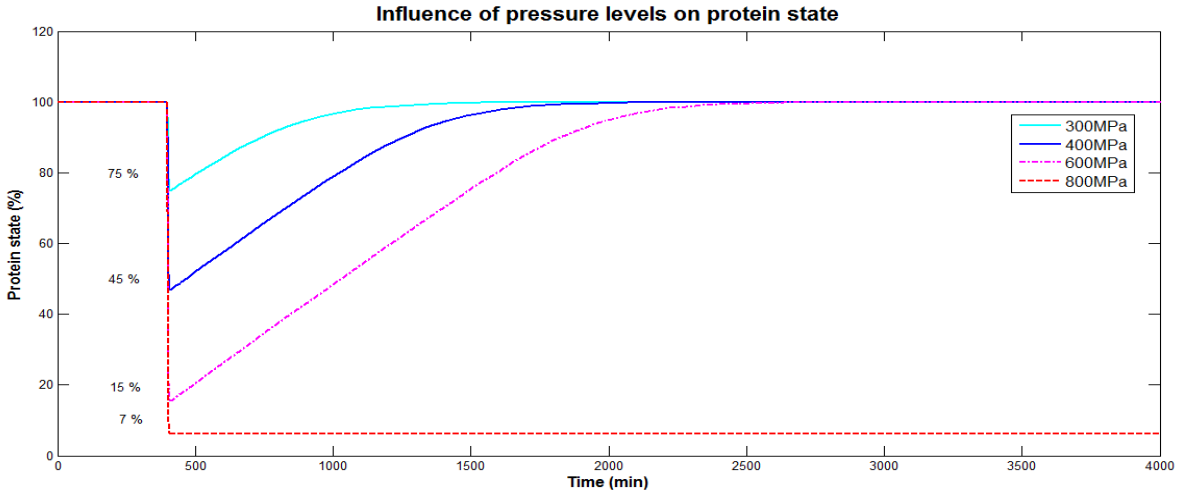



**Figure 4.19: Pressure levels with different impact on the envelope associated proteins. Pressure applied after 400 minutes. Pressures of 300, 400, 600 and 600 MPa will reduce the protein state to 75, 45, 15 and 7 % respectively.**

Pressures of 300, 400 and 600 MPa will reduce the state of the functional proteins down to 75 %, 45 % and 15 % respectively. Pressures over this level, such as 800 MPa, will reduce the state to such a low level that proteins cannot be repair anymore and will therefore stay at a state below 10 % and not reach back to 100 %.

### 4.3.4 Cell Envelope Repair Model

The mathematical model of the Cell envelope system used in Matlab is, as the original envelope model, an assemblage of the three repair models developed in this thesis. For the simulations, it is the simplified models that has been combined. The assemblage of models can be uncoupled or

coupled. An uncoupled system gathers the models, but do not connect them. Making each system independent from each other. A coupled model however, both gathers and links the systems together. Such a model is more interactive, as the state of one component may be able to affect all parts in the system.

#### 4.3.4.a *Uncoupled System*

The simulation of the uncoupled cell envelope system is presented in Figure 4.20. By combining the three models into one, the differences in repair dynamics are more apparent. The cell wall is least affected and the quickest to be repaired. The membrane is more complex than the cell wall and needs more time to rejuvenate. The repair of proteins has a delayed activation and is therefore the slowest to be repaired. All three components manages to repair however, meaning that the cell envelope is fully recovered after nearly 2 days (2000 minutes).

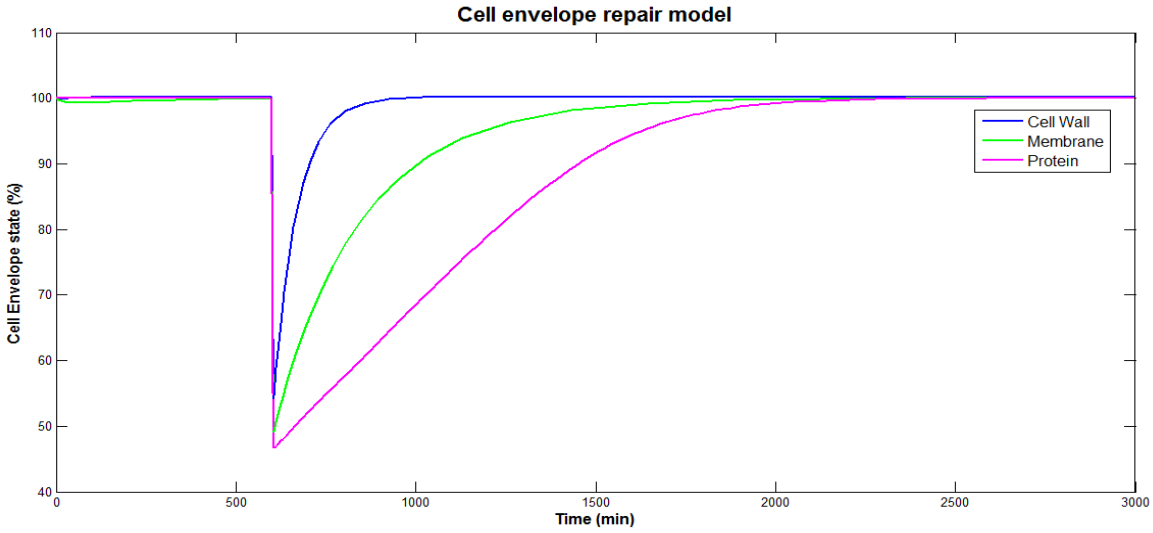



**Figure 4.20: Combination of the cell wall, membrane and protein model. After 200 minutes, pressure of 400 MPa is applied for 5 minutes. After 2000 minutes, all three components has reached optimum state (100 %).**

#### 4.3.4.b *Coupled System*

The coupled system combines the cell wall, membrane and protein repair models into one, while taking into account the influence they have on each other's state. This makes the coupled system a closer approximation of real events. The cell wall and membrane models are connected by having a common regulator, meaning that when RpoN changes expression it will affect both systems. The membrane and protein models are connected because the functional protein must be inserted before the membrane can reach its optimum state. The state of the membrane is therefore dependent on the protein as it must comprise a certain amount of proteins. The cell wall and protein models are not connected. The combination of the three models is showed in Figure 4.21.

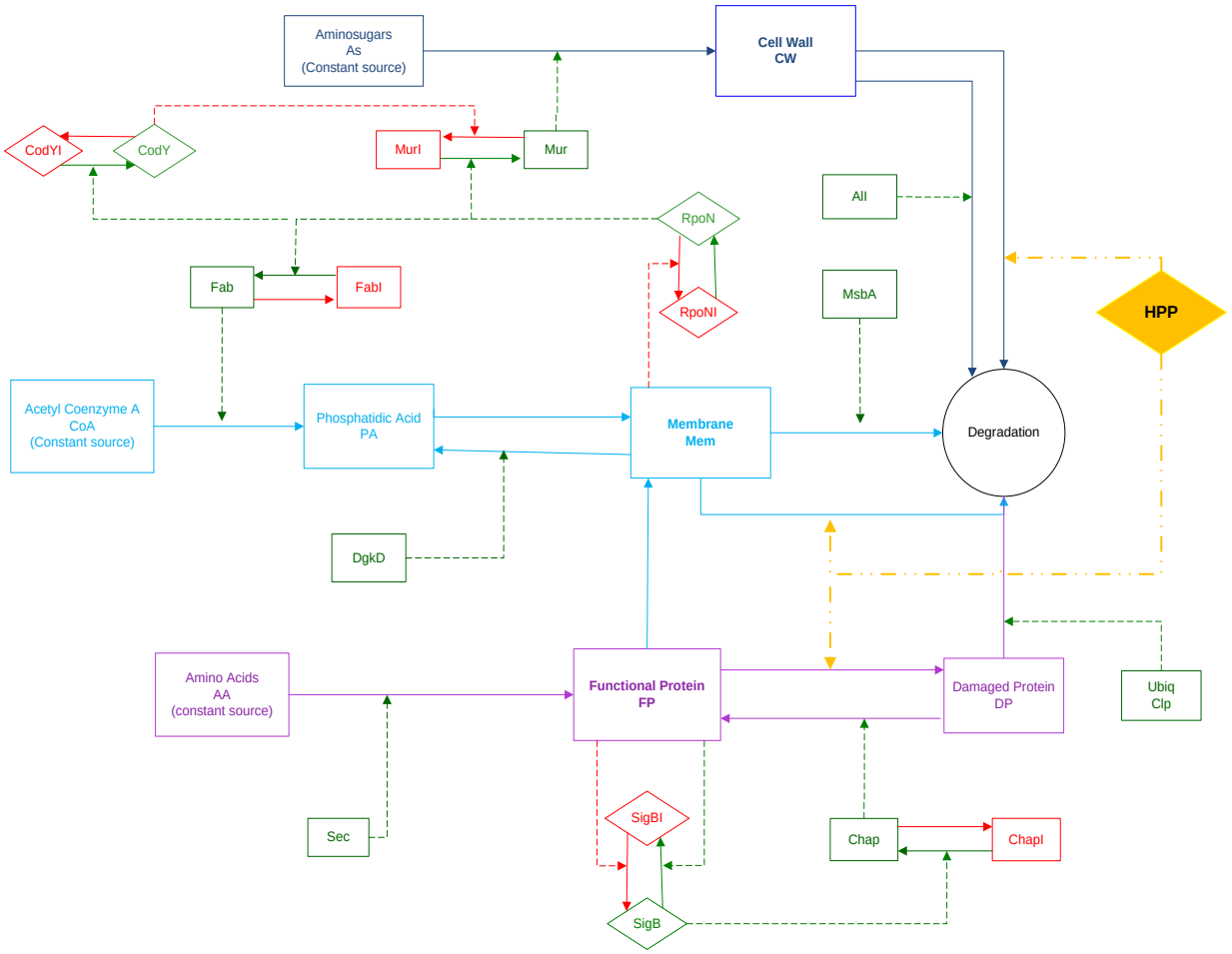



**Figure 4.21: The coupled envelope model used for simulations. Dark blue nodes belongs to cell wall system, light blue nodes belongs to membrane system and purple nodes belongs to protein system.**

The simulations for the envelope model are developed to show how the state of the cell wall, membrane and protein state are affected by a pressure of 400 MPa, but also by each other. Originally, CodY was not included as a co-regulator for the cell wall model. However, a lack of co-regulation results in an overshoot in the cell wall state. By introducing CodY, which controls overproduction by being a transcriptional repressor, the overshoot is avoided. The difference between co-regulation with CodY and lack of CodY regulation is showed in Figure 4.22.

The overshoot is a result of the cell wall being affected by the membrane from their common regulator, RpoN. RpoN regulates its activity according to the membrane state, so that when the membrane state is low, the RpoN signal is high. During the first 200 minutes of reparation, the membrane state is low, due to lack of functional proteins in its matrix. As long as the membrane cannot rejuvenate, RpoN will send elevated signals to the cell wall even though it repairs quite quickly. By introducing the CodY regulator, the cell wall state is not allowed to go over 100 % and stops repair activity when it reaches this level. In a real biological system, it would be a waste of energy to produce more cell wall than what is strictly necessary, so production must stop in time.

The difference appearing with and without CodY regulation, simulated by this model, reflects well the proper behaviour in nature. Co-regulation is often necessary for fine-tuning of signals, meaning that *in vivo* systems often are dependent of several regulators for one function. The fact that the developed model shows the same tendencies as the system it reflects, strengthens its authenticity.

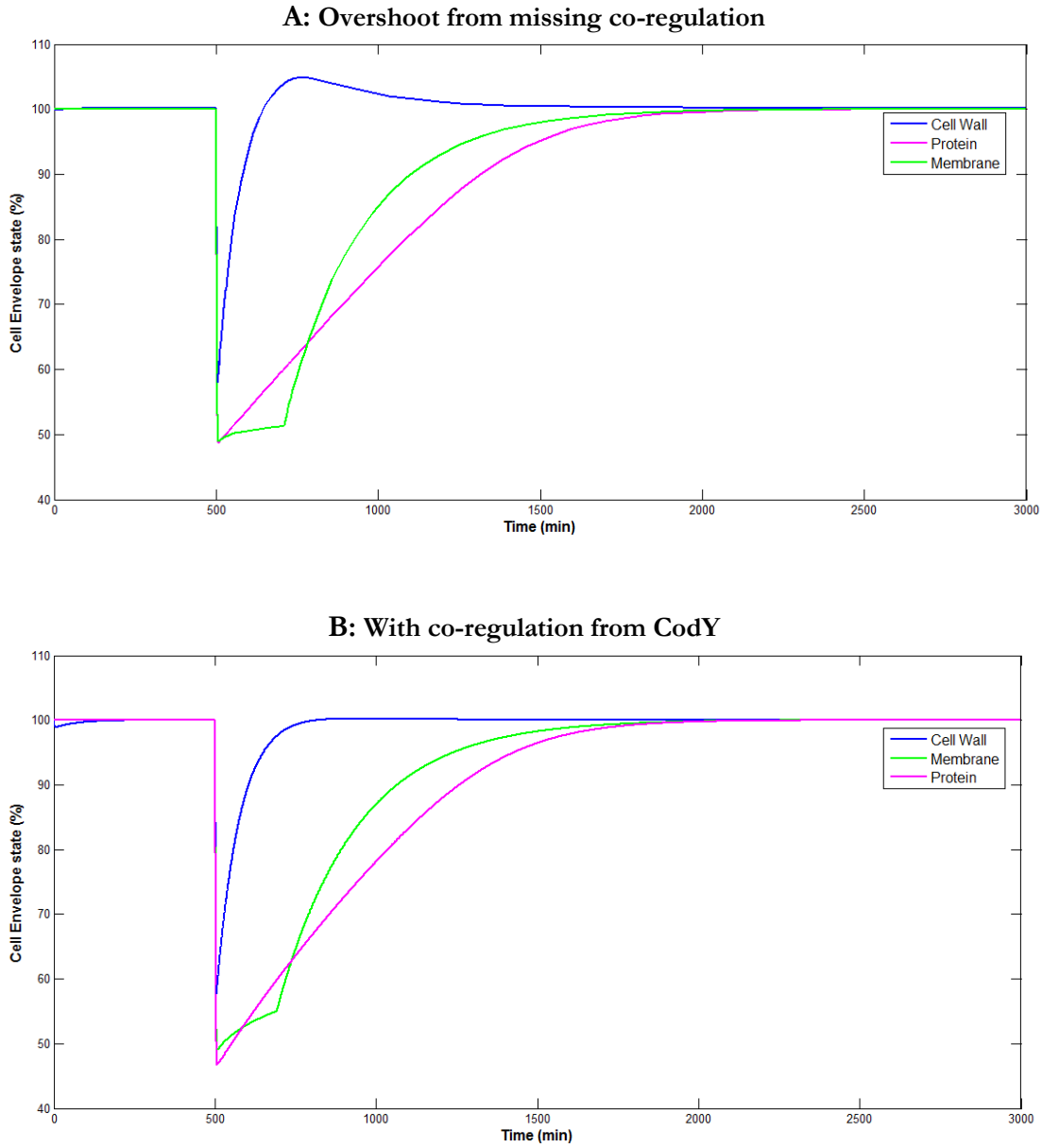



**Figure 4.22: Simulations of cell envelope repair with and without CodY, showing the importance of co-regulation. After 500 min, pressure of 400 MPa applied for 5 min.**

The cell envelope repair simulations shows that the start of reparation goes fast, seen by the initial steep curve in all of the three components being repaired. This shows that major leakages are quickly patched, but after the rough work is done, it takes more time to repair. This is probably because reparation is not that pressing anymore, allowing the bacteria to focus on other parts of the cell that

have a bigger need to be repaired. This is why the simulated curves levels out approximately after reaching a state over 90 %. The more the cell envelope is repaired, the more time it takes to continue repairing.

### 4.3.5 **Sensitivity Analysis**

The sensitivity analysis was initially applied on the coupled cell envelope model that has been described in the previous section. The sensitivity analysis made it possible to detect incorrect behaviours and necessary changes could be determined. The models were therefore modified and a second sensitivity analysis was done in order to investigate the correctness of the changes. The results are shown in section 4.3.5.c Analysis Results, to give the opportunity to compare the old and new version with each other.

#### 4.3.5.a ***Analysis of Original Model***

The three systems comprising the envelope repair model were systematically tested for their sensitivities. The subsequent response of the models were thereafter detected. Any change in rate constant that introduces a deviation from the original steady state, means that this variable has an impact on the homeostasis of the cell. Applying a perturbation on rate constant k1, gives the following sensitivity coefficients on the three models, found with Equation 3.4:

$$|S_{CW1}| = \frac{CWState_{(k_1)} - CWState_{(k_1+k_1*\Delta k_1)}}{k_1 * \Delta k_1} = \frac{100{,}0217 - 101{,}0219}{1{,}751 * 0{,}01} = |\mathbf{57, 121}|$$

$$|S_{Mem1}| = \frac{MemState_{(k_1)} - MemState_{(k_1+k_1*\Delta k_1)}}{k_1 * \Delta k_1} = \frac{100{,}0533 - 100{,}0533}{1{,}751 * 0{,}01} = |\mathbf{0}|$$

$$|S_{FP1}| = \frac{FPState_{(k_1)} - FPState_{(k_1+k_1*\Delta k_1)}}{k_1 * \Delta k_1} = \frac{100{,}00 - 100{,}00}{1{,}751 * 0{,}01} = |\mathbf{0}|$$

Such sensitivity result indicates that k1 has a significant impact on the cell wall model, but no impact on neither the membrane model, nor the protein model. Graphs showing the sensitivity coefficient for all the parameters in each system, are given from Figure 4.27 to Figure 4.32. Only the parameters creating a change in the systems are given. This means that the more rate constants being included in the graph, the more susceptible is the system to changes. Two different simulations were conducted on each system. One simulation gave the obtained steady state value of the model without any pressurization, while the other simulation gave the obtained steady state of the system after being exposed to pressure with a following pressure relief. Pressure exposure reduces the state index, meaning that the simulation starts at the point where the cell is in its recovery phase and must reach back to its optimum state.

From the sensitivity analysis, the cell wall model showed to be very sensitive to perturbations as it was significantly affected by 11 of the parameters. The protein model was very little susceptible to

perturbations by hardly being affected by two parameters. The resulting sensitivity of the membrane model was more expected. It was affected by 10 parameters, but only significantly sensitive to d2 and kR1. The membrane system is connected to both the cell wall and protein system and should therefore be easily influenced by perturbations. On the other hand, the path of influence in the envelope model is that proteins affect membrane state and membrane affects cell wall state. This means that the protein system is not affected by any of the other systems while the cell wall is directly or indirectly affected by all systems. In the protein model, it is the damaged protein (DP) node that is susceptible to degradation, while the functional protein (FP) node is more shielded to influences. This means that FP, which is the node used for sensitivity measurements, turns out to be very robust and it takes a lot to change its steady state. This lack of sensitivity is not correct to what the protein system would be in reality and should therefore be changed. The Cell wall model is the simplest system, reflected by the few rate constants included in this system, and easily influenced by changes in the other models, making its steady state very susceptible to changes. This is not correct concerning the robustness that the cell wall should have in reality. The lack of robustness should therefore be changed to make the model more authentic.

#### 4.3.5.b ***New Version of Model***

From the sensitivity analysis, it was discovered that the functional protein node was not subjected to any natural degradation in the form of protein turnover. The only way that the state of functional protein could be reduced in the created model was through high pressure. This is not the correct biological phenomenon as there will always occur turnover of proteins. A degradation/recycling reaction was therefore added to the protein model, both in the original model and the simplified Matlab system. The modified protein repair models are shown in Figure 4.23.

The cell wall model was changed to a more robust system by changing the way of influence on RpoN. In the new model, the activity of RpoN is regulated by the state of the cell wall instead of the membrane state. It is assumed that the cell wall is resealed before the membrane. By resealing the cell wall first, the immediate leakage emergency is eliminated and the time required for repairing the membrane and proteins can be used without resulting in fatal leakage consequences. An effect of changing the component that regulates the activity of RpoN was that CodY regulation was not needed anymore. In the old model, the cell wall reached optimum state before the membrane, but the activity of RpoN did not go down before the membrane reached optimum state. This caused the overshoot in the cell wall repair. In the new model, there is no overshoot in the membrane repair as the activity of the RpoN regulator has reduced before the membrane state is back to optimum. Meaning that there is no need for co-regulation of repair activity. Nevertheless, the co-regulation proved to be necessary in the original model, showing that it can be important in biological systems. It was not crucial in this model, but this is most likely due to the simplifications that were done to create it, making some reactions that are important in the real life redundant in a simplified model. The new simulation of the cell envelope behaviour is provided in Figure 4.24.

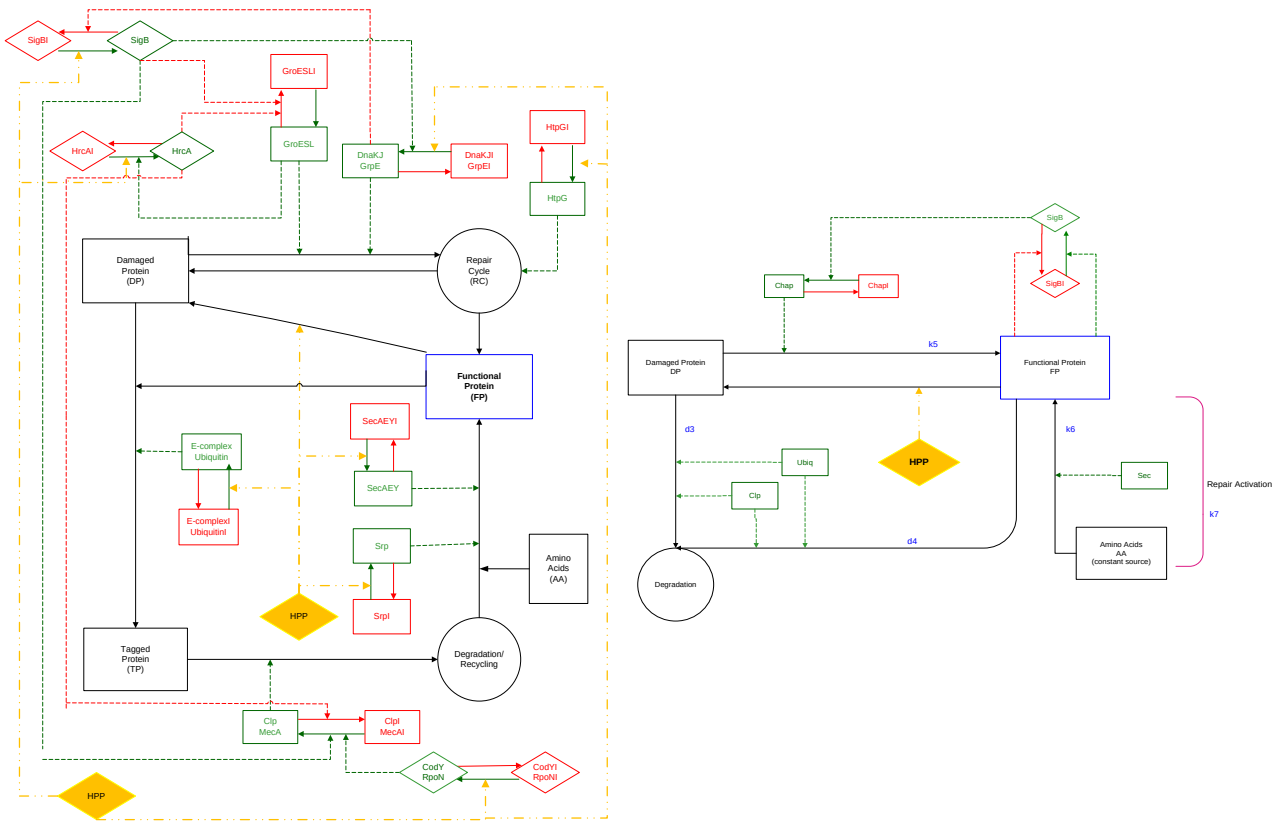



**Figure 4.23: Modification of the protein repair models by the addition of a degradation reaction. Left: The whole model. Right: The simplified model.**

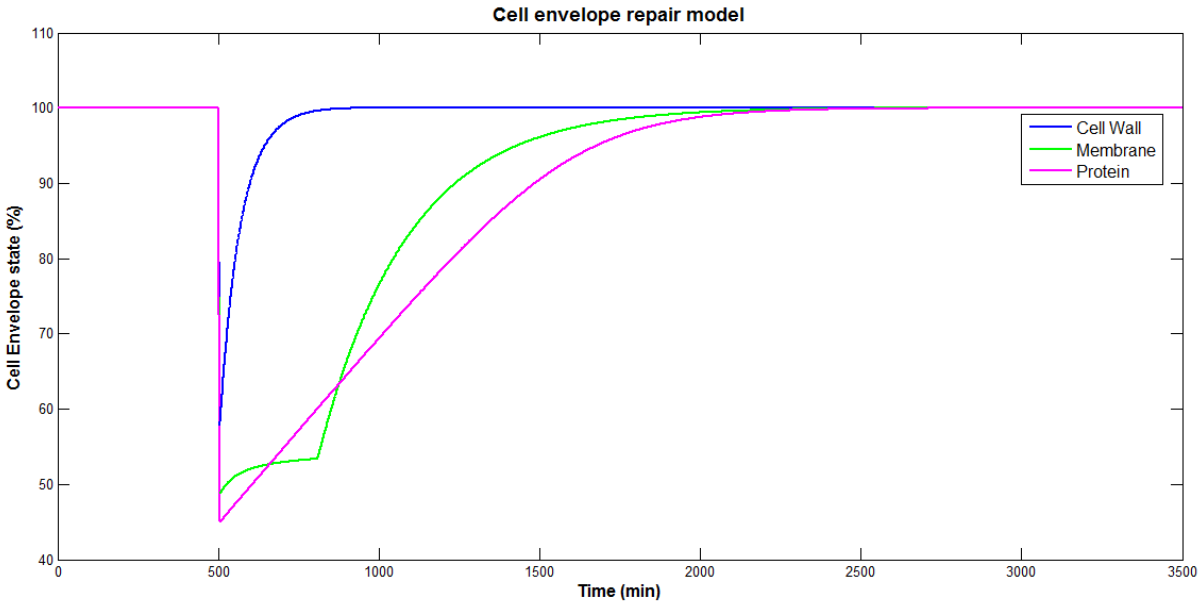



**Figure 4.24: Cell envelope simulation of the modified model. Pressure applied after 500 minutes and relieved after 505 minutes.**

As can be seen from the simulation of the new model, no significant difference has appeared from the changes applied on the model. However, the sensitivity analysis can reveal that changes has been done. These are discussed under the next section, 4.5.5.c Analysis Results.

### 4.3.5.c *Analysis Results*

The independent variables used for analysis were the rate constants for the main reactions and for the enzyme reactions. These constants are given in Figure 4.25 and Figure 4.26.

**Parameters in original model:**

| Rate constant (main rx) | In reacion | Value in system |
|---|---|---|
| k1 | As-->CW | 1.751 |
| d1 | CW-->Deg | 0.014 |
| k2 | CoA-->PA | 0.0785 |
| k3 | PA-->Mem | 0.0382 |
| k4 | Mem-->PA | 0.0012 |
| d2 | Mem-->Deg | 0.003706 |
| k5 | DP-->FP | 0.0005 |
| k6 | DP-->FP | 0.06 |
| k7 | RepAct-->Deg | 1.1 |
| d3 | DP-->Deg | 0.0036 |

**Parameters in modified model:**

| Rate constant (main rx) | In reaction | Value in system |
|---|---|---|
| k1 | As-->CW | 2.944 |
| d1 | CW-->Deg | 0.0139 |
| k2 | CoA-->PA | 0.0785 |
| k3 | PA-->Mem | 0.0382 |
| k4 | Mem-->PA | 0.0012 |
| d2 | Mem-->Deg | 0.003706 |
| k5 | DP-->FP | 0.005 |
| k6 | DP-->FP | 0.4 |
| k7 | RepAct-->Deg | 8.1 |
| d3 | DP-->Deg | 0.36 |
| d4 | FP-->Deg | 0.000009 |

**Figure 4.25: List of rate constants in the main reactions. Numbers marked in green represents the constants with changed value in the new model.**

**Parameters in original model:**

| Rate constant (enzyme rx) | In reaction | Value in system |
|---|---|---|
| kM1 | MurI-->Mur | 10 |
| kM2 | Mur-->MurI | 0.5 |
| kC1 | CodYI-->CodY | 1 |
| kC2 | CodY-->CodYI | 0.1 |
| kR1 | RpoNI-->RpoN | 1.08 |
| kR2 | RpoN-->RponI | 0.1 |
| kF1 | FabI-->Fab | 10 |
| kF2 | Fab-->FabI | 0.5 |
| kH1 | ChapI-->Chap | 0.4 |
| kH2 | Chap-->ChapI | 0.1 |
| kS1 | SigBI-->SigB | 5 |
| kS2 | SigB-->SigBI | 0.5 |

**Parameters in modified model:**

| Rate constant (enzyme rx) | In reaction | Value in system |
|---|---|---|
| kM1 | MurI-->Mur | 10 |
| kM2 | Mur-->MurI | 0.5 |
| kR1 | RpoNI-->RpoN | 1.08 |
| kR2 | RpoN-->RponI | 0.1 |
| kF1 | FabI-->Fab | 10 |
| kF2 | Fab-->FabI | 0.5 |
| kH1 | ChapI-->Chap | 0.04 |
| kH2 | Chap-->ChapI | 1 |
| kS1 | SigBI-->SigB | 5 |
| kS2 | SigB-->SigBI | 0.5 |

**Figure 4.26: List of enzymatic rate constants.**

In the original model there were in total 10 rate constants for the main reactions and 12 rate constants for the enzyme reactions. The modified model increased to 11 rate constants for the main reaction, by the addition of the protein degradation constant (d4). The enzyme reactions decreased to 10 rate constants, by the elimination of CodY regulation.

The sensitivity coefficients have been normalized relative to each other by dividing them with the highest coefficient value and then multiplying by 1000. The highest possible value is therefore 1000. The sensitivity coefficients before normalization are given in Appendix 4. Because of large deviations between values, the resulting coefficients are presented in log scale. Numbers between 1 and 1000 point upwards while numbers between 0 and 1 point downwards. Only parameters inducing a change is presented in the respective models.

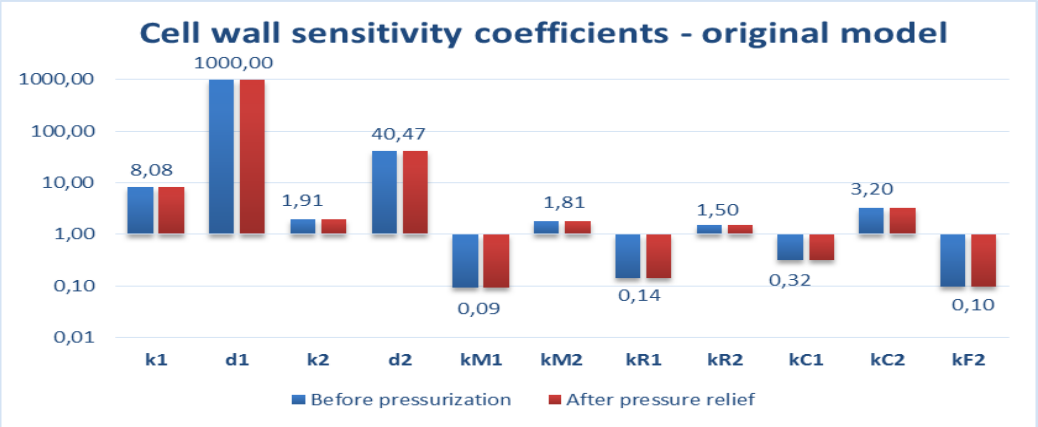



**Figure 4.27: Sensitivity coefficients for parameters in the original cell wall model. There are 11 influencing variables where rate constant d1 has the most significant impact.**

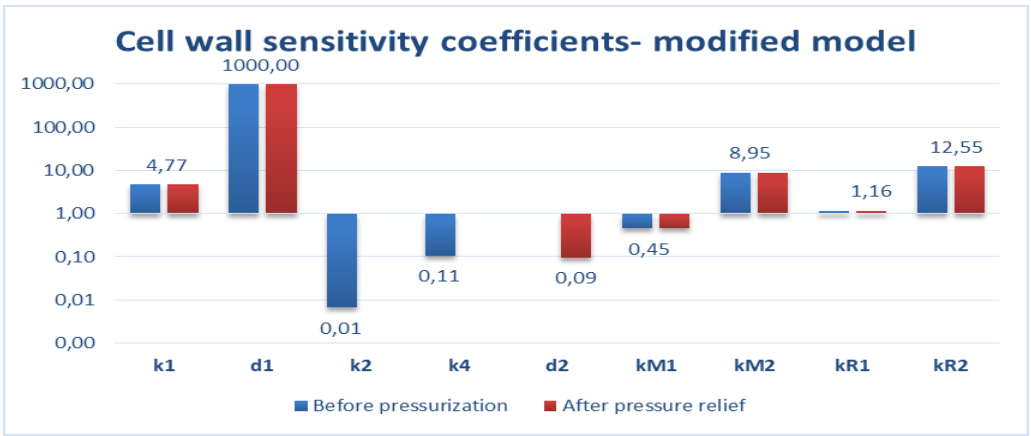



**Figure 4.28: Sensitivity coefficients for parameters in the modified cell wall model. There are 9 influencing variables, but not in both conditions. Rate constant d1 has the most significant impact.**

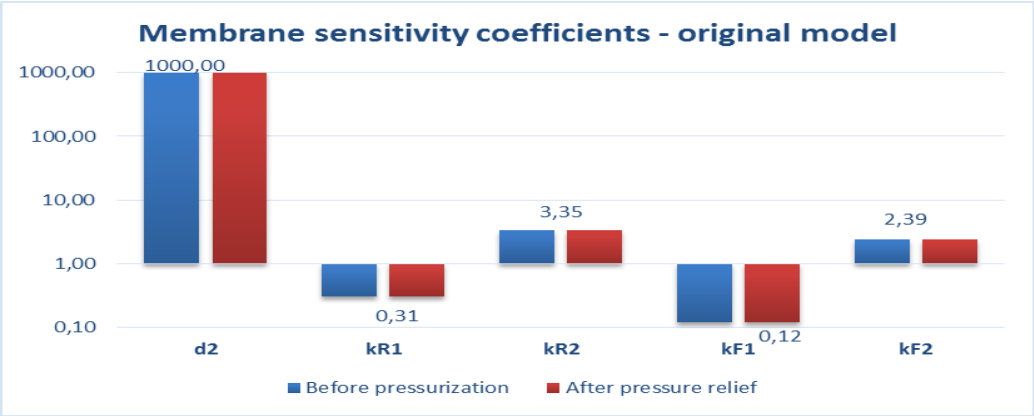



**Figure 4.29: Sensitivity coefficients for parameters in the original membrane model. There are 5 influencing variables, but not in both conditions. Rate constant d2 has the most significant impact.**

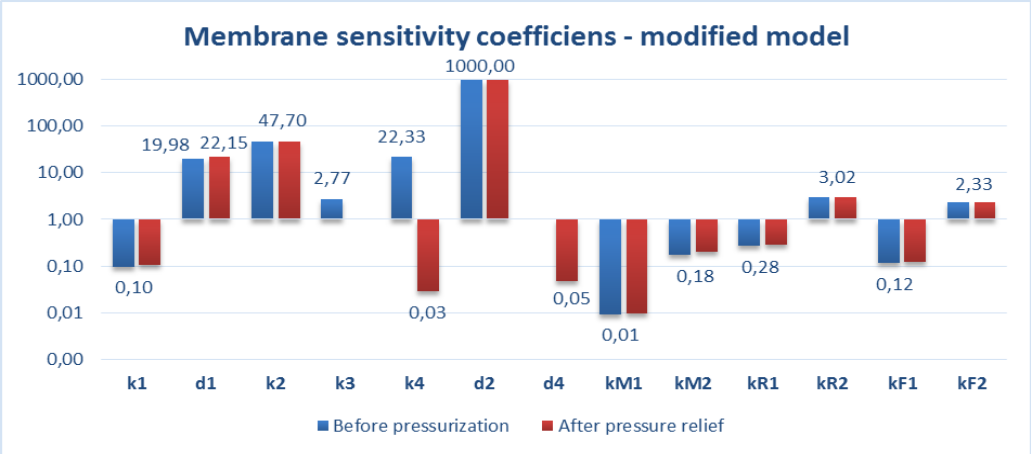



**Figure 4.30: Sensitivity coefficients for parameters in the modified membrane model. There are 13 influencing variables, but not in both conditions. Rate constant d2 has the most significant impact.**

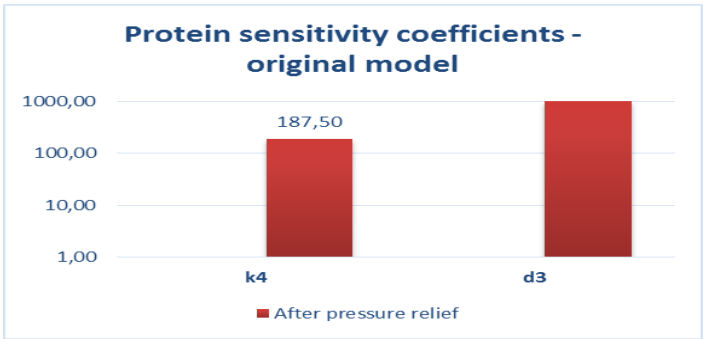



**Figure 4.31: Sensitivity coefficients for parameters in the original protein model. There are 2 influencing variables, but only after pressure relief. Rate constant d3 has the most significant impact.**

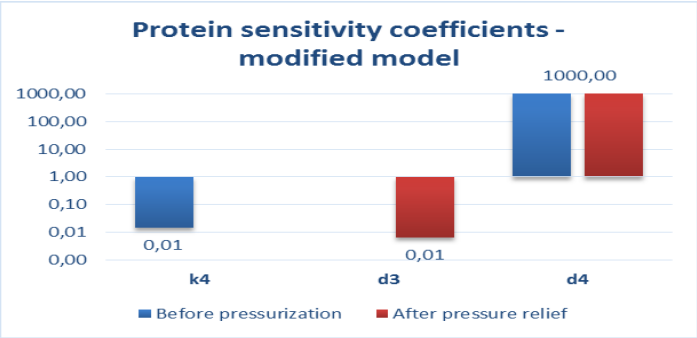



**Figure 4.32: Sensitivity coefficients for parameters in the modified protein model. There are 3 influencing variables, but not in both conditions. Rate constant d4 has the most significant impact.**

The sensitivity coefficients have been normalized with respect to the most significant rate constant as the coefficients do not have an absolute value. The sensitivity coefficients only provide meaningful data when they are compared relative to each other. The highest sensitivity coefficient in each model (except for the original protein model) had a noteworthy high value compared to the other coefficients. The highest coefficient could have a value of several thousand, while the rest could have values around 10 or lower. The coefficients with a very low value should already be regarded upon as not significant, but when they were normalized with respect to a significantly higher number, many of the constants appeared very low and were disregarded. This might give a different result than if the numbers were not normalized, but as long as there is one value that outcompete all the others, the low coefficients will always appear low.

The resulting sensitivity coefficients showed significantly different outcome between the old and new model. In the modified model, the cell wall appeared more robust by being influenced by less variables as well as getting lower sensitivity coefficient values from these variables. This is mainly because the cell wall is not directly affected by the membrane state in the new model, meaning that the sensitivity towards the membrane parameters are significantly reduced. In addition, removal of the co-regulator, CodY, also reduced the number of influencing variables. By removing the rate constants kC1 and kC2, the number of independent variables affecting the cell wall reduced from 11 to 9. The protein model on the other hand, became more sensitive in the new model. The newly introduced rate constant, d4, had a significant impact on the protein state, resulting in an increase from 2 to 3 influencing parameters. The sensitivity of the membrane also changed, as it went from being only affected by the protein model to be sensitive towards both systems. This resulted in the model being affected by more variables, increasing from 5 to 13 influencing rate constants with higher values. The new interconnection setup seems more reasonable since it is assumed that, *in vivo,* both the cell wall and the proteins are more independent in their reparation, while the membrane relies on having functional proteins in its structure. Presumably, it is also the initial resealing of the cell wall that should determine how the bacteria cell prioritize energy used on repair activity. Since the immediate action must be to regain the integrity of the cell, the repair of membrane relies on the capability to repair the cell wall.

The sensitivity analysis made it possible to find details in the created repair model that did not correspond well enough with the system it was reflecting. This means that such analysis is a good tool to test out the quality of a developed model. There are still much room for improvements in the repair model as it is still lacks accuracy. The protein model is not sensitive enough to influences; The SigB regulator with the Chap complex should have more impact on the protein state than they currently do. Regulating activity from RpoN or CodY should also be included in the protein model. The enzyme complexes Mur and Fab, should be more sensitive towards their regulators, as they were not respondent enough. These are only a handful of the improvements that should be made to the repair model, but the sensitivity analysis showed how the needed improvements can be found. Many more creation cycles of the mathematical model, as shown in Figure 3.4, should be conducted before the repair model can have any practical value. Nevertheless, this thesis shows the methodology of how the procedure can be executed along with a suggestion of an initial model that can be further modified.

As the mathematical model builds upon so many assumptions and uncertainties, a good recommendation for possible target components in the genetic network is difficult to provide. Having this said, the rate constants associated with degradation had undeniably the highest influence in all of the three systems. They are probably not pressure specific since the mechanisms used to explain the reparation process is taken from natural synthesis and turnover. Though they might not be pressure specific, they may be the key elements for recovery after pressure induced damage. These elements are responsible for degradation or recycling of damaged components to be replaced by functional ones. Without this mechanism, un-functional parts may not be removed and the pressure induced holes cannot be resealed. The degradation/recycling responsible components found in this thesis are the autolysins (lmo0717 and lmo1521) in the cell wall and the transmembrane proteins MsbA, LplT and TscA, in the membrane. There are many components involved in degradation of the un-reparable proteins. The ubiquitin ligases (lmo2190, lmo1374, lmo1371 and lmo1052-5) as well as the peptidases (lmo0232, lmo0997, lmo1138, lmo1268, lmo2206 and lmo1278-90) all have a role in the removal of un-functional proteins. In addition, the enzymes responsible for natural turnover of proteins should be found, as the system is very sensitive to the degradation rate constant, d4. Not many of the degradation responsible components in the protein system has shown to be much up-regulated in the experiment from Bowman, Claudio et al. (2007), meaning that follow-up work should be done in order to investigate their activity during pressure exposure. In general, all the proteins mentioned here should be examined for their importance during pressure stress recovery and most likely there are many more components responsible for degradation in the cell envelope, then enlisted here. The regulators also have an important role in bacteria response to pressure and should be examined further, in order to find out about their specific importance for repair of pressure induced injuries.

Magnitude of pressure, pressurization time and temperature, type of bacteria, cell growth phase, suspending media and the presence of microbial substances have been shown to influence the efficacy of HPP. With so many influencing factors, a versatile mathematical model that can simulate

the behaviour of a pressurized bacteria cell is almost impossible to do. That requires a very complex model that can take into account every possible factor that may influence the result. The mathematical model created in this thesis is limited to some defined conditions and cannot fully represent the behaviour of a bacteria cell that, as an example, is pressurized for 10 minutes instead of 5 minutes. In addition, the recovery time is based on very vague data and can probably not even completely simulate the correct behaviour for the given conditions in this thesis. The mathematical models needs modifications and correct data of the kinetics before they can be used. The state index being used to measure the state of the cell envelope should also be improved. A self-defined index was applied in this thesis, but with the lack of a proper measurable value, this was the preferred scheme.

Though there may be much room for improvement in this study, the methodology is a good basis for further work. By doing the experiments, actual damages can be detected and a relationship between pressure level and cell envelope injury can be found. Experiments detects if envelopes are damaged and storage will say something about the dynamics of repair. The proper bacterium*, L. monocytogenes* should however be used as no substitutes can give the exact details. After that, the relationship between envelope damage and activated repair mechanisms through mathematical modelling can be found.

# 5 Concluding Remarks

This report has given an introduction to the cell envelope repair in bacteria after exposure to high pressure processing, an issue given more concern after the development of the high pressure processing technique. A brief introduction to the cell envelope repair system has been given, where possible repair mechanisms used for reparation of it have been introduced. Experimental procedures used to investigate and quantify the extent of damage on pressurized cells have been presented and their results are given and discussed. Lastly, mathematical models with simulations based on the developed models are provided where the results are presented and discussed. The combination of this work results in a hypothetic repair model that shows how the state of the cell envelope is affected by high pressure and what genes the bacterium activates in order to regain its desired state, being a cell envelope with full integrity.

A hypothesis was made, saying that pressure induced leakages in the cell envelope is one of the main reasons for cell death. Not particularly because of the created holes itself, but because of the possible loss of vital components from the bacterium, as well as the entering of undesired components. The developed repair model therefore dealt with the resealing of holes in the cell envelope. The developed hypothesis said that the cell wall and membrane, being the two main structures in the cell envelope, were repaired by replacement of damaged components with new and functional components. These mechanisms were assumed to happen through the same pathway as the natural turnover and synthesis of peptidoglycan and phosphatidic acid. The proteins, which is a large constituent in the cell membrane, were assumed to either become un-functional or squeezed out of the membrane structure, as a result of pressurization. The repair hypothesis of the proteins involved a repair cycle where they either would become functional again or go through a degradation or recycling process. Functional proteins, either repaired or newly synthesised, would thereafter be inserted where they were missing.

The experiments consisted of two different methods: staining and recovery. The staining experiment verified the actual occurrence of pressure induced leakages in the cell envelope and the recovery experiment detected the viability of bacteria throughout a period of recovery time. From the staining experiment, it was found that leakages do occur. Visualized by the PMA stain that only can enter a leaking cell. However, fast resealing capability of the cell envelope was detected by the difference between the addition of stain before and after pressurization. This means that the cell is able to reseal during a couple of minutes, given mild enough processing conditions. The cell recovery experiment did not give enough valuable information to make it suitable for use with great confidence. However, the results could indicate that it may take almost 2 days for a typical bacterium to recover and start growing again after pressurization of the given conditions.

The mathematical models were made to simulate the repair dynamics of a pressure damaged bacteria cell. The models were based on the findings from the pathway analysis and high pressure experiments. The conditions that the model was simulating was pressurized fish soup, contaminated by *L. monocytogenes*. The process conditions were 5 minutes exposure time, 20 °C of processing

temperature and a pressure level of mainly 400 MPa, though this could vary. Recovery temperature was 4 °C. The mathematical model showed to correspond with the real life system, though many improvements of its accuracy proved to be needed. The areas of improvement became especially apparent from the stability analysis, showing that the model should go through more cycles of refining before it can be considered as a practical mathematical model. The exact features of repair cannot be generalized, as varied treatment conditions will give too different responses. However, the results indicates that repair of cell wall and membrane is activated immediately, while the protein repair is subsequently activated.

A suggestion of key elements in the repair system is given. These may be the genes responsible for degradation or recycling of damaged components in the cell envelope. The degradation responsible protein are the autolysins, some specific transmembrane proteins, peptidases and the ubiquitin ligases. The key elements can be used for further studies on the bacterium's resilience towards high pressure. However, these elements may not be pressure specific and this suggestion is based upon many uncertainties.

# 6 Future Work

The developed cell envelope repair model has increased the understanding of the recovery process for pressure exposed *L. monocytogenes*, but more work needs to be done to the model in order to obtain more precise answers. There are many possibilities for further work on this thesis, both when it comes to improvement areas and follow-up work.

Genetic analysis with the use of PCR (polymerase chain reaction), would make it possible to closer investigate the genes determined to be involved in the cell envelope repair. More accurate data of the specific genes are needed in order to provide the proper protein interaction system for cell envelope repair. Flow cytometry experiments should also be conducted to get more insight on how the single cell actually behaves. The obtainable insight from such experiments would give a major contribution in the investigation of the damage effects that pressure has on a bacteria and could contribute to the creation of a much more accurate repair model.

More experiments should be done in order to verify the PMA staining results. As the conducted staining experiment only involved the addition of stain after pressurization, the method of adding stain prior to pressurization should also be done. This is because the assumption that more cells would be stained with the latter method needs to be verified. More pressure levels should also be used in conjunction with the staining experiment and especially a higher level than 400 MPa. The reason for this is because intensified conditions can give a more efficient outcome of leakage occurrence. The second experiment, involving cell recovery detection, have much room for improvement. The obtainable results did not give any valuable information about the time consumption of cell envelope repair. First of all, the measurement intervals should be much shorter, by investigating the cell growth on a daily rate at minimum instead of a weekly rate. Flow cytometry should also be used in combination with these measurements to give the possibility of visualizing the state of the cells throughout the recovery period. There is a high chance that the obtainable results from such an experimental procedure would be very valuable in the research on cell repair and especially the dynamics of the repair. Lastly it must be emphasized that all these experiments should be conducted on the bacteria of interest, *L. monocytogenes*. Even though *L. innocua* and L. *monocytogenes* have many similarities by belonging in the same genus, a substitution will never be as accurate as using the proper specie.

As the mathematical model created in this thesis can be regarded upon as only a start of what the model can be, there is naturally further work that can be done to it. The envelope repair model could be more complex so that all aspects of the repair mechanism is included. As the biological system involves so many interactions, pathways and components, the simplifications done in this thesis are probably too many to make the model realistic enough. A complex system is particularly necessary when it comes to targeting of key components that are crucial for high pressure recovery. If the model is too simple, the real sensitivity a system has to a component may not be detected. As an example, if one component shows to be very important in the repair pathway it would not have such a great effect to inhibit it if there is a backup protein that can do the exact same job. It is not only

the components that needs to be clarified, the proper parameters should also be known. In the model development, the parameters have been estimated by assigning them values that gives the desired response, but the model would be much more accurate if the proper values were known and could be used. However, such limitations in the mathematical model mainly comes from the incomplete knowledge about the biological system. The whole system with all its components should be more sensitive to perturbations and many of the components should be more responsive to each other. The developed model should go through more refining cycles with tuning of parameters, in order to become a practical model.

A suggestion of key elements in the repair system was given. There were however many uncertainties around this recommendation, meaning that more research should be done in order to verify the assumption. More theoretical foundation is needed to verify the proposal of key elements. Experiments that investigates their actual importance, for example by gene knock-outs, would be particularly valuable.

The main objective of this thesis was to show the method of how simulations can be used in order to find out more about bacteria repair. Therefore, it is encouraged to continue this work in order to make the models good enough for practical use. The work has been started on, but if the project is continued, a new approach in the research on biological systems may be developed and can potentially contribute to more knowledge about the unknown systems in nature. By being such an unexplored field, the results from this thesis are based on a lot of uncertainties and there is obviously a lot of improvements needed. However, this thesis introduces a suggestion of how this work can be done and can hopefully help as a guidance for how to start and continue such work.

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# 8 Appendix

## 8.1 Appendix A: Repair pathway models with references

The following repair pathways are the same models as provided in Results and Discussion. These are however enlarged for better overview and with references included. The references are numbered and gives the source of each connection in the pathways. If there is a question mark behind a reference, it means that this source has not fully proved the actual occurrence, but given an indication that this connection might exist.

### 8.1.1 **Model References**

The numbered pathways represents the following references:
[1] (Bowman, Claudio et al. 2007)
[2] (STRING 2013)
[3] (Heijenoort 2007)
[4] (Sieger, Schubert et al. 2013)
[5] (Vicente, Rico et al. 2006)
[6] (KEGG 2014)
[7] (Barton 2005)
[8] (Gindsburg 2002)
[9] (Zhang and Rock 2008)
[10] (Ha, Johnson et al. 1999)
[11] (Chaturongakul, Raengpradub et al. 2008)
[12] (Chaturongakul, Raengpradub et al. 2010)
[13] (Kerrigan 2011)
[14] (Maillard, Chan et al. 2000)
[15] (Qian 2009)
[16] (Lodish, Berk et al. 2004)
[17] (Kampinga and Craig 2010)

### 8.1.2 Cell Wall Repair Model

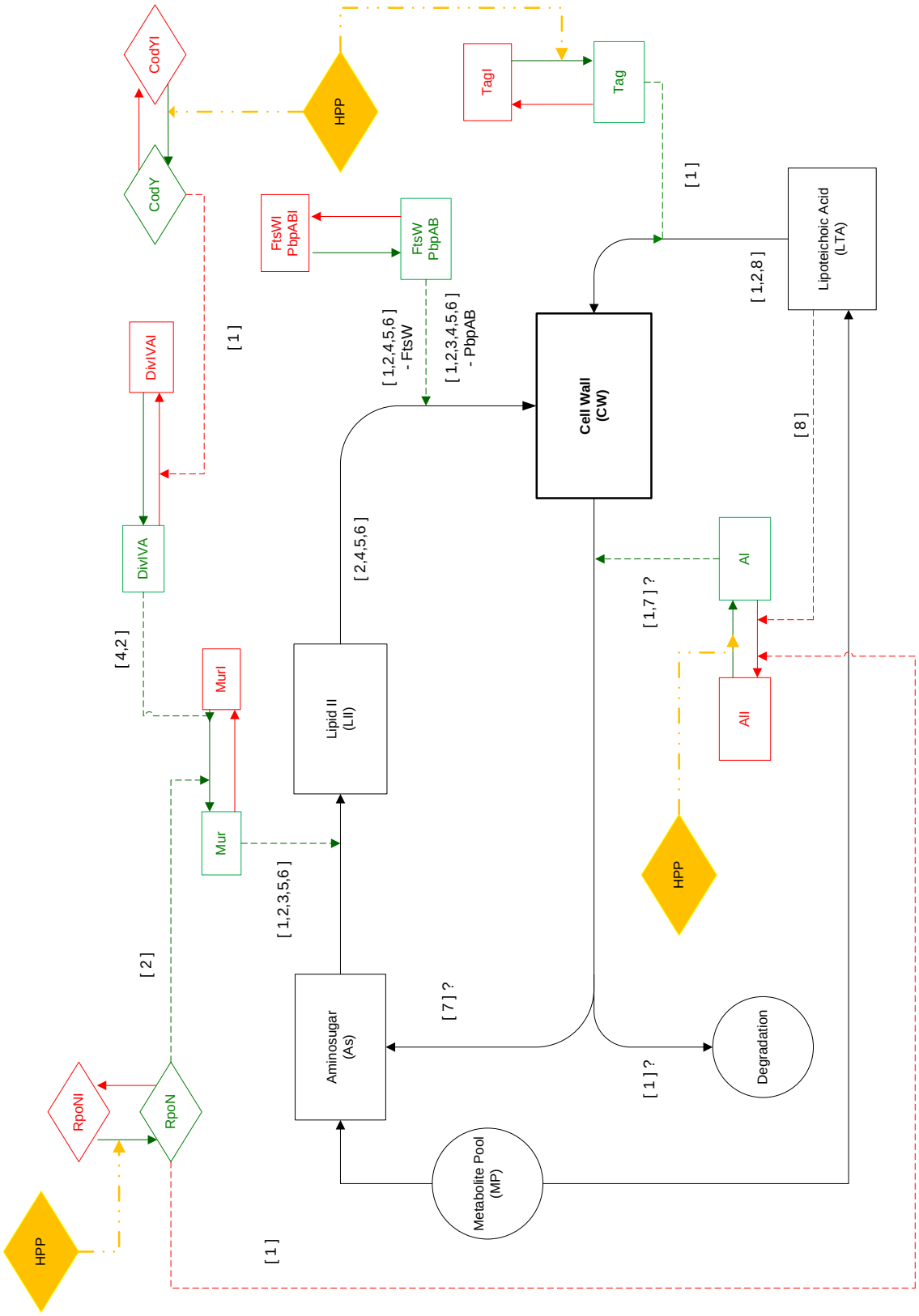


**Figure 8.1: Cell wall repair model with references**

### 8.1.3 Membrane Repair Model

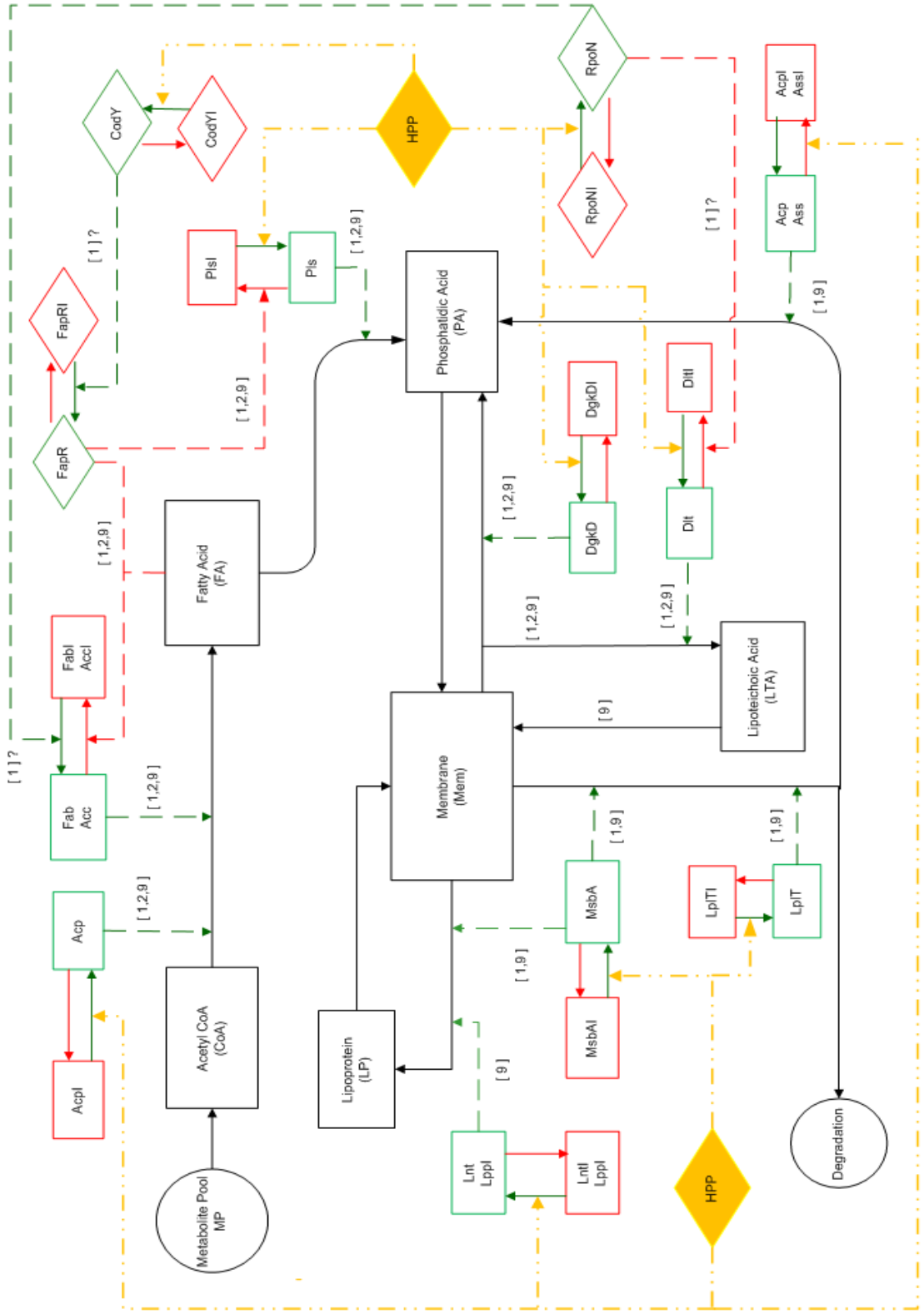


**Figure 8.2: Membrane repair model with references**

### 8.1.4 **Protein Repair Model**

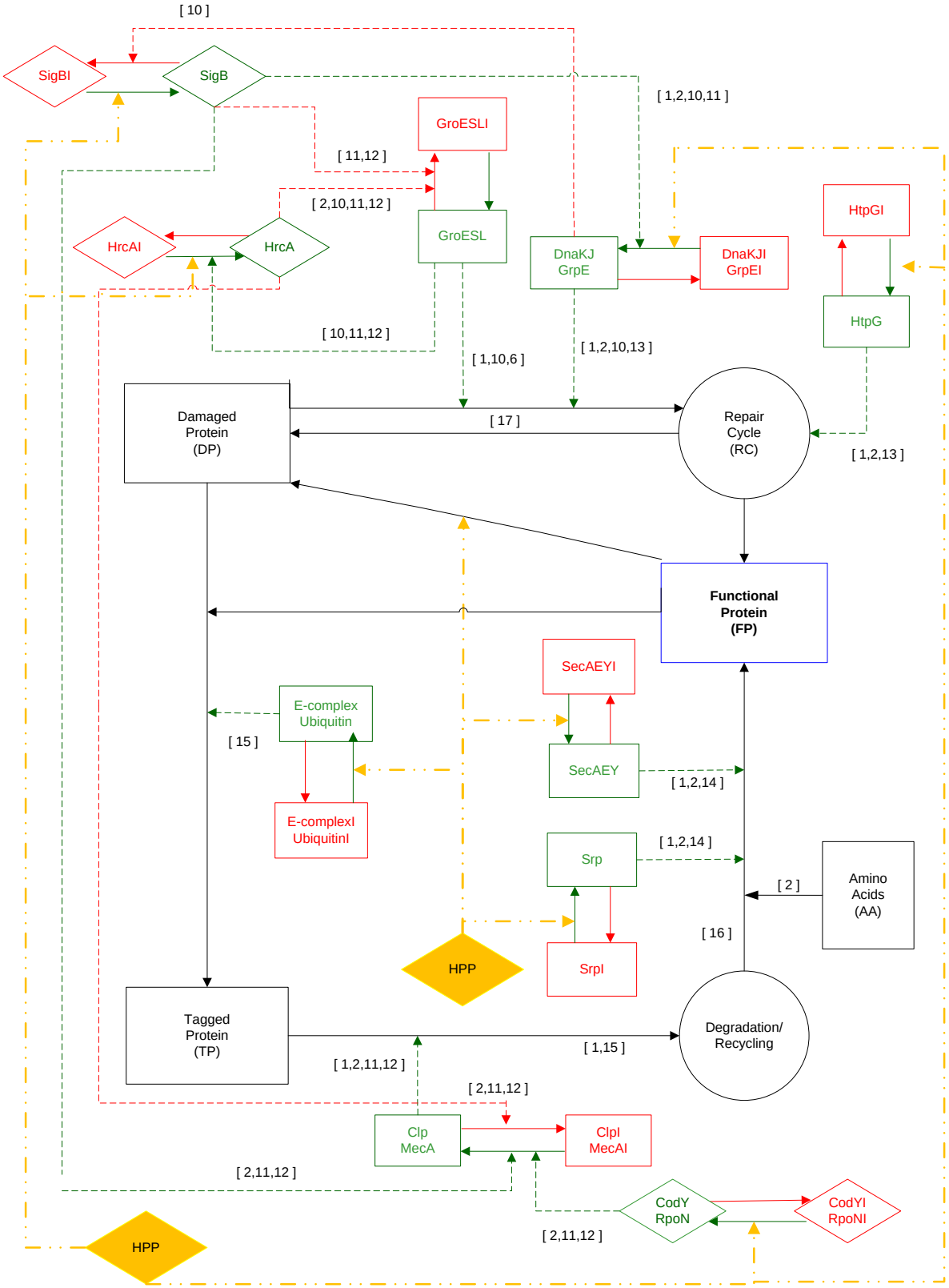


**Figure 8.3: Protein repair model with references**

### 8.1.5 Envelope Repair Model

The envelope repair model comprises the cell wall, membrane and protein repair model. References are therefore not necessary in this model, but it is provided in a bigger format for overview. The extra degradation reaction for the functional protein is also included.

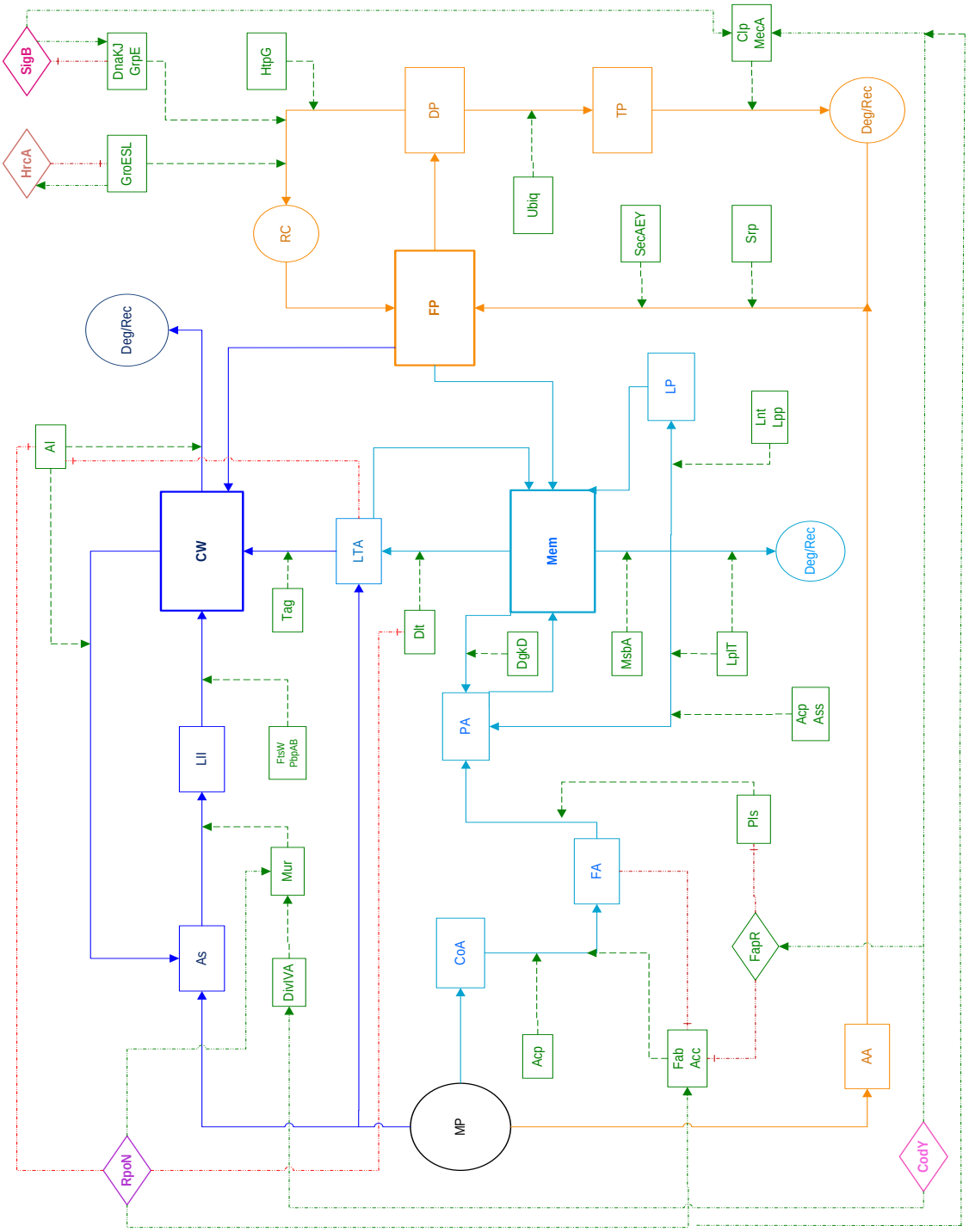


**Figure 8.4: Cell envelope repair model with references**

## 8.2 Appendix B: Microscope images from staining experiment

During the PMA staining experiment, 9 samples were made:
3 replicates of pressurized-stained cells, 1 untreated-stained sample, 1 untreated-unstained sample, 3 replicates of pressurized-unstained cells and 1 sample of heat treated-stained cells.
All representative pictures from these samples are provided in this chapter, Appendix 2.
The order of pictures on all samples are as follows:
Left picture: both Hoechst 33342 stained cells (purple) and PMA stained cells (red). Middle picture: cells stained only with Hoechst 33342 (purple). Right picture: cells stained only with PMA (red).

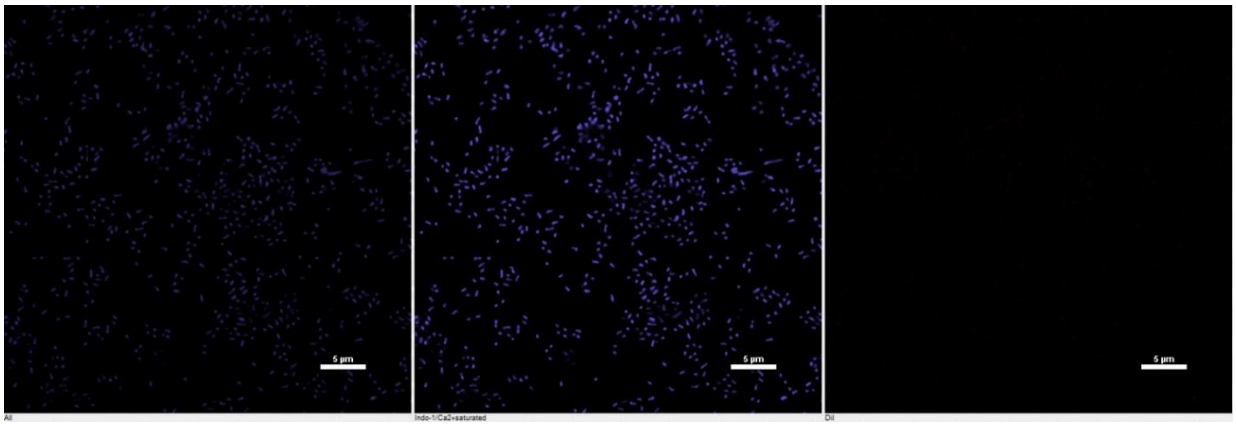


**Figure 8.5: Pressurized sample, unstained (control)**

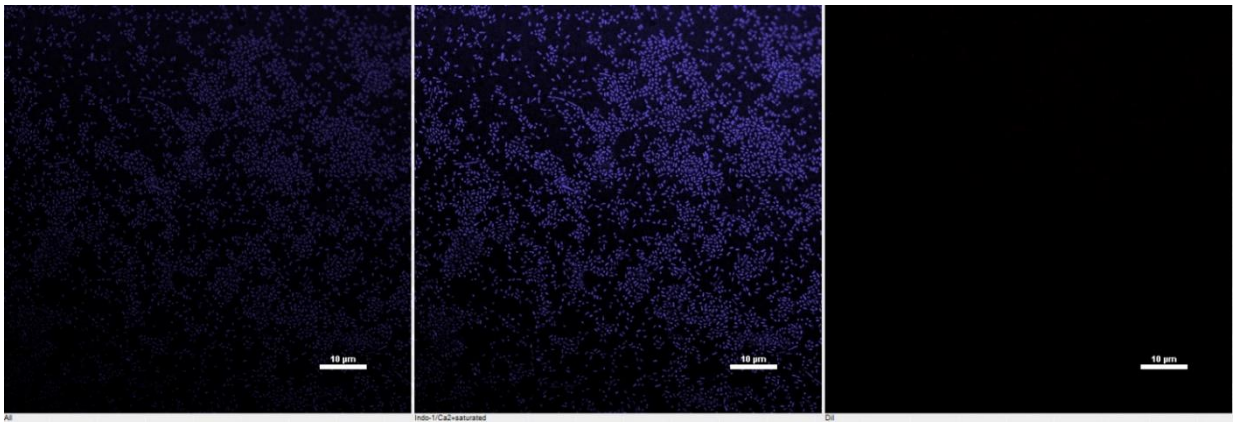


**Figure 8.6: Unstained sample, unstained (control)**

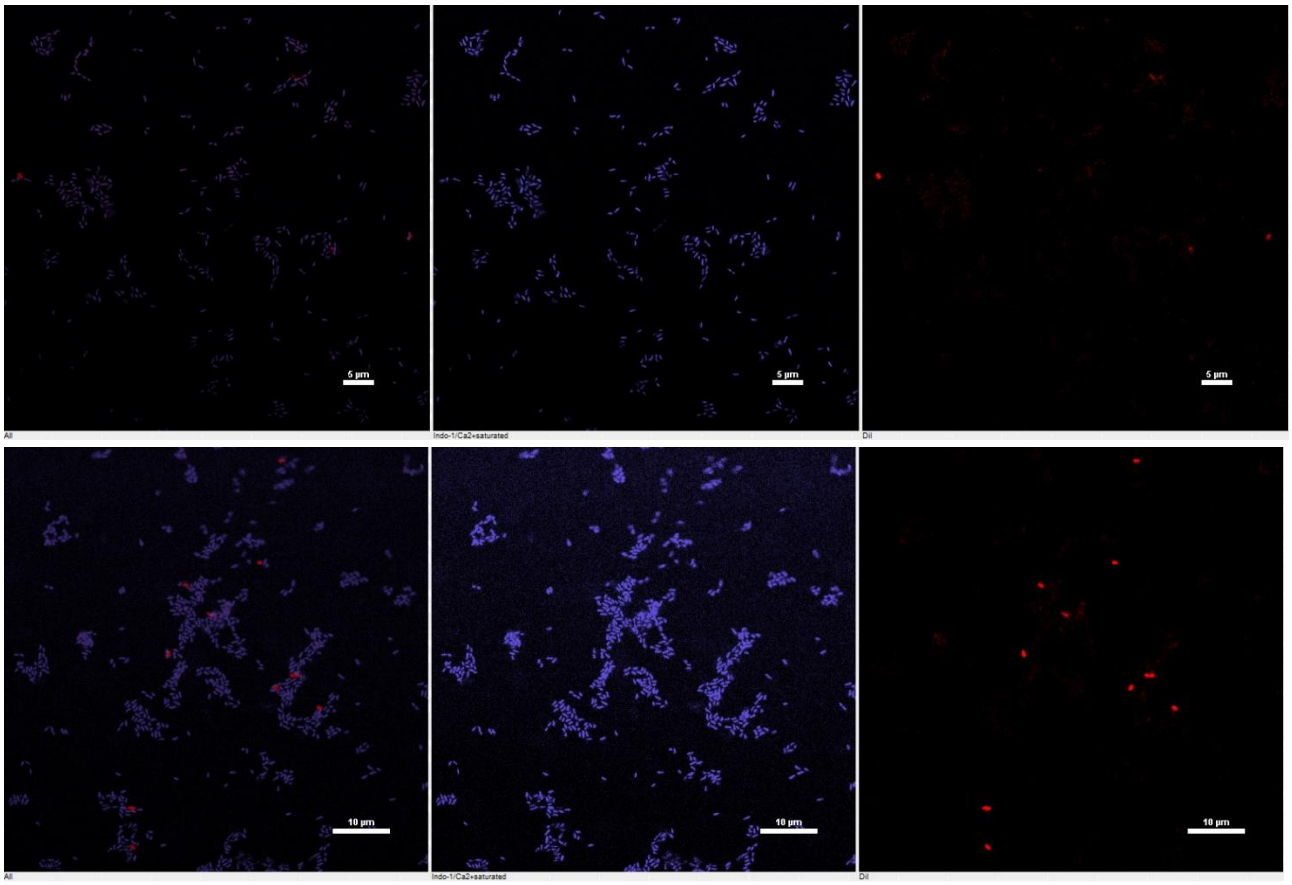


**Figure 8.7: Heated sample 2, stained**

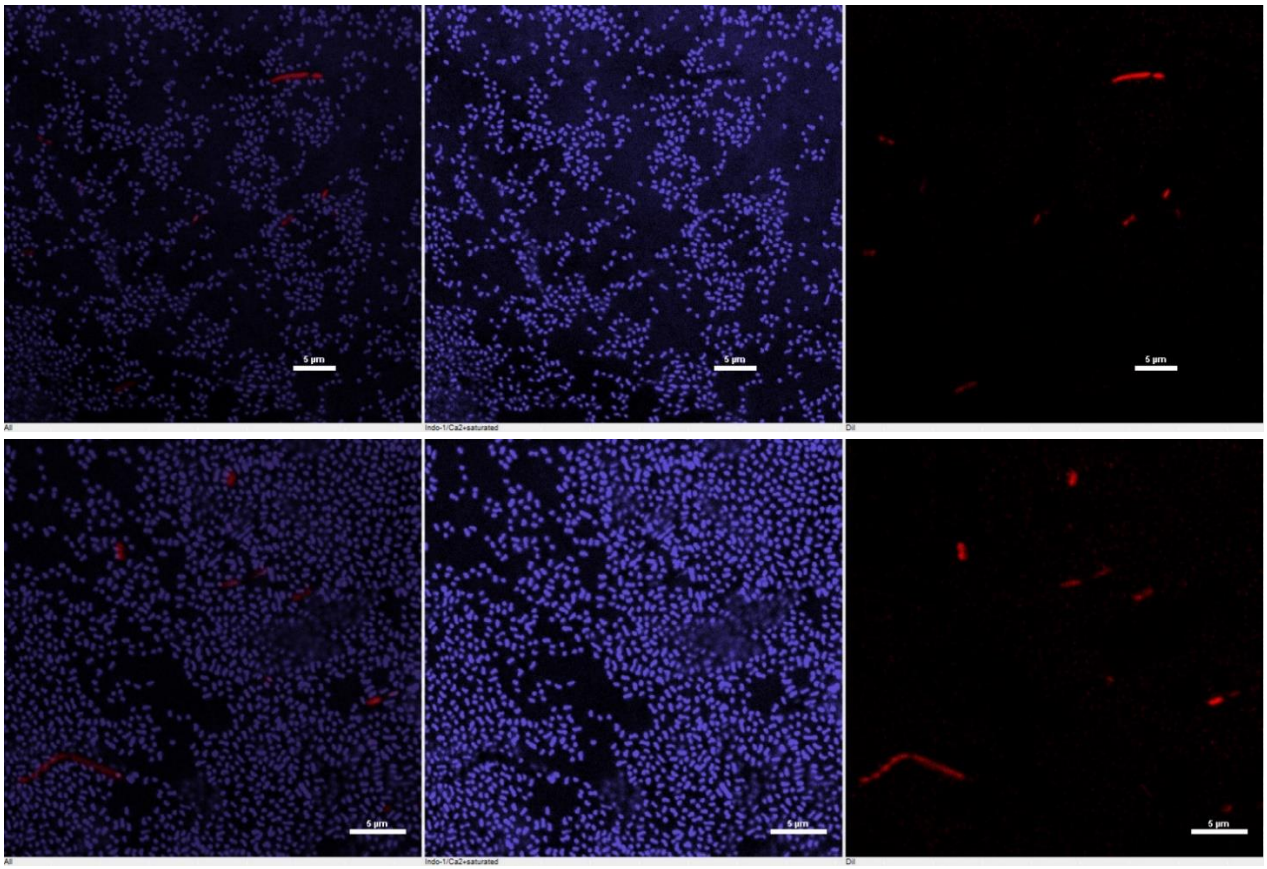

**Figure 8.8: Pressurized samples, stained**

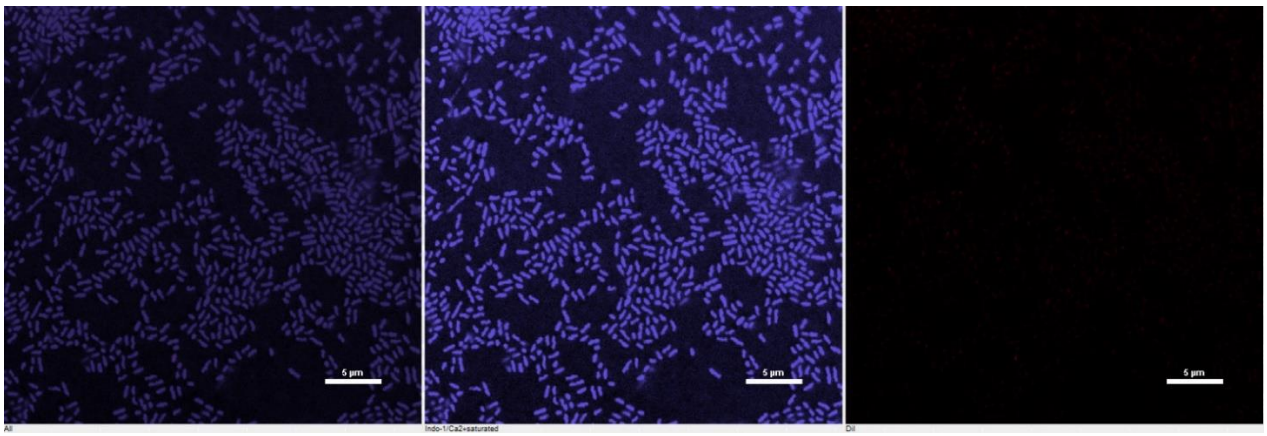

**Figure 8.9: Untreated sample, stained.**

## 8.3 Appendix 3: Matlab scripts

### 8.3.1 Cell Wall, Membrane and Protein State Simulations

A model to simulate the state of the cell wall has been developed during this project work. The model consists mainly of three scripts; CWPar.m, CW_ODE.m and CW.m. The first contains all the parameter values, the second contains all the ordinary differential equations that describes the model and the third takes in these settings and use the ODE solver to simulate the model. CWPressurLevel.m is used to simulate the various outcomes of cell wall state at different pressure levels. The same procedure has been applied for the membrane and protein models. For the membrane model the scripts are; MembranePar.m, Membrane_ODE.m , Membrane.m and MembranePressureLevels.m. For the protein models. For the protein model, the scripts are ProteinPar.m, Protein_ODE.m, Protein.m and ProteinPressureLevels.m

**CW.m**

```
function []=CW()% Main function for cell wall simulations
clc
close all
clear all
par=CWPar();% Retrieving the parameters

%Initial conditions:
CW0=100;          % Initial cell wall state
Mur0=0.47;        % Initial Mur-complex value
RpoN0=0.029;      % Initial RpoN regulator value
options=[];

%ODE functions:
par.P=0;        % Setting pressure parameter to normal pressure
[t0,x0]=ode23s(@CW_ODE,[0 150],[CW0 Mur0 RpoN0],options,par);
% Simulating the three components at normal pressure conditions for 150 min
 xa=x0(end,:);
 par.P=400;    % Setting a new pressure state. Here equal to 400MPa
 [t,x]=ode23s(@CW_ODE,[t0(end) 155],xa,options,par);
% Simulating the states at new pressure conditions.  Mimicking a HPP that
runs for 5 min
 xb=x(end,:);
 par.P=0;      % Pressure relief, back to normal pressure conditions
[t1,x1]=ode23s(@CW_ODE,[t(end) 800],xb,options,par);
% Simulation of the three components, getting CW state back to optimum

% Plot settings, for simulations to be visualized
PlotTime(t0,x0,par);
hold on
PlotTime(t,x,par);
PlotTime(t1,x1,par);
end

function []=PlotTime(t,x,par)
```

```
figure(1)
subplot(1,3,1);              %Plot of the cell wall state
plot(t,x(:,1),'b-');
xlabel('Time (min)')
ylabel('CW state (%)');
hold on
subplot(1,3,2);              %Plot of Mur complex activity
plot(t,x(:,2),'g-');
xlabel('Time (min)')
ylabel('Mur');
hold on
subplot(1,3,3);             %Plot of RpoN activity
plot(t,x(:,3),'g-');
xlabel('Time (min)')
ylabel('RpoN');
hold on
figure(2)                  % New picture showing clearly the cell wall state
plot(t,x(:,1),'b-');
xlabel('Time (min)')
ylabel('CW state (%)');
hold on
end
```

**CW_ODE.m**

```
function CellWall_dot=CW_ODE(tCW,xCW,par) % All the ODEs are defined here

% Defining the position of components in function
CW=xCW(1);
Mur=xCW(2);
RpoN=xCW(3);

% Reactions
% 1. As + Mur --> PG
% 2. PG + Al --> As + Deg
% 3. PG + HPP --> HPP + Deg
%% CodY + MurI --> CodY + Mur

%Hill Function. Applied pressure is input. Output is the value of influence
%it has on cell wall degradation
HPP=(par.P.^par.nP)/(par.P.^par.nP+par.KmCW.^par.nP);

% Differential equations, substrate, enzyme and regulator
CW_dot = par.k1*par.As*Mur -(HPP.^2.95*CW)- par.d1*par.Al*CW;
if CW<10.1
    CW_dot=0; %When the cell wall reaches a certain level, it will not be
able to repair itself anymore
end
Mur_dot = (par.kM1*(par.MurT-Mur).^par.n*RpoN)./(par.kMm1.^par.n+(par.MurT-
Mur).^par.n) - par.kM2*Mur.^par.n./(par.kMm2.^par.n+Mur.^par.n);
```

```
RpoN_dot= ((par.kR1*(par.RpoNT-RpoN).^par.n)./(par.kRm1.^par.n+(par.RpoNT-
RpoN).^par.n) - par.kR2*RpoN.^par.n*CW./(par.kRm2.^par.n+RpoN.^par.n));

CellWall_dot = [CW_dot;Mur_dot;RpoN_dot];
% Transferring the differential equations to the main function
```

**CW_Par.m**

```
function par=CWPar()
% Defining all parameters to be used
par.k1=2.948;    % As --> PG
par.d1=0.0138;   % PG --> Deg
par.n=4;         % Exponent in Michaelis Menten equations

par.As=1;        % Aminosugar source, defined as constant
par.Al=1;        % Autolysin, defined as a constant

par.kM1=10;      %induction of Mur
par.kM2=0.5;     %Repression of Mur
par.kMm1=0.4;
par.kMm2=0.5;
par.MurT=1;       % Total amount of Mur complex

par.kR1=0.98;     % Induction of RpoN
par.kR2 =0.1;     % Repression of RpoN
par.kRm1=0.05;
par.kRm2 = 0.05;
par.RpoNT=1;      % Total amount of RpoN regulator

%Hill function coefficients for HPP
par.KmCW=400;    % Repressor coefficient
par.nP=2;        % Hill coefficien
end
```

**CWPressureLevels.m**

```
function []=CWPressureLevels() % Function used to simulate different
pressure conditions
clc
close all
clear all
par=CWPar(); % Retrieving the parameters
%Initial conditions:
CW0=100;
Mur0=0.4;
CodY0=0;
options=[];

%ODE function - Pressure applied:
par.P=0; % Setting pressure parameter to normal pressure.
% During the first simulation, all systems behaves similar:
```

```
[t03,x03]=ode23s(@CW_ODE,[0 100],[CW0 Mur0 CodY0],options,par); % Used to
simulate cell wall state when exposed to 300 MPa
 xa3=x03(end,:);
 [t04,x04]=ode23s(@CW_ODE,[0 100],[CW0 Mur0 CodY0],options,par); % Used to
simulate cell wall state when exposed to 400 MPa
 xa4=x04(end,:);
  [t06,x06]=ode23s(@CW_ODE,[0 100],[CW0 Mur0 CodY0],options,par); % Used to
simulate cell wall state when exposed to 600 MPa
 xa6=x06(end,:);
  [t08,x08]=ode23s(@CW_ODE,[0 100],[CW0 Mur0 CodY0],options,par); % Used to
simulate cell wall state when exposed to 800 MPa
 xa8=x08(end,:);
 par.P=300; % Setting pressure parameter to 300MPa
 [t3,x3]=ode23s(@CW_ODE,[t03(end) 105],xa3,options,par);
 xb3=x3(end,:);
 par.P=400;  % Setting pressure parameter to 400MPa
 [t4,x4]=ode23s(@CW_ODE,[t04(end) 105],xa4,options,par);
 xb4=x4(end,:);
 par.P=600;  % Setting pressure parameter to 600MPa
 [t6,x6]=ode23s(@CW_ODE,[t03(end) 105],xa6,options,par);
 xb6=x6(end,:);
 par.P=800; % Setting pressure parameter to 800MPa
 [t8,x8]=ode23s(@CW_ODE,[t04(end) 105],xa8,options,par);
 xb8=x8(end,:);
 par.P=0; % Pressure relief, no pressure exposure
[t13,x13]=ode23s(@CW_ODE,[t3(end) 600],xb3,options,par);
[t14,x14]=ode23s(@CW_ODE,[t4(end) 600],xb4,options,par);
[t16,x16]=ode23s(@CW_ODE,[t6(end) 600],xb6,options,par);
[t18,x18]=ode23s(@CW_ODE,[t4(end) 600],xb8,options,par);

% Plots to visualize simulations:
PlotTime3(t03,x03,par);
hold on
PlotTime4(t04,x04,par);
hold on
PlotTime6(t06,x06,par);
hold on
PlotTime8(t08,x08,par);
hold on
PlotTime3(t3,x3,par);
PlotTime3(t13,x13,par);
PlotTime4(t4,x4,par);
PlotTime4(t14,x14,par);
PlotTime6(t6,x6,par);
PlotTime6(t16,x16,par);
PlotTime8(t8,x8,par);
PlotTime8(t18,x18,par);
end

% Plots:
function []=PlotTime3(t3,x3,par)
```

```
figure(1)
plot(t3,x3(:,1),'c-','linewidth',2);
hold on
end
function []=PlotTime4(t4,x4,par)
figure(1)
plot(t4,x4(:,1),'b-','linewidth',2);
hold on
end
function []=PlotTime6(t6,x6,par)
figure(1)
plot(t6,x6(:,1),'m-.','linewidth',2);
hold on
end
function []=PlotTime8(t8,x8,par)
figure(1)
plot(t8,x8(:,1),'r--','linewidth',2);
leg1=legend('300MPa','400MPa','600MPa','800MPa');
hold on
end
```

**Membrane.m**

```
function []=Membrane() % Main function for membrane simulations
clc
close all
clear all
par=MembranePar(); % Retrieving the parameters

%Initial conditions:
PA0=12.84;      % Initial value of phosphatidic acid source
Mem0=100;       % Initial membrane state
Fab0=0.469;     % Initial Fab complex value
RpoN0=0.0288;   % Initial RpoN regulator value
options=[];

%ODE functions:
par.P=0; % Setting pressure parameter to normal pressure
[t0,x0]=ode23s(@Membrane_ODE,[0:1:500],[PA0 Mem0 Fab0 RpoN0],options,par);
% Simulating the four components at normal pressure conditions for 500 min
 xa=x0(end,:);
 par.P=400; % Setting a new pressure state. Here equal to 400MPa
 [t,x]=ode23s(@Membrane_ODE,[t0(end):1:505],xa,options,par);
% Simulating the states at new pressure conditions.  Mimicking a HPP that
runs for 5 min
 xb=x(end,:);
 par.P=0;
% Pressure relief, back to normal pressure conditions
[t1,x1]=ode23s(@Membrane_ODE,[t(end):1:3000],xb,options,par);
% Simulation of the four components, getting membrane state back to optimum

% Plot settings, for simulations to be visualized
```

```
PlotTime(t0,x0,par);
hold on
PlotTime(t,x,par);
PlotTime(t1,x1,par);
end

%% plot time
function []=PlotTime(t,x,par)
figure(1)                          % Plot of the membrane state
subplot(2,2,1);
plot(t,x(:,2),'b-');
xlabel('Time (min)')
ylabel('Membrane state(%)');
hold on
subplot(2,2,2);                    % Plot of the phosphatidic acid usage
plot(t,x(:,1),'c-');
xlabel('Time (min)')
ylabel('PA');
hold on
subplot(2,2,3);                    %Plot of Fab complex activity
plot(t,x(:,3),'g-');
xlabel('Time (min)')
ylabel('Fab');
hold on
subplot(2,2,4);                    % Plot of RpoN activity
plot(t,x(:,4),'g-');
xlabel('Time (min)')
ylabel('RpoN');
hold on
figure(2)                          % New picture showing clearly the membrane
state
plot(t,x(:,2),'b-');
xlabel('Time (min)')
ylabel('Membrane state(%)');
hold on
end
```

**Membrane_ODE.m**

```
function Membrane_dot=Membrane_ODE(t,x,par) % All the ODEs are defined here

% Defining the position of components in function
PA=x(1);
Mem=x(2);
Fab=x(3);
RpoN=x(4);

% Reactions
% 1. CoA + Fab --> PA
% 2. nyeprot+PA --> Mem
% 3. Mem + DgkD --> Pa + DgkD
```

```
% 4. Mem + MsbA --> Deg + MsbA
% 5. Mem + HPP --> Deg + HPP
% RpoN + FabI --> RpoN + Fab

%Hill Function.
% Applied pressure is input. Output is the value of influence it has on cell
wall degradation
HPP=(par.P.^par.nP)/(par.P.^par.nP+par.Km.^par.nP);

%Differential equations, substrates, enzyme and regulator
PA_dot = par.k2*par.Coa*Fab + par.k4*par.DgkD*Mem - par.k3*PA;
Mem_dot = par.k3*PA - par.d2*par.MsbA*Mem - par.k4*par.DgkD*Mem -
(HPP.^3.4*Mem);
if Mem<7.1
    Mem_dot=0;  %When the membrane reaches a certain level, it will not be
able to repair itself anymore
end
Fab_dot = (par.kF1*(par.FabT-Fab).^par.n*RpoN)./(par.kFm1.^par.n+(par.FabT-
Fab).^par.n) - par.kF2*Fab.^par.n./(par.kFm2.^par.n+Fab.^par.n);
RpoN_dot= ((par.kR1*(par.RpoNT-RpoN).^par.n)./(par.kRm1.^par.n+(par.RpoNT-
RpoN).^par.n) - par.kR2*RpoN.^par.n*Mem./(par.kRm2.^par.n+RpoN.^par.n));

Membrane_dot=[PA_dot;Mem_dot;Fab_dot;RpoN_dot]; % Transferring the
differential equations to the main function
```

**MembranePar.m**

```
function par=MembranePar()

par.k2=0.079;       % CoA + Fab --> PA
par.k3=0.0382;      % PA --> Mem
par.k4=0.0012;      % Mem + DgkD --> Pa + DgkD
par.d2=0.003706;    %Mem + MsbA --> Deg + MsbA
par.n=4;

par.Coa=10;         % Acetyl Coa source, defined as constant
par.MsbA=1;         % Degradation protein MsbA, defined as a constant
par.DgkD=1;         % Recycling protein DgkD, defined as a constant

par.kR1=1;          % Induction of RpoN
par.kR2 =0.1;       % Repression of RpoN
par.kRm1=0.05;
par.kRm2 = 0.05;
par.RpoNT=1;        % Total amount of RpoN regulator

par.kF1=10;         % Induction of Fab complex
par.kF2 =0.5;       % Repression of Fab complex
par.kFm1= 0.4;
par.kFm2 = 0.5;
par.FabT=1;         % Total amount of Fab complex
```

```
%Hill function coefficients for HPP
par.Km=350;         % Repressor coefficient
par.nP=2;           % Hill coefficien for pressure
end
```

**MembranePressureLevels.m**

```
function []=MembranePressureLevels()
clc
close all
clear all
par=MembranePar();
%Initial conditions:
PA0=12.5;
Mem0=100;
Fab0=0.469;
RpoN0=0.0288;
options=[];

%ODE function - Pressure applied:
par.P=0; % Setting pressure parameter to normal pressure
% During the first simulation, all systems behaves similar:
[t03,x03]=ode23s(@Membrane_ODE,[0 500],[PA0 Mem0 Fab0 RpoN0],options,par); %
Used to simulate membrane state when exposed to 300 MPa
 xa3=x03(end,:);
 [t04,x04]=ode23s(@Membrane_ODE,[0 500],[PA0 Mem0 Fab0 RpoN0],options,par);
% Used to simulate membrane state when exposed to 400 MPa
 xa4=x04(end,:);
  [t06,x06]=ode23s(@Membrane_ODE,[0 500],[PA0 Mem0 Fab0 RpoN0],options,par);
% Used to simulate membrane state when exposed to 600 MPa
 xa6=x06(end,:);
  [t08,x08]=ode23s(@Membrane_ODE,[0 500],[PA0 Mem0 Fab0 RpoN0],options,par);
% Used to simulate membrane state when exposed to 800 MPa
 xa8=x08(end,:);
 par.P=300;  % Setting pressure parameter to 300MPa
 [t3,x3]=ode23s(@Membrane_ODE,[t03(end) 505],xa3,options,par);
 xb3=x3(end,:);
 par.P=400;  % Setting pressure parameter to 400MPa
 [t4,x4]=ode23s(@Membrane_ODE,[t04(end) 505],xa4,options,par);
 xb4=x4(end,:);
 par.P=600;  % Setting pressure parameter to 600MPa
 [t6,x6]=ode23s(@Membrane_ODE,[t03(end) 505],xa6,options,par);
 xb6=x6(end,:);
 par.P=800; % Setting pressure parameter to 800MPa
 [t8,x8]=ode23s(@Membrane_ODE,[t04(end) 505],xa8,options,par);
 xb8=x8(end,:);
 par.P=0; % Pressure relief, no pressure exposure
[t13,x13]=ode23s(@Membrane_ODE,[t3(end) 3000],xb3,options,par);
[t14,x14]=ode23s(@Membrane_ODE,[t4(end) 3000],xb4,options,par);
[t16,x16]=ode23s(@Membrane_ODE,[t6(end) 3000],xb6,options,par);
[t18,x18]=ode23s(@Membrane_ODE,[t4(end) 3000],xb8,options,par);
```

```
% Plots to visualize simulations:
PlotTime3(t03,x03,par);
hold on
PlotTime4(t04,x04,par);
hold on
PlotTime6(t06,x06,par);
hold on
PlotTime8(t08,x08,par);
hold on
PlotTime3(t3,x3,par);
PlotTime3(t13,x13,par);
PlotTime4(t4,x4,par);
PlotTime4(t14,x14,par);
PlotTime6(t6,x6,par);
PlotTime6(t16,x16,par);
PlotTime8(t8,x8,par);
PlotTime8(t18,x18,par);
end

% Plots:
function []=PlotTime3(t3,x3,par)
figure(1)
plot(t3,x3(:,2),'c-','linewidth',2);
hold on
end
function []=PlotTime4(t4,x4,par)
figure(1)
plot(t4,x4(:,2),'b-','linewidth',2);
hold on
end
function []=PlotTime6(t6,x6,par)
figure(1)
plot(t6,x6(:,2),'m-.','linewidth',2);
hold on
end
function []=PlotTime8(t8,x8,par)
figure(1)
plot(t8,x8(:,2),'r--','linewidth',2);
xlabel('Time (min)')
ylabel('Membrane state (%)');
leg1=legend('300MPa','400MPa','600MPa','800MPa');
end
```

**Protein.m**

```
function []=Protein() % main function for protein simulations
clc
close all
clear all
color='b';
par=ProteinPar(); % Retrieving the parameters
```

```
%Initial conditions:
FP0=100;         % Initial state of functional proteins
DP0=0;           % Initial value of damaged proteins
Chap0=0.4;       % Initial Chaperone value
SigB0=0;         % Initial SigB regulator value
RepAct0=0;       % Initial value of the repair activation step
options=[];

%ODE functions:
par.P=0;          % Setting pressure parameter to normal pressure
[t0,x0]=ode15s(@Protein_ODE,[0 200],[FP0 DP0 Chap0 SigB0
RepAct0],options,par);    % Simulating the five components at normal
pressure conditions for 200 min
 xa=x0(end,:);
 par.P=400;       % Setting a new pressure state. Here equal to 400MPa
 [t,x]=ode15s(@Protein_ODE,[t0(end) 205],xa,options,par);
% Simulating the states at new pressure conditions.  Mimicking a HPP that
runs for 5 min
 xb=x(end,:);
 par.P=0;         % Pressure relief, back to normal pressure conditions
[t1,x1]=ode15s(@Protein_ODE,[t(end) 4000],xb,options,par);
% Simulation of the five components, getting protein state back to optimum

% Plot settings, for simulations to be visualized
PlotTime(t0,x0,par);
hold on
PlotTime(t,x,par);
PlotTime(t1,x1,par);
end

%% plot time
function []=PlotTime(t,x,par)
figure(1)
subplot(2,3,1);                  % Plot of the protein state
plot(t,x(:,1),'b-');
xlabel('Time (min)')
ylabel('Protein state (%)');
hold on
subplot(2,3,2);                  %Plot of the damaged protein state
plot(t,x(:,2),'c-');
xlabel('Time (min)')
ylabel('DP');
hold on
subplot(2,3,3);                 % Plot of chaperone activity
plot(t,x(:,3),'g-');
xlabel('Time (min)')
ylabel('Chap');
hold on
subplot(2,3,4);                  % Plot of SigB activity
plot(t,x(:,4),'g-');
xlabel('Time (min)')
```

```
ylabel('SigB');
hold on
subplot(2,3,5);
plot(t,x(:,5),'g-');
xlabel('Time (min)')
ylabel('RepActivation');
hold on
figure(2)                          % New picture showing clearly the protein
state
plot(t,x(:,1),'b-');
xlabel('Time (min)')
ylabel('Protein state(%)');
hold on
end
```

**Protein_ODE.m**

```
function Protein_dot=Protein_ODE(t,x,par) % All the ODEs are defined here

% Defining the position of components in function
FP=x(1);
DP=x(2);
Chap=x(3);
SigB=x(4);
RepAct=x(5);

% Reactions
% 1. FP+HP --> DP + HPP
% 2. DP + Hcp --> FP + Hcp
% 3. AA + Sec --> FP + Ubiq + AA
% 4. DP + Ubiq + Clp --> Deg + Ubiq + Clp

%Hill Function.
% Applied pressure is input. Output is the value of influence it has on cell
wall degradation
HPP=(par.P.^par.nP)/(par.P.^par.nP+par.Km.^par.nP);
Damage=(100.^par.n1-FP.^par.n1)/(100.^par.n1);

%Differential equations, substrate, enzyme and regulator
FP_dot = par.k5*DP*Chap + RepAct - par.k7*FP*HPP.^3.3;
if FP<6.2
    FP_dot=0;    %When the cell wall reaches a certain level, it will not be
able to repair itself anymore
end
RepAct_dot=par.k6*par.AA*par.Sec*Damage-par.k8*RepAct;  %Representing the
delay in activation of genes
DP_dot = par.k7*FP*HPP.^3.3-par.k5*DP*Chap-par.d3*DP*par.Ubiq*par.Clp;
Chap_dot=(par.kH1*(par.ChapT-Chap).^par.n*SigB)./(par.kHm1.^par.n
+(par.ChapT-Chap).^par.n)-par.kH2*Chap.^par.n./(par.kHm2.^par.n+Chap.^par.n)
```

```
SigB_dot= ((par.kS1*(par.SigBT-SigB).^par.n)./(par.kSm1.^par.n+(par.SigBT-
SigB).^par.n) -
par.kS2*SigB.^par.n*FP./(par.kSm2.^par.n+SigB.^par.n))*Damage;

Protein_dot=[FP_dot;DP_dot;Chap_dot;SigB_dot;RepAct_dot]; % Transferring the
differential equations to the main function
```

**ProteinPar.m**

```
  function par=ProteinPar()

par.k5=0.00005;       % DP + Hcp --> FP
par.k6=0.06;          % DP + Sec --> FP
par.k7=1;             % FP --> DP
par.k8=1.1;           % RepairActivation --> Deg
par.d3=0.0036;        % DP --> Deg
par.n=4;

par.Ubiq=1;           % Tagging complex Ubiq, defined as constant
par.AA=1;             % Amino acid source, defined as constant
par.Sec=1;            % Insertion protein complex sec, defined as constant
par.Clp=1;            % Degradation complex Clp, defined as a constant

par.kH1=0.4;          % Induction of Chap complex
par.kH2 =0.1;         % Repression of Chap complex
par.kHm1=0.05;
par.kHm2 = 0.05;
par.ChapT=1;          % Total amount of Chap complex

par.kS1=5;            % Induction of SigB regulator
par.kS2 =0.5;         % Repression of SigB regulator
par.kSm1= 0.4;
par.kSm2 = 0.5;
par.SigBT=1;          % Total amount of SigB regulator

%Hill function coefficients for HPP
par.Km=350;          % Repressor coefficient
par.nP=2;            % Hill coefficient for pressure
par.n1=10;            % Damage signal to SigB
end
```

**ProteinPressureLevels.m**

```
function []=ProteinPressureLevels()
clc
close all
clear all
par=MembranePar();
%Initial conditions:
par=ProteinPar();
FP0=100;
DP0=0;
```

```
Chap0=0.4;
SigB0=0;
RepAct0=0;
options=[];

%ODE function - Pressure applied:
par.P=0; % Setting pressure parameter to normal pressure
% During the first simulation, all systems behaves similar:
[t03,x03]=ode23s(@Protein_ODE3,[0 100],[FP0 DP0 Chap0 SigB0
RepAct0],options,par); % Used to simulate protein state when exposed to 300
MPa
 xa3=x03(end,:);
 [t04,x04]=ode23s(@Protein_ODE3,[0 100],[FP0 DP0 Chap0 SigB0
RepAct0],options,par); % Used to simulate protein state when exposed to 400
MPa
 xa4=x04(end,:);
  [t06,x06]=ode23s(@Protein_ODE3,[0 100],[FP0 DP0 Chap0 SigB0
RepAct0],options,par); % Used to simulate protein state when exposed to 600
MPa
 xa6=x06(end,:);
  [t08,x08]=ode23s(@Protein_ODE3,[0 100],[FP0 DP0 Chap0 SigB0
RepAct0],options,par); % Used to simulate protein state when exposed to 800
MPa
 xa8=x08(end,:);
 par.P=300;  % Setting pressure parameter to 300MPa
 [t3,x3]=ode23s(@Protein_ODE3,[t03(end) 105],xa3,options,par);
 xb3=x3(end,:);
 par.P=400;  % Setting pressure parameter to 400MPa
 [t4,x4]=ode23s(@Protein_ODE3,[t04(end) 105],xa4,options,par);
 xb4=x4(end,:);
 par.P=600; % Setting pressure parameter to 600MPa
 [t6,x6]=ode23s(@Protein_ODE3,[t03(end) 105],xa6,options,par);
 xb6=x6(end,:);
 par.P=800; % Setting pressure parameter to 800MPa
 [t8,x8]=ode23s(@Protein_ODE3,[t04(end) 105],xa8,options,par);
 xb8=x8(end,:);
 par.P=0; % Pressure relief, no pressure exposure
[t13,x13]=ode23s(@Protein_ODE3,[t3(end) 3000],xb3,options,par);
[t14,x14]=ode23s(@Protein_ODE3,[t4(end) 3000],xb4,options,par);
[t16,x16]=ode23s(@Protein_ODE3,[t6(end) 3000],xb6,options,par);
[t18,x18]=ode23s(@Protein_ODE3,[t4(end) 3000],xb8,options,par);

% Plots to visualize simulations:
PlotTime3(t03,x03,par);
hold on
PlotTime4(t04,x04,par);
hold on
PlotTime6(t06,x06,par);
hold on
PlotTime8(t08,x08,par);
hold on
```

```
PlotTime1(t01,x01,par);
hold on
PlotTime3(t3,x3,par);
PlotTime3(t13,x13,par);
PlotTime4(t4,x4,par);
PlotTime4(t14,x14,par);
PlotTime6(t6,x6,par);
PlotTime6(t16,x16,par);
PlotTime8(t8,x8,par);
PlotTime8(t18,x18,par);
end

% Plots:
function []=PlotTime3(t3,x3,par)
figure(1)
plot(t3,x3(:,1),'c-','linewidth',2);
hold on
end
function []=PlotTime4(t4,x4,par)
figure(1)
plot(t4,x4(:,1),'b-','linewidth',2);
hold on
end
function []=PlotTime6(t6,x6,par)
figure(1)
plot(t6,x6(:,1),'m-.','linewidth',2);
hold on
end
function []=PlotTime8(t8,x8,par)
figure(1)
plot(t8,x8(:,1),'y','linewidth',2);
hold on
leg1=legend('300MPa','400MPa','600MPa','800MPa');
end
```

### 8.3.2 Uncoupled Cell Envelope Simulations

The uncoupled cell envelope simulations gathers the cell wall, membrane and protein models without coupling them. The setup is similar to the scripts previously described with the model consisting of three scripts; Envelope.m, Envelope_ODE.m and EnvelopePar.m.

**Envelope.m**

```
function []=Envelope() % Main function for uncoupled envelope simulations
clc
close all
clear all
par=EnvelopePar();  % Retrieving the parameters
% Initial conditions:
CW0=100;
Mur0=0.4;
```

```
CodY0=0.023;
PA0=12;
Mem0=100;
Fab0=0.4;
RpoN0=0;
FP0=100;
DP0=0;
Chap0=0.4;
SigB0=0;
RepAct0=0;
options=[];

%ODE functions:
par.P=0;    % Setting pressure parameter to normal pressure
% Simulating the cell wall system at normal pressure conditions for 200 min
[tCW0,xCW0]=ode15s(@CW_ODE,[0 200],[CW0 Mur0 CodY0],options,par);
 xCWa=xCW0(end,:);
% Simulating the membrane system at normal pressure conditions for 200 min
[tMem0,xMem0]=ode15s(@Membrane_ODE,[0 200],[PA0 Mem0 Fab0
RpoN0],options,par);
xMema=xMem0(end,:);
% Simulating the protein system at normal pressure conditions for 200 min
[tProt0,xProt0]=ode15s(@Protein_ODE,[0 200],[FP0 DP0 Chap0 SigB0
RepAct0],options,par);
xProta=xProt0(end,:);
 par.P=400;   % Setting a new pressure state. Here equal to 400MPa
% Simulating the states at new pressure conditions.  Mimicking a HPP that
runs for 5 min
 [tCW,xCW]=ode15s(@CW_ODE,[tCW0(end) 205],xCWa,options,par);
xCWb=xCW(end,:);
 [tMem,xMem]=ode15s(@Membrane_ODE,[tMem0(end) 205],xMema,options,par);
 xMemb=xMem(end,:);
 [tProt,xProt]=ode15s(@Protein_ODE,[tProt0(end) 205],xProta,options,par);
 xProtb=xProt(end,:);
 par.P=0;    % Pressure relief, back to normal pressure conditions
% Simulation of the three systems, striving to get the states back to
optimum
[tCW1,xCW1]=ode15s(@CW_ODE,[tCW(end) 3000],xCWb,options,par);
 [tMem1,xMem1]=ode23s(@Membrane_ODE,[tMem(end) 3000],xMemb,options,par);
[tProt1,xProt1]=ode15s(@Protein_ODE,[tProt(end) 3000],xProtb,options,par);

% Plot settings, for simulations to be visualized
PlotTimeCW(tCW0,xCW0,par);
PlotTimeMem(tMem0,xMem0,par);
PlotTimeProt(tProt0,xProt0,par);
hold on
PlotTimeCW(tCW,xCW,par);
PlotTimeMem(tMem,xMem,par);
PlotTimeProt(tProt,xProt,par);
hold on
PlotTimeCW(tCW1,xCW1,par);
```

```
PlotTimeMem(tMem1,xMem1,par);
PlotTimeProt(tProt1,xProt1,par);
end

function []=PlotTimeCW(tCW,xCW,par) % Plot of the cell wall state
figure(1)
plot(tCW,xCW(:,1),'b-','linewidth',2);
hold on
end

function []=PlotTimeMem(tMem,xMem,par)  % Plot of the membrane state
figure(1)
plot(tMem,xMem(:,2),'g-','linewidth',2);
hold on
end

function []=PlotTimeProt(tProt,xProt,par)
figure(1)
plot(tProt,xProt(:,1),'y-','linewidth',2);  % Plot of the protein state
xlabel('Time (min)')
ylabel('Cell Envelope');
legend('Cell Wall','Membrane','Protein');
hold on
end
```

**EnvelopePar.m**

```
function par=EnvelopePar()
%All reactions:
par.k1=2.97;    % As --> PG
par.d1=0.014;   % PG --> Deg

par.k2=0.077;   % CoA + Fab --> PA
par.k3=0.0382;  % PA --> Mem
par.k4=0.0012;  % Mem + DgkD --> Pa + DgkD
par.d2=0.0036;  % Mem + MsbA --> Deg + MsbA

par.k5=0.00005;  % DP + Hcp --> FP
par.k6=0.06;     %  DP + Sec --> FP
par.k7=1;        % FP --> DP
par.k8=1.1;
par.d3=0.0036;  % DP --> Deg
par.n=4;

% Proteins/Substrates made as Constants:
par.n=4;
par.As=1;
par.Al=1;
par.Coa=10;
par.MsbA=1;
par.DgkD=1;
```

```
par.Ubiq=1;
par.AA=1;
par.Sec=1;
par.Clp=1;

par.kM1=10;      %induction of Mur
par.kM2=0.5;     %Repression of Mur
par.kMm1=0.4;
par.kMm2=0.5;
par.MurT=1;

par.kC1=1.03;    % Induction of CodY
par.kC2=0.1;     % Repression of CodY
par.kCm1=0.05;
par.kCm2=0.05;
par.CodYT=1;

par.kR1=1;       % Induction of RpoN
par.kR2 =0.1;    % Repression of RpoN
par.kRm1=0.05;
par.kRm2 = 0.05;
par.RpoNT=1;

par.kF1=10;       % Induction of Fab
par.kF2 =0.5;     % Repression of Fab
par.kFm1= 0.4;
par.kFm2 = 0.5;
par.FabT=1;

par.kH1=0.4;      % Induction of Hcp
par.kH2 =0.1;     % Repression of Hcp
par.kHm1=0.05;
par.kHm2 = 0.05;
par.ChapT=1;

par.kS1=5;        % Induction of SigB
par.kS2 =0.5;     % Repression of SigB
par.kSm1= 0.4;
par.kSm2 = 0.5;
par.SigBT=1;

% Hill function coefficients for HPP
par.Km=350;      % Repressor coefficient
par.KmCW=400;    % Repressor coefficient specifically for cell wall
par.nP=2;        % Hill coefficient for pressure
par.n1=10;       % Hill coefficiant for damage signal
end
```

### 8.3.3 Coupled Cell Envelope Simulations

The coupled cell envelope simulations gathers the cell wall, membrane and protein models with coupling of the three systems. The setup is similar to the scripts previously described with the model consisting of three scripts; CWMemProt.m, CWMemProt_ODE.m and CWMemProtPar.m. Both original and modified version is given here.

**CWMemProt.m**

```
 function [T0,X0]=CWMemProt(par) % Main function for coupled envelope model
if nargin<1 % Used for sensitivity analysis
     par.k1=1.751;
     par.k2=0.0785;
     noplot=1;
 else
     noplot=0;
 end
clc
close all
par=CWMemProtPar(par); % Retrieving the parameters
% Initial conditions:
CW0=100;
Mur0=0.4;
CodY0=0.0402;
FP0=100;
RepAct0=0;
DP0=0;
Hcp0=0.4;
SigB0=0;
PA0=12.84;
Mem0=100;
Fab0=0.4;
RpoN0=0.0295;
options=[];

% ODE functions
par.P=0;    % Setting pressure parameter to normal pressure
% Simulating the envelope model at normal pressure conditions for 500 min:
[t0,x0]=ode15s(@CWMemProt_ODE,[0:0.5:500],[CW0 Mur0 CodY0 FP0 RepAct0 DP0
Hcp0 SigB0 PA0 Mem0 Fab0 RpoN0],options,par);
 xa=x0(end,:);
 par.P=400; % Setting a new pressure state. Here equal to 400MPa
% Simulating the states at new pressure conditions.  Mimicking a HPP that
runs for 5 min
 [t,x]=ode15s(@CWMemProt_ODE,[t0(end):0.5:505],xa,options,par);
 xb=x(end,:);
 par.P=0; % Pressure relief, back to normal pressure conditions
% Simulation of the envelope model, striving to get the states back to
optimum
[t1,x1]=ode15s(@CWMemProt_ODE,[t(end):0.5:3000],xb,options,par);
xc=x1(end,:);
T0=t0;
```

```
X0=x0;

if noplot % For sensitivity analysis
PlotTime(t0,x0,par);
hold on
PlotTime(t,x,par);
PlotTime(t1,x1,par);
end
end

% Plot settings, for simulations to be visualized
function []=PlotTime(t,x,par)
% Simulations of cell wall, membrane and protein states
figure(1)
subplot(2,4,1);
plot(t,x(:,1),'b-');
xlabel('Time (min)')
ylabel('Cell wall');
hold on
subplot(2,4,2);
plot(t,x(:,10),'y-');
xlabel('Time (min)')
ylabel('Membrane');
hold on
subplot(2,4,3);
plot(t,x(:,4),'g-');
xlabel('Time (min)')
ylabel('Functional protein (FP)');
hold on
% Simulations of damaged protein and activity of CodY, RpoN, Mur and Fab
subplot(2,4,4);
plot(t,x(:,6),'y-');
xlabel('Time (min)')
ylabel('Damaged protein (DP)');
hold on
subplot(2,4,5);
plot(t,x(:,3),'m-');
xlabel('Time (min)')
ylabel('CodY');
hold on
subplot(2,4,6);
plot(t,x(:,12),'m-');
xlabel('Time (min)')
ylabel('RpoN');
hold on
subplot(2,4,7);
plot(t,x(:,2),'m-');
xlabel('Time (min)')
ylabel('Mur');
hold on
subplot(2,4,8);
```

```
plot(t,x(:,11),'m-');
xlabel('Time (min)')
ylabel('Fab');
hold on
% Combination of cell wall, membrane and protein simulations
figure(2)
plot(t,x(:,1),'b-','linewidth',2);
hold on
plot(t,x(:,10),'g-','linewidth',2);
plot(t,x(:,4),'y-','linewidth',2);
xlabel('Time (min)')
ylabel('Cell Envelope state (%)');
legend('Cell Wall','Membrane','Protein');
hold on
end
```

**CWMemProt_ODE.m**

```
function MemProt_dot=CWMemProt_ODE(t,x,par) % All the ODEs are defined here

% Defining the position of components in function
CW=x(1);
Mur=x(2);
CodY=x(3);
FP=x(4);
RepAct=x(5);
DP=x(6);
Hcp=x(7);
SigB=x(8);
PA=x(9);
Mem=x(10);
Fab=x(11);
RpoN=x(12);

%Hill Functions
HPP=(par.P.^par.nP)/(par.P.^par.nP+par.Km.^par.nP); % Input = Applied
processing pressure. Output = value of influence on envelope
DamageProt =(100.^par.n1-FP.^par.n1)/(100.^par.n1); % Input = State of
functional protein. Output = Protein damage signal
TransProt = 0.17; % Delay applied on membrane state when lacking a certain
amount of functional proteins
if FP > 60        % Threshold value for functional protein is a state of 60%
    TransProt=0;
end

%Differential equations, substrates and enzymes
CW_dot = par.k1*par.As*Mur -  (HPP.^3.8*CW)- par.d1*par.Al*CW;
if CW<10
    CW_dot=0;
end
```

```
Mur_dot = (par.kM1*(par.MurT-Mur).^par.n*RpoN)./(par.kMm1.^par.n+(par.MurT-
Mur).^par.n) - par.kM2*Mur.^par.n*CodY./(par.kMm2.^par.n+Mur.^par.n);
CodY_dot =(par.kC1*(par.CodYT-
CodY).^par.n)*RpoN./(par.kCm1.^par.n+(par.CodYT-CodY).^par.n) -
par.kC2*CodY.^par.n./(par.kCm2.^par.n+CodY.^par.n);

FP_dot = par.k5*DP*Hcp + RepAct - FP*HPP.^3.3;
if FP<6.2
    FP_dot=0;
end
RepAct_dot=par.k6*par.Ubiq*par.AA*par.Sec*DamageProt-par.k7*RepAct; %This
should really be par.k5*DP*Hcp - par.k8*RepActivation
DP_dot = FP*HPP.^3.3-par.k5*DP*Hcp-par.d3*DP*par.Ubiq*par.Clp;
Hcp_dot = (par.kH1*(par.HcpT-Hcp).^par.n*SigB)./(par.kHm1.^par.n+(par.HcpT-
Hcp).^par.n) - par.kH2*Hcp.^par.n./(par.kHm2.^par.n+Hcp.^par.n);
SigB_dot= ((par.kS1*(par.SigBT-SigB).^par.n)./(par.kSm1.^par.n+(par.SigBT-
SigB).^par.n) -
par.kS2*SigB.^par.n*FP./(par.kSm2.^par.n+SigB.^par.n))*DamageProt;

PA_dot = par.k2*par.Coa*Fab + par.k4*par.DgkD*Mem - par.k3*PA;
Mem_dot = par.k3*PA - TransProt - par.d2*par.MsbA*Mem  - par.k4*par.DgkD*Mem
- (HPP.^3.4*Mem);
if Mem<7.1
    Mem_dot=0;
end
Fab_dot = (par.kF1*(par.FabT-Fab).^par.n*RpoN)./(par.kFm1.^par.n+(par.FabT-
Fab).^par.n) - par.kF2*Fab.^par.n./(par.kFm2.^par.n+Fab.^par.n);
RpoN_dot=((par.kR1*(par.RpoNT-RpoN).^par.n)./(par.kRm1.^par.n+(par.RpoNT-
RpoN).^par.n) - par.kR2*RpoN.^par.n*Mem./(par.kRm2.^par.n+RpoN.^par.n));


MemProt_dot=[CW_dot;Mur_dot;CodY_dot;FP_dot;RepAct_dot;DP_dot;Hcp_dot;SigB_d
ot;PA_dot;Mem_dot;Fab_dot;RpoN_dot];
```

### CWMemProtPar.m

```
function par=CWMemProtPar(par)
%All reactions:
par.k1=1.751;     % 2.97 As --> PG
par.d1=0.014;     % 0.014PG --> Deg

par.k2=0.0785;    % CoA + Fab --> PA
par.k3=0.0382;    % PA --> Mem
par.k4=0.0012;    % Mem + DgkD --> Pa + DgkD
par.d2=0.003706; % Mem + MsbA --> Deg + MsbA

par.k5=0.0005;    % DP + Hcp --> FP
par.k6=0.06;      % DP + Sec --> FP
par.k7=1.1;       % RepAct --> Deg
par.d3=0.0036;    % DP --> Deg
par.n=4;
```

```
% Proteins/Substrates made as Constants:
par.As=1;
par.Al=1;
par.Coa=10;
par.MsbA=1;
par.DgkD=1;
par.Ubiq=1;
par.AA=1;
par.Sec=1;
par.Clp=1;
par.n=4;

par.kM1=10;     %Induction of Mur
par.kM2=0.5;    %Repression of Mur
par.kMm1=0.4;
par.kMm2=0.5;
par.MurT=1;

par.kR1=1.08;   % Induction of RpoN
par.kR2 =0.1;   % Repression of RpoN
par.kRm1=0.05;
par.kRm2 = 0.05;
par.RpoNT=1;

par.kC1=1;       % Induction of CodY
par.kC2=0.1;     % Repression of CodY
par.kCm1=0.05;
par.kCm2=0.05;
par.CodYT=1;

par.kF1=10;      % Induction of Fab
par.kF2 =0.5;    % Repression of Fab
par.kFm1= 0.4;
par.kFm2 = 0.5;
par.FabT=1;

par.kH1=0.4;     % Induction of Hcp
par.kH2 =0.1;    % Repression of Hcp
par.kHm1=0.05;
par.kHm2 = 0.05;
par.HcpT=1;

par.kS1=5;       % Induction of SigB
par.kS2 =0.5;    % Repression of SigB
par.kSm1= 0.4;
par.kSm2 = 0.5;
par.SigBT=1;

% Hill function coefficients for HPP
```

```
par.Km=350;      % Repressor coefficient
par.KmCW=400;    % Repressor coefficient specifically for cell wall
par.nP=2;        % Hill coefficient for pressure
par.n1=10;       % Hill coefficiant for damage signal
end
```

**CWMemProtChange.m**

```
function [T0,X0]=CWMemProtChange(par) % Main function for coupled envelope
model, modified version
% Used for sensitivity analysis:
 if nargin<1
     par.k1=1.751;
     noplot=1;
 else
     noplot=0;
 end
clc
close all
par=CWMemProtParChange(par);% Retrieving the parameters
% Initial conditions:
CW0=100;
Mur0=0.47;
CodY0=0.0402;
FP0=100;
RepAct0=0;
DP0=0;
Chap0=0.4;
SigB0=0;
PA0=12.84;
Mem0=100;
Fab0=0.47;
RpoN0=0.0295; %0.023
options=[];

% ODE functions
par.P=0;     % Setting pressure parameter to normal pressure
% Simulating the envelope model at normal pressure conditions for 500 min:
[t0,x0]=ode15s(@CWMemProtChange_ODE,[0:1:500],[CW0 Mur0 CodY0 FP0 RepAct0
DP0 Chap0 SigB0 PA0 Mem0 Fab0 RpoN0],options,par);
 xa=x0(end,:);
 par.P=400; % Setting a new pressure state. Here equal to 400MPa
% Simulating the states at new pressure conditions.  Mimicking a HPP that
runs for 5 min
 [t,x]=ode15s(@CWMemProtChange_ODE,[t0(end):1:505],xa,options,par);
 xb=x(end,:);
 par.P=0; % Pressure relief, back to normal pressure conditions
% Simulation of the envelope model, striving to get the states back to
optimum
[t1,x1]=ode15s(@CWMemProtChange_ODE,[t(end):1:3500],xb,options,par);
T0=t0;
X0=x0;
```

```
if noplot % For sensitivity analysis
PlotTime(t0,x0,par);
hold on
PlotTime(t,x,par);
PlotTime(t1,x1,par);
end
end

% Plot settings, for simulations to be visualized
function []=PlotTime(t,x,par)
% Simulating acticity of Mur, Fab, Chap, SigB and RpoN
figure(1)
subplot(2,3,1);
plot(t,x(:,2),'b-');
xlabel('Time (min)')
ylabel('Mur');
hold on
subplot(2,3,2);
plot(t,x(:,11),'m-');
xlabel('Time (min)')
ylabel('Fab');
hold on
subplot(2,3,3);
plot(t,x(:,7),'y-');
xlabel('Time (min)')
ylabel('Chap');
hold on
subplot(2,3,4);
plot(t,x(:,8),'y-');
xlabel('Time (min)')
ylabel('SigB');
hold on
subplot(2,3,5);
plot(t,x(:,12),'m-');
xlabel('Time (min)')
ylabel('RpoN');
hold on

% Combination of cell wall, membrane and protein simulations
figure(2)
plot(t,x(:,1),'b-','linewidth',2);
hold on
plot(t,x(:,10),'g-','linewidth',2);
plot(t,x(:,4),'y-','linewidth',2);
xlabel('Time (min)')
ylabel('Cell Envelope state (%)');
legend('Cell Wall','Membrane','Protein');
hold on
end
```

**CWMemProtChange1.m**

```
function [T1,X1]=CWMemProtChange1(par) % Main function for coupled envelope
model, modified version. Only used in combination with sensitivity analysis
if nargin<1 % Used for sensitivity analysis:
     par.k1=1.751;
     noplot=1;
 else
     noplot=0;
 end
clc
close all
color='b';
par=CWMemProtParChange(par);% Retrieving the parameters
% Initial conditions:
CW0=100;
Mur0=0.47;
CodY0=0.0402;
FP0=100;
RepAct0=0;
DP0=0;
Chap0=0.4;
SigB0=0;
PA0=12.84;
Mem0=100;
Fab0=0.47;
RpoN0=0.0295;
options=[];

% ODE functions
par.P=0;    % Setting pressure parameter to normal pressure
% Simulating the envelope model at normal pressure conditions for 500 min:
[t0,x0]=ode15s(@CWMemProtChange_ODE,[0:1:500],[CW0 Mur0 CodY0 FP0 RepAct0
DP0 Chap0 SigB0 PA0 Mem0 Fab0 RpoN0],options,par);
 xa=x0(end,:);
 par.P=400; % Setting a new pressure state. Here equal to 400MPa
% Simulating the states at new pressure conditions.  Mimicking a HPP that
runs for 5 min
 [t,x]=ode15s(@CWMemProtChange_ODE,[t0(end):1:505],xa,options,par);
 xb=x(end,:);
 par.P=0; % Pressure relief, back to normal pressure conditions
% Simulation of the envelope model, striving to get the states back to
optimum
[t1,x1]=ode15s(@CWMemProtChange_ODE,[t(end):1:3500],xb,options,par);
T1=t1;
X1=x1;

if noplot % For sensitivity analysis
PlotTime(t0,x0,par);
hold on
PlotTime(t,x,par);
PlotTime(t1,x1,par);
```

```
end
end

% Plot settings, for simulations to be visualized
function []=PlotTime(t,x,par)
% Simulating acticity of Mur, Fab, Chap, SigB and RpoN
figure(1)
subplot(2,3,1);
plot(t,x(:,2),'b-');
xlabel('Time (min)')
ylabel('Mur');
hold on
subplot(2,3,2);
plot(t,x(:,11),'m-');
xlabel('Time (min)')
ylabel('Fab');
hold on
subplot(2,3,3);
plot(t,x(:,7),'y-');
xlabel('Time (min)')
ylabel('Chap');
hold on
subplot(2,3,4);
plot(t,x(:,8),'y-');
xlabel('Time (min)')
ylabel('SigB');
hold on
subplot(2,3,5);
plot(t,x(:,12),'m-');
xlabel('Time (min)')
ylabel('RpoN');
hold on

% Combination of cell wall, membrane and protein simulations
figure(2)
plot(t,x(:,1),'b-','linewidth',2);
hold on
plot(t,x(:,10),'g-','linewidth',2);
plot(t,x(:,4),'y-','linewidth',2);
xlabel('Time (min)')
ylabel('Cell Envelope state (%)');
legend('Cell Wall','Membrane','Protein');
hold on
end
```

**CWMemProtChange_ODE.m**

```
function MemProt_dot=CWMemProtChange_ODE(t,x,par) % All the ODEs are defined
here

% Defining the position of components in function
CW=x(1);
```

```
Mur=x(2);
CodY=x(3);
FP=x(4);
RepAct=x(5);
DP=x(6);
Chap=x(7);
SigB=x(8);
PA=x(9);
Mem=x(10);
Fab=x(11);
RpoN=x(12);

%Hill Functions
HPP=(par.P.^par.nP)/(par.P.^par.nP+par.Km.^par.nP); % Input = Applied
processing pressure. Output = value of influence on envelope
DamageProt =(100.^par.n1-FP.^par.n1)/(100.^par.n1); % Input = State of
functional protein. Output = Protein damage signal
TransProt = 0.17; % Delay applied on membrane state when lacking a certain
amount of functional proteins
if FP > 60        % Threshold value for functional protein is a state of 60%
    TransProt=0;
end

%Differential equations, substrates and enzymes
CW_dot = par.k1*par.As*Mur -  (HPP.^3.8*CW)- par.d1*par.Al*CW;
if CW<10
    CW_dot=0;
end
Mur_dot = (par.kM1*(par.MurT-Mur).^par.n*RpoN)./(par.kMm1.^par.n+(par.MurT-
Mur).^par.n) - par.kM2*Mur.^par.n/(par.kMm2.^par.n+Mur.^par.n);
CodY_dot =(par.kC1*(par.CodYT-
CodY).^par.n)*RpoN./(par.kCm1.^par.n+(par.CodYT-CodY).^par.n) -
par.kC2*CodY.^par.n./(par.kCm2.^par.n+CodY.^par.n);

FP_dot = par.k5*DP*Chap + RepAct - par.k7*FP*HPP.^6.9-
par.d4*par.Ubiq*par.Clp*FP;
if FP<6.2
    FP_dot=0;
end
RepAct_dot=par.k6*par.AA*par.Sec*DamageProt-par.k7*RepAct;
DP_dot = FP*HPP.^6.9-par.k5*DP*Chap-par.d3*DP*par.Ubiq*par.Clp;
Chap_dot = (par.kH1*(par.ChapT-
Chap).^par.n*SigB)./(par.kHm1.^par.n+(par.ChapT-Chap).^par.n) -
par.kH2*Chap.^par.n./(par.kHm2.^par.n+Chap.^par.n);
SigB_dot= ((par.kS1*(par.SigBT-SigB).^par.n)./(par.kSm1.^par.n+(par.SigBT-
SigB).^par.n) -
par.kS2*SigB.^par.n*FP./(par.kSm2.^par.n+SigB.^par.n));%*DamageProt;

PA_dot = par.k2*par.Coa*Fab + par.k4*par.DgkD*Mem - par.k3*PA;
Mem_dot = par.k3*PA - TransProt - par.d2*par.MsbA*Mem  - par.k4*par.DgkD*Mem
- (HPP.^3.4*Mem);
```

```
if Mem<7.1
    Mem_dot=0;
end
Fab_dot =(par.kF1*(par.FabT-Fab).^par.n*RpoN)./(par.kFm1.^par.n+(par.FabT-
Fab).^par.n) - par.kF2*Fab.^par.n./(par.kFm2.^par.n+Fab.^par.n);
RpoN_dot=((par.kR1*(par.RpoNT-RpoN).^par.n)./(par.kRm1.^par.n+(par.RpoNT-
RpoN).^par.n) - par.kR2*RpoN.^par.n*CW./(par.kRm2.^par.n+RpoN.^par.n));

MemProt_dot=[CW_dot;Mur_dot;CodY_dot;FP_dot;RepAct_dot;DP_dot;Chap_dot;SigB_
dot;PA_dot;Mem_dot;Fab_dot;RpoN_dot];
```

**CWMemProtParChange.m**

```
function par=CWMemProtParChange(par)
%All reactions:
par.k1=2.944;       %  As --> PG
par.d1=0.0139;      % PG --> Ø

par.k2=0.0785;    % CoA + Fab --> PA
par.k3=0.0382;    % PA --> Mem
par.k4=0.0012;    % Mem + DgkD --> Pa + DgkD
par.d2=0.003706; % Mem + MsbA --> Deg + MsbA

par.k5=0.005;       % DP + Chap --> FP
par.k6=0.4;        % DP + Sec --> FP
par.k7=8.1;        % RepAct --> Deg
par.d3=0.36;       % DP --> Deg
par.d4=0.000009;    % FP --> Deg
par.n=4;

% Proteins/Substrates made as Constants:
par.As=1;
par.Al=1;
par.Coa=10;
par.MsbA=1;
par.DgkD=1;
par.Ubiq=0.1;
par.AA=1;
par.Sec=1;
par.Clp=0.1;
par.n=4;

par.kM1=10;      %induction of Mur
par.kM2=0.5;     %Repression of Mur
par.kMm1=0.4;
par.kMm2=0.5;
par.MurT=1;

par.kR1=1.08;    % Induction of RpoN
par.kR2 =0.1;    % Repression of RpoN
```

```
par.kRm1=0.05;
par.kRm2 = 0.05;
par.RpoNT=1;

par.kC1=1;        % Induction of CodY
par.kC2=0.1;      % Repression of CodY
par.kCm1=0.05;
par.kCm2=0.05;
par.CodYT=1;

par.kF1=10;       % Induction of Fab
par.kF2 =0.5;     % Repression of Fab
par.kFm1= 0.4;
par.kFm2 = 0.5;
par.FabT=1;

par.kH1=0.04;      % 0.04Induction of Chap
par.kH2=1;     % 0.1Repression of Chap
par.kHm1=0.05;
par.kHm2 = 0.05;
par.ChapT=1;

par.kS1=5;        % Induction of SigB
par.kS2 =0.5;     % Repression of SigB
par.kSm1= 0.4;
par.kSm2 = 0.5;
par.SigBT=1;

% Hill function coefficients for HPP
par.Km=350;      % Repressor coefficient
par.KmCW=400;    % Repressor coefficient specifically for cell wall
par.nP=2;        % Hill coefficient for pressure
par.n1=10;       % Hill coefficiant for damage signal
end
```

### 8.3.4 Sensitivity Analysis

A script has been developed in order to test the sensitivity of the model by introducing a perturbation to all rate constants and then detecting the resulting states.

**SensitivityAnalysis.m**

```
% Sensitivity Analysis, to check all rate constants
clear all
kS2=0.5; % The rate constant of interest, changes
```

```
Ki=[kS2,(kS2+kS2*0.01)];  % Checking the value of rate constant with and
without 1% perturbation
for z=1:length(Ki)                    % First iteration, testing the initial
steady states
    par.kS2=Ki(z);
    [t0,x0]=CWMemProtChange(par);     % Taking in the function to be tested
    CW(:,z)=x0(:,1);                  % Checking the initial cell wall values
    Membrane(:,z)=x0(:,10);           % Checking the initial membrane values
    FP(:,z)=x0(:,4);                  % Checking the initial protein values
end

for zz = 1:length(Ki)                 % Second iteration, testing the steady
states after pressure relief
    par.kS2=Ki(zz);
    [t1,x1]=CWMemProtChange1(par);    % Taking in the function to be tested
    CW1(:,zz)=x1(:,1);                % Checking the cell wall state after
pressure relief
    Membrane1(:,zz)=x1(:,10);         % Checking the membrane state after
pressure relief
    FP1(:,zz)=x1(:,4);                % Checking the protein state after
pressure relief
end

% Output that provides the states:
CWconst=CW(end,:)
CWconst1=CW1(end,:)
Membraneconst=Membrane(end,:)
Membraneconst1=Membrane1(end,:)
Proteinconst=FP(end,:)
Proteinconst1=FP1(end,:)

% Sensitivity coefficients obtained from the perturbations
Scw=(CWconst(1)-(CWconst(2)))/(Ki(2)-Ki(1));
Scw1=(CWconst1(1)-(CWconst1(2)))/(Ki(2)-Ki(1));
SCW=[Scw,Scw1]
SMem=(Membraneconst(1)-(Membraneconst(2)))/(Ki(2)-Ki(1));
SMem1=(Membraneconst1(1)-(Membraneconst1(2)))/(Ki(2)-Ki(1));
SMEM=[SMem,SMem1]
Sfp=(Proteinconst(1)-(Proteinconst(2)))/(Ki(2)-Ki(1));
Sfp1=(Proteinconst1(1)-(Proteinconst1(2)))/(Ki(2)-Ki(1));
SFP=[Sfp,Sfp1]

% Visualization of the initial states
figure(1)
subplot(2,2,1)
plot(CW)
xlabel('Time (min)')
ylabel('CW');
subplot(2,2,2)
plot(Membrane)
xlabel('Time (min)')
```

```
ylabel('Membrane');
subplot(2,2,3)
plot(FP)
xlabel('Time (min)')
ylabel('FP');
legend(num2str(Ki(1)),num2str(Ki(2)));

% Visualization of the states after pressure relief
figure(2)
subplot(2,2,1)
plot(CW1)
xlabel('Time (min)')
ylabel('CW');
subplot(2,2,2)
plot(Membrane1)
xlabel('Time (min)')
ylabel('Membrane');
subplot(2,2,3)
plot(FP1)
xlabel('Time (min)')
ylabel('FP');
legend(num2str(Ki(1)),num2str(Ki(2)));
```

## 8.4 Appendix 4: Sensitivity coefficients

Sensitivity coefficients from the original and modified model where 1 % perturbation is applied. The given numbers are before normalization, which results in a higher number of influencing rate constants on each model.

**Original models:**

| *Parameter sensitivity cell wall model* | | |
|---|---|---|
| ***Variable*** | **Before pressurization** | **After pressure relief** |
| ***k1*** | 57,12 | 57,12 |
| ***d1*** | 7073,70 | 7073,70 |
| ***k2*** | 13,52 | 13,52 |
| ***d2*** | 286,30 | 286,46 |
| ***kM1*** | 0,64 | 0,64 |
| ***kM2*** | 12,81 | 12,81 |
| ***kR1*** | 0,98 | 0,98 |
| ***kR2*** | 10,62 | 10,62 |
| ***kC1*** | 2,27 | 2,27 |
| ***kC2*** | 22,62 | 22,62 |
| ***kF1*** | 0,03 | 0,03 |
| ***kF2*** | 0,68 | 0,68 |
| ***Sum*** | 12 rate constants | |

**Modified models:**

| *Parameter sensitivity cell wall model* | | |
|---|---|---|
| ***Variable*** | **Before pressurization** | **After pressure relief** |
| ***k1*** | 31,15 | 31,17 |
| ***d1*** | 6536,80 | 6540,50 |
| ***k2*** | 0,04 | 0,00 |
| ***k3*** | 0,03 | 0,00 |
| ***k4*** | 0,70 | 0,00 |
| ***d2*** | 0,00 | 0,62 |
| ***kM1*** | 2,93 | 2,94 |
| ***kM2*** | 58,50 | 58,55 |
| ***kR1*** | 7,61 | 7,61 |
| ***kR2*** | 82,04 | 82,08 |
| ***Sum*** | 10 rate constants | |

| *Parameter sensitivity membrane model* | | |
|---|---|---|
| ***Variable*** | **Before pressurization** | **After pressure relief** |
| ***d1*** | 0,00 | 0,01 |
| ***k3*** | 0,01 | 0,00 |
| ***k4*** | 0,05 | 0,11 |
| ***d2*** | 24523,00 | 24533,00 |
| ***k5*** | 0,00 | 0,01 |
| ***d3*** | 0,00 | 0,03 |
| ***kR1*** | 7,61 | 7,61 |
| ***kR2*** | 82,05 | 82,09 |
| ***kF1*** | 2,93 | 2,94 |
| ***kF2*** | 58,52 | 58,55 |
| ***Sum*** | 10 rate constants | |

| *Parameter sensitivity membrane model* | | |
|---|---|---|
| ***Variable*** | **Before pressurization** | **After pressure relief** |
| ***k1*** | 2,09 | 2,79 |
| ***d1*** | 443,54 | 591,81 |
| ***k2*** | 1022,10 | 1274,60 |
| ***k3*** | 61,45 | 0,03 |
| ***k4*** | 495,70 | 0,78 |
| ***d2*** | 22199,00 | 26722,00 |
| ***d3*** | 0,00 | 0,05 |
| ***d4*** | 0,00 | 1,26 |
| ***kM1*** | 0,20 | 0,26 |
| ***kM2*** | 3,92 | 5,28 |
| ***kR1*** | 6,20 | 7,62 |
| ***kR2*** | 67,01 | 82,09 |
| ***kF1*** | 2,58 | 3,20 |
| ***kF2*** | 51,65 | 63,81 |
| ***kH1*** | 0,02 | 0,00 |
| ***kS2*** | 0,02 | 0,00 |
| ***Sum*** | 16 rate constants | |

| *Parameter sensitivity protein model* | | |
|---|---|---|
| ***Variable*** | **Before pressurization** | **After pressure relief** |
| ***k4*** | 0,00 | 0,01 |
| ***d3*** | 0,00 | 0,03 |
| ***Sum*** | 2 rate constants | |

| *Parameter sensitivity protein model* | | |
|---|---|---|
| ***Variable*** | **Before pressurization** | **After pressure relief** |
| ***d1*** | 0,01 | 0,01 |
| ***k2*** | 0,02 | 0,00 |
| ***k3*** | 0,01 | 0,00 |
| ***k4*** | 0,27 | 0,01 |
| ***k5*** | 0,00 | 0,01 |
| ***k6*** | 0,00 | 0,03 |
| ***d3*** | 0,00 | 0,13 |
| ***d4*** | 18529,00 | 20272,00 |
| ***kR2*** | 0,01 | 0,00 |
| ***Sum*** | 9 rate constants | |