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REGULATORY FUNCTIONS OF SIGMA FACTORS OF RNA POLYMERASE SIGH AND SIG E IN *CORYNEBACTERIUM GLUTAMICUM*

Relatório de dissertação do Mestrado em Engenharia Biológica e Química

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JÚRI

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Dezembro 2019

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ABSTRACT

The study presented in this document was developed in the laboratory of molecular genetics of bacteria from the Institute of Microbiology of the Czech Academy of Sciences.

With the main objective of studying the structure of the promoters associated to the different sigma factors, the bacterium *Corynebacterium glutamicum* was used, a non-pathogenic bacterium widely used industrially in the production of amino acids. To this end were used four different promoters, two recognized by the sigma factor H and the other two by sigma factor E.

To make it possible to obtain the strains are necessary for the development of the study, first it was necessary to proceed with the preparation of plasmids pEC-XT99A and the pEPRI who bore the different sigma factors and the different promoters, respectively, being then necessary to carry out the processing of cells with plasmids.

The results of the activity of the promoters were obtained through fluorescence assays, through the pEPRI plasmid that contained the reporter gene that encodes for the green fluorescent protein (GFP).

KEYWORDS: *Corynebacterium glutamicum,* Transformation, Sigma Factor, Fluorescence Assay.

RESUMO

O estudo apresentado no presente documento foi desenvolvido no laboratório de genética molecular de bactérias do Instituto de Microbiologia da Academia Checa de Ciências.

Com o principal objetivo de estudar a estrutura dos promotores que se encontra associada aos diferentes fatores sigma, foi utilizada a bactéria *Corynebacterium glutamicum,* uma bactéria não patogénica bastante usada industrialmente na produção de aminoácidos. Para este fim foram utilizados quatro promotores diferentes, dois reconhecidos pelo fator sigma H e outros dois pelo fator sigma E.

Para que fosse possível obter as estirpes necessárias para o desenvolvimento do estudo, primeiramente foi necessário proceder à preparação dos plasmídeos pEC-XT99A e o pEPRI que carregavam os diferentes fatores sigma e os diferentes promotores, respetivamente, sendo em seguida necessário proceder à transformação das células com os plasmídeos.

Os resultados da atividade dos promotores foram obtidos através de ensaios de fluorescência, através do plasmídeo pEPRI que continha o gene repórter que codifica para a proteína verde fluorescente (GFP).

PALAVRAS-CHAVE: *Corynebacterium glutamicum,* Transformação, Fator Sigma, Ensaio de Fluorescencia.

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1. INTRODUCTION

The Czech Academy of Sciences hosts different institutes and one of them is Institute of Microbiology. The experimental work on this thesis was done in the Laboratory of Molecular Genetics of Bacteria of the Institute. Work in this Laboratory is focused on regulation of transcription in bacteria used in environmental and synthetic biotechnology, particularly corynebacteria and rhodococci.

The study presented in this thesis starts with the transformation of a wild type bacterial strain, into a double plasmid strain, with the principal objective of study in the structure of the promoters associated with different sigma factors. The transformation of bacteria is really useful in order to study the impact that certain changes have in the genome. In order to reach these aims, plasmids with different characteristics are introduced into the bacteria.

The aim of this thesis was to analyze relationship between the function of two important stress sigma factors in *Corynebacterium glutamicum*, SigH and SigE and to define important specific sequences in the promoters recognized by these sigma factors. Homology modeling showed that the two most important amino acids for recognition of promoters by these sigma factors are Met170 within SigH and Arg185 within SigE. To prove that these amino acids are those which allow different binding of the respective sigma factors to specific promoters *in vivo*, the mutation in the positions Arg185 and Met170 were done in such a way, that the amino acids were exchanged in SigH-mut170R and SigE-mut185M (Table 3). Two SigH-dependent and two SigE-dependent promoters were used in the study. The homology modeling suggested that in addition to the -35 region GGAA, which is present in both classes of promoters, the 5 nucleotides downstream of the -35 region affect the specificity of the promoters. These 5-nt sequences were exchanged in the promoters PtrxB1, PrshA (both SigH-dependent), PcseA and Pcg3309 (both SigE-dependent) (Table 2). The activity of all combinations of sigma factors and promoters were used for measuring promoter activity using a two-plasmid system and fluorescence assay of promoter activity (Section 3.6).

For the study developed and reported in the present document *Corynebacterium glutamicum* was used. It is a non-pathogenic bacterium, mainly used in industrial production of amino acids, which have its gene expression regulated by different sigma factors, that are strongly associated with the different types of stresses.

As mentioned, the principal aim of the project was to study the structure of four different promoters, which are preferentially recognized by sigma H and sigma E. Four original promoters and four others with a mutation were used, to better understand the dependence of each promoter on these different sigma factors.

Along with the wild type strains (*C. g.* pEC-XT99AsigH and *C. g.* pEC-XT99AsigE) two mutated strains (*C. g.* pEC-XT99AsigHmut170R and *C. g.* pEC-XT99AsigEmut185M) were used, to get a clear confirmation of the results. Two other strains, *C. g.* pEC-XT99AsigA and *C. g.* pEC-XT99A, were used as control. The activity of the promoters was studied by measurements of fluorescence produced by the reporter, green fluorescent protein (Gfp).

2. STATE OF KNOWLEDGE

This chapter presents the theoretical foundations, necessary to have a better understanding of the work done during the research, as well as of the results, are presented.

2.1. Cell transformation

Both prokaryotic and eukaryotic cells have specific functions and their own mechanisms, that allow them to produce all the intermediates which are necessary to perform these functions. Some bacterial species are receptive to new DNA, *i.e.*, to receive new DNA that gives them new characteristics that originally, they did not have. This process of the insertion of new genetic material into cells is called cell transformation. The most common method for the transformation of bacteria uses circular DNA molecules as vectores, which can be isolated from bacteria, redesigned and re-introduced into cells. This type of DNA molecules is called plasmids [1].

This type of molecules does not integrate into the chromosomal DNA of the cell but uses specific mechanism to replicate itself. Since the plasmids don't interfere with the DNA, they will not cause any inconvenience to the host, making it possible to the cell to acquire the characteristics that are in the plasmid without changing its original chromosomal DNA [1,2].

2.2. Cells transformation using plasmids

Plasmids are molecules of circular DNA, which can be found in bacteria, that can replicate by themselves and which are dispensable to the genome of the organism, *i.e.*, it uses the host resources to replicate themselves, but since they are not in the genome, can play this role alone. This is a huge advantage to science, since it can therefore rely on these molecules to transform different organisms, causing them to produce, for example proteins, that they do not usually produce. Another great advantage of the utilization of plasmids is the fact that a single bacterium may harbor different plasmids [3].

The fundamental characteristics of plasmid DNA as vector molecules are the existence of an origin of replication, a multiple cloning site and a marker of selection, for example a gene for resistance to a particular antibiotic, such as tetracycline [3].

The cell transformation using plasmid consists in three fundamental steps. At the first step it is necessary to make competent cells, the second is to prepare the plasmid with all desired characteristics, and after these two steps the transformation is performed, as illustrated in Figure 1 [4].

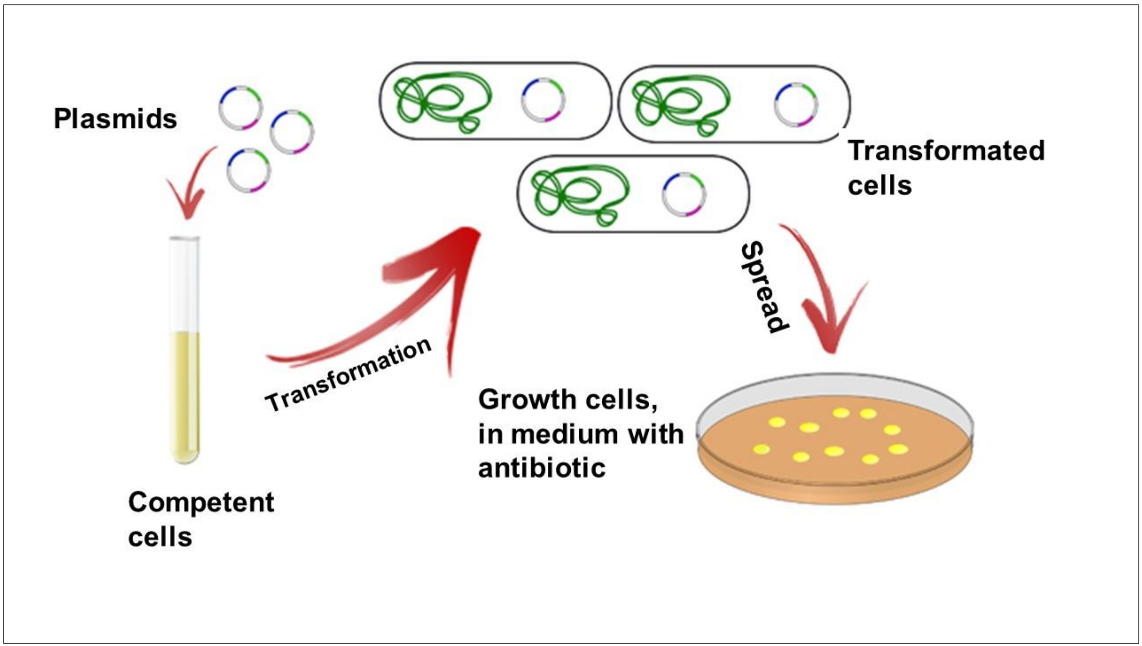

Figure 1- Transformation steps.

2.3. What are sigma factors

Sigma factors, also known as bacterial transcription factors, are the subunits of the RNA polymerase, responsible for the recognition of the promoter region of DNA, *i.e.* are responsible for recognizing the place where the transcription of the DNA starts. The first sigma factor discovered was in *Escherichia coli* and it was the sigma 70, which can bind to the RNA polimerase core, forming a holoenzyme that can make a specific interaction with the DNA and initiate the transcription [5,6].

These subunits are consequently the key of the regulatory elements that control different classes of promoters and activate expression of their respective groups of genes, or regulons, a type of operons all under the control of the same regulator [7,8].

Under specific nutritional composition of the growth medium and environmental conditions, sigma factors can control different functions of the bacterial cells. There are also the anti-sigma factors, which are responsible for the inhibition of sigma factors [5,6].

2.4. *Corynebacterium glutamicum*

Corynebacterium glutamicum is a gram-positive bacterium, known as non-pathogenic and non-sporulating, that belongs to the order Actinomycetales. In this order are also included other bacteria such as *Mycobacterium, Rhodococcus, Streptomyces* among others. [9]

This bacterium has an important role in industrial production of various amino acids as well as other metabolites. *C. glutamicum* genome encodes seven sigma factors of RNA polymerase: the primary sigma factor SigA (σ^A), the primary-like σ^B and five alternative sigma factors (σ^C, σ^D, σ^E, σ^H and σ^M) [5,8].

The sigma factor A is an essential primary sigma factor which is responsible for the transcription of the largest part of genes that are expressed during exponential growth, called the housekeeping genes (σA-dependent)- Their promoter sequences (particularly regions −35 and −10) match the generally accepted consensus of housekeeping promoters. The consensus sequence of σ^A-dependent promoters was deduced from a large number of defined promoters which are believed to be σA-dependent [5,8].

The sigma factor B is a non-essential primary-like σ factor that is present in the highest levels on *C. glutamicum* cells at the transition growth phase as well as in the stationary phase, it is involved in responses to various stresses, for example acid and heat stress. In addition to its involvement in stress-protective functions, σ^B is responsible for the transcription of the genes active in glucose utilization during exponential growth. Sigma factor B can be considered a σ factor necessary for proper slow growth and general stress conditions [5,8,10].

The sigma factor H is the most studied *C. glutamicum*'s sigma factor, and is the one which controls a transcriptional regulatory network enabling the *C. glutamicum* cell to respond to temperature, oxidative and growth-phase induced stresses. The consensus sequence −35 (g/tGGAAt) and −10 (t/cGTTgaa) was defined based on the 45 proposed σH-regulated promoters [5,8,10].

The sigma factor E was found to be involved in heat and cell surface stress responses. It is known that there is an overlap in promoter recognition specificity for σ^H and σ^E. The consensus sequence of σE-specific promoters has not yet been determined [5,8].

3. MATERIAL AND METHODS

3. 1. Strains and plasmids

Corynebacterium glutamicum ATCC 13032 was used as a basic strain. The strains used for measuring promoter activity contained two plasmids: pECXT-99A (carrying tetracycline resistance marker) was the vector for overexpression of the *sig* genes, that encode the sigma factors. pEPR1 (kanamycin resistance marker) was the promoter-test vector carrying the tested promoter. Each *C. glutamicum* strain harbored two plasmids, the pEC-XT99A (containing the gene encoding a sigma factor) and the pEPR1 (containing different promoters).

Table 1 shows combinations of plasmids harboured by the tested two-plasmid *C. glutamicum* strains.

Table 1- Combinations of plasmids harboured by two-plasmid C. glutamicum strains used in this thesis.

Promotor	pEC-XT99AsigH	pEC-XT9AsigE	pEC-XT99AsigH mut170R	pEC-XT99AsigE mut185M	pEC-XT99AsigA	pEC-XT99A
PtrxB1	X	X	this study	this study	X	X
PtrxB1-mut -35	X	X	this study	this study	X	X
PrshA	X	X	this study	this study	X	X
PrshA-mut -35	X	X	this study	this study	X	X
Pcg3309	X	X	this study	this study	X	X
Pcg3309-mut -31	this study	X	this study	this study	X	X
PcseA	X	X	this study	this study	-	X
PcseA-mut -31	X	X	this study	this study	X	X

[1]*The strains marked by "X" were already available in the Laboratory*

Table 2 presents the wild-type sequences of the promoters as well as sequences of the mutant promoters.The mutants have the same sequences as the original promoters except for the part that is underlined in Table 2. The underlined parts within the mutant promoter sequences, dependent originally on sigma H, came from the parts of the sigma E dependent promoters, shown in italics and vice versa.

Table 2- Promoters and their mutants used in the study.

Promoter	Sigma Dependence[1]	Sequence[2]
PtrxB1	H	…TGGCCC**GGAA***TAACT*ACAGTCCGCTGAAA**GTT**GTCTATATA…
PtrxB1-mut	N	…TGGCCC**GGAA**CTTTCACAGTCCGCTGAAA**GTT**GTCTATATA…
PrshA	H	…ATCGT**GGAA***GAAAA*CAGCTCCGAGGAAT**GTT**AAAGGAAGTAG…
PrshA-mut	N	…ATCGT**GGAA**CTTATCAGCTCCGAGGAAT**GTT**AAAGGAAGTAG…
Pcg3309	E	…CCCGTG**GGAA***CTTTC*CTCTAACTAAGTGC**GTT**AAGGAAGGTA...
Pcg3309-mut	N	…CCCGTG**GGAA**TAACTCTCTAACTAAGTGC**GTT**AAGGAAGGTA...
PcseA	E	…GGACAG**GGAA***CTTAT*CACAGGCGACATCC**GTT**TTGAGTAGTA...
PcseA-mut	N	…GGACAG**GGAA**GAAAACACAGGCGACATCC**GTT**TTGAGTAGTA...

[1]N marks the unknown dependence of the mutant promoters under study.
[2]Sequences in bold mark the promoter consensus elements -35 and -10 which are identical in SigH- and SiE-dependent promoters.

The used two plasmids, pEC-XT99A and pEPR1, confer resistance for two different antibiotics, tetracycline and kanamycin, respectively. In culture they should be used in specific concentrations, 30 µg/ml for kanamycin and 10 µg/ml for tetracycline. The antibiotics must be used accordingly with the plasmid(s) that are in the cells.

In table 3 are presented the original amino acids sequences of gene-encoding sigma factors E and H, as well as the mutations made in both sequences.

Table 3- Segments of the amino acids sequences of each original sigma factor and their respective mutations.

Sigma Factor	Amino acids sequence[1]
SigH	MDVPLGTVMSRLHRGRK
SigH-mut170R	MDVPLGTVRSRLHRGRK
SigE	ETLGVKMGTVRSRIHRGR
SigE-mut185M	ETLGVKMGTVMSRIHRGR

[1]*The amino acids marked in green are the original amino acids. Only one amino acid was mutated in each sequence (SigH: M => R; SigE: R => M) which is marked in yellow.*

3.2. Preparation of *C. glutamicum* competent cells

To prepare the *C. glutamicum* competent cells it was necessary to put 10 ml of 2 x TY complex liquid medium (Annex I) on a 100ml flask inoculated with one colony from a plate and let to grow on shaker, overnight at 30 ºC. If the cells already had any plasmid it was necessary to add the corresponding antibiotics in their respective concentrations, tetracycline (10 µg/ml) kanamycin (30 µg/ml).

After overnight incubation, optical density (O.D.) of the culture was measured and the appropriate volume of the overnight culture in order to obtain an O.D. of 0.2.was added into two 500-ml flasks with 65 ml of 2 x TY complex medium.

The volume of overnight culture which is necessary, to reach the O.D. of 0.2 was calculated using the equation 1.

$$Vc = \frac{Vf * O.D.f}{O.D.i} \tag{1}$$

Where:
Vc corresponds to the volume of culture wich is necessary; Vf representing the final volume of culture that will be used, which in this case is 65 ml; $O.D._f$ is the final O.D., which in this case is 0.2; and $O.D._i$ that corresponds to the O.D. of the overnight culture.

The cultures were incubated on a shaker with vigorous shaking at 30 ºC until they reached an O.D. between 0.4 and 0.6, which usually corresponded to approximately 2.5 hours.

At this point, the cultures where palced into ice to rest for 15 minutes, to stop the growth. Cells were centrifuged, 30 ml each time, in 50 ml falcon tubes, for 10 min at 4 ºC and at 4500 rpm. The supernatant was discarded and the pellet was washed, and resuspended in 15 ml of cold (4 °C) GT solution (Annex I) and the suspensions were joined into only one tube and centrifuged again at the same conditions. The supernatant was removed again, and the washing step was repeated.

The pellet was resuspended in 1 ml of cold G (Annex I) solution and transferred into a 2ml Eppendorf tube. This Eppendorf tubes were centrifuged at 4 ºC, with at 10 000 rpm, for 3 min, and the supernatant was again discarded. To complete the preparation of the competent cells, the cells were resuspended in 1 ml of cold GT solution and divided into aliquots of 170 µl. The competent cells could be used immediately after this procedure for transformation or stored at - 80 ºC.

3.3. Mutagenesis of plasmid pEC-XT99sigH

For the mutagenesis of the plasmid pEC-XT99AsigH into plasmid pEC-XT99AsigHmut170R, the kit “Q5 Site-Directed Mutagenesis Kit” from BioLabs, was used. This kit allows to make any site-specific mutations using the oligonucleotide primers. With this kit it is possible to create insertions, deletions and substitutions, quickly and efficiently.

The kit contains three essential steps, exponential PCR amplification, Kinase-Ligase-DpnI (KLD) Reaction and transformation as illustrated in Figure 2. The primers should be diluted fifty times to use on the first step, and the DNA template used in this step was the plasmid pEC-XT99AsigH.

The aim was to exchange a single amino acid, a methionine (M) into an arginine (R), as shown in table 3.

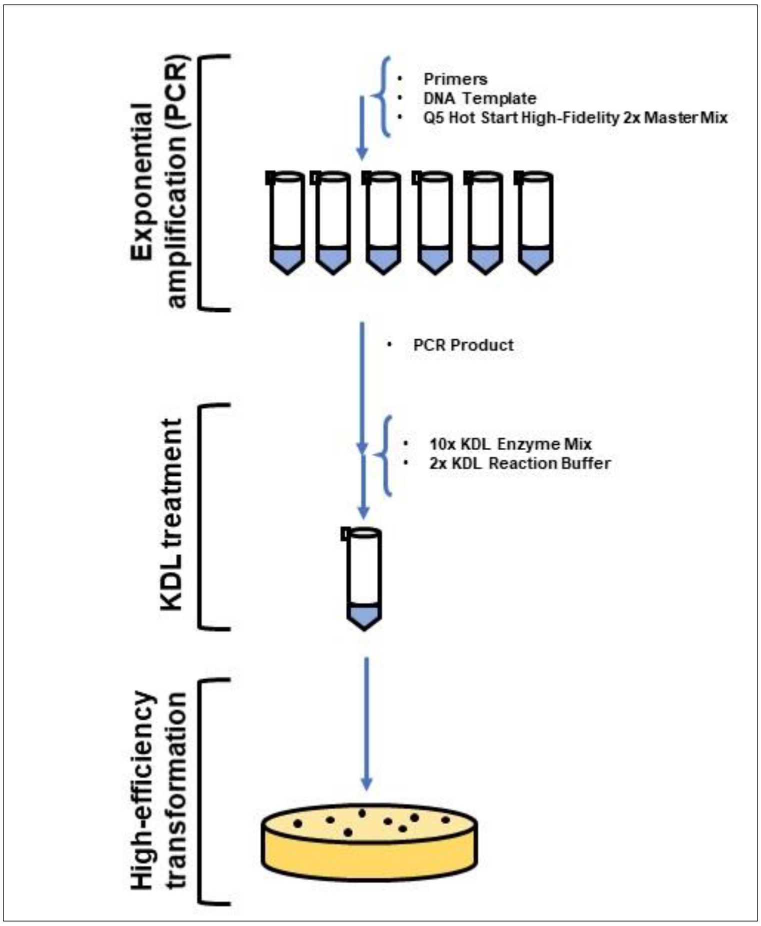

Figure 2- Q5 Site Mutagenesis Kit Overview.
At the first step it is done the exponential amplification, using primers, DNA template and the master mix Q5 Hot Start High-Fidelity DNA Polymerase. The second step is the KDL treatment, where the PCR Product is incubated with kinase, ligase and Dpnl, which provides its rapid circularization and the elimination of DNA template. The last step is a high-efficiency transformation of competent cells.

The primers should be designed according to the intended mutation, because these are used to incorporate into the plasmid DNA the substitutions, deletions or insertions (Figure 2).

3.2.1. Substitutions

To create substitutions, the forward primer should have a more than ten nucleotides and should contain the alteration (Figure 2). The reverse primer should start on the nucleotide immediately before of the first nucleotide of the forward primer and proceed in the opposite direction. Similarly, to the forward primer, the reverse primer has more than ten nucleotides and contains the complementary strand.

3.2.2. Deletions

To delete a part of the plasmid, the primers should be designed in order to flank the region that should be deleted (Figure 2). Both primers have to be designed in on opposite directions with the 5' ends adjacent to the deleted region, and they could contain mismatches or insertions if desired.

3.2.3. Insertions

The insertions could be considered small or largen depending on the number of nucleotides to insert (Figure 2). To insert 6 nucleotides, or less, the sequence should be added on the 5' end of the primer forward. Both primers anneal back-to-back. If the sequence has more than 6 nucleotides it should be split and added to the 5' end of the both primers.

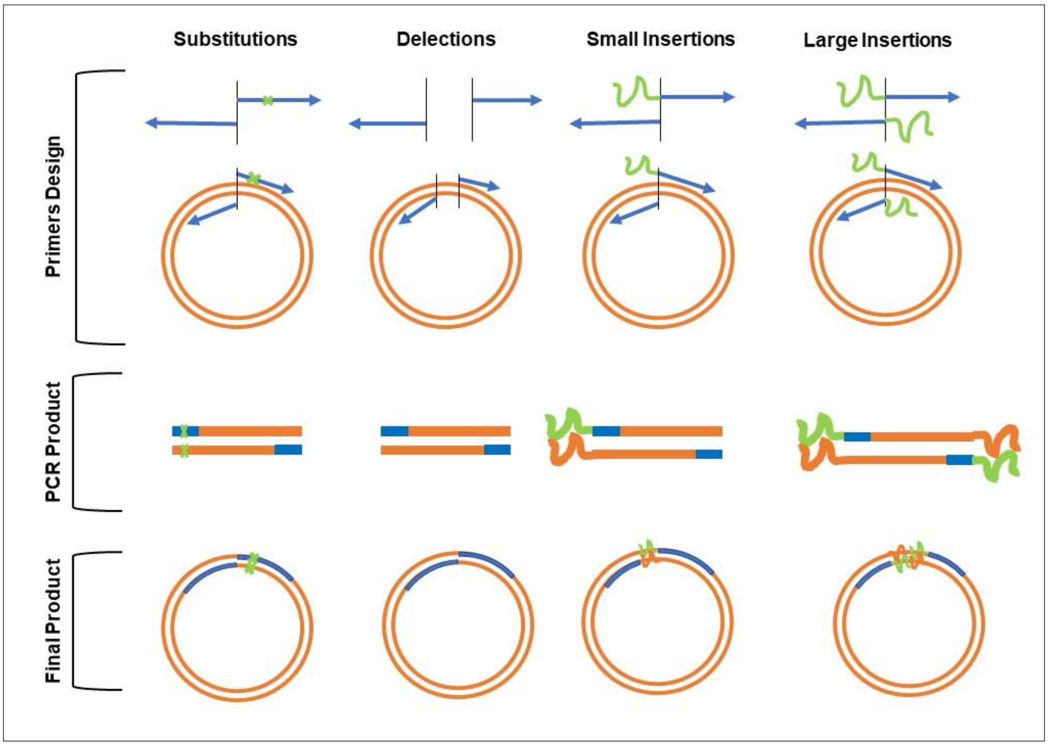

Figure 3- Primers design to use on Q5 Site Mutagenesis Kit from BioLabs.
To create Substitutions, the desire nucleotide changes (denoted by green X) should be in included on primer forward (blue arrow pointing to the right) and have at least 10 nucleotides on the 3' end. The primer reverse (blue arrow pointing to the left) should be designed so that the 5' ends of the two primers anneal back-to-back; To make Deletions the primers should be designed by standard strategy and flank the region that should be deleted; The small insertions (with less or equal to 6 nucleotides) should be incorporated on primer forward, and similarly to Substitutions, both primers should anneal back-to-back at 5' ends. Large Insertions are created incorporating half of the desire insertion into the 5' ends of both primers.

In Table 4 it is possible to find the original sequence of *sigH* gene encoding, as well as the primers used for the site mutagenesis. As it is possible to verify, the mutation is present on the forward primers, SIGHmutM-RF and SIGHmutM-RF2.

In this study, to create the desired mutation (M → R) two different combinations of primers were used:

- SIGHmutM-RF + SIGHmutM-RRa
- SIGHmutM-RF2 + SIGHmutM-RR2

Table 4- Original sequence of sigH gene and primers used for site-directed mutagenesis.

Original Sequence	**5'– GATCATGGACGTTCCACTCGGAACTGTG**ATG**TCCCGACTCCATC -3'**
SIGHmutM-RF	5' - CGGAACTGTG**CGC**TCCCGACTCCATC - 3'
SIGHmutM-RRa	5' - AGTGGAACGTCCCTGATCTCGG - 3'
SIGHmutM-RF2	5' - G**CGC**TCCCGACTCCATCGTGG - 3'
SIGHmutM-RR2	5' - ACAGTTCCGAGTGGAACGTCCAT - 3'

3.3. Plasmid DNA isolation

To initiate the isolation of plasmid DNA from *E. coli* (or *C. glutamicum*) a cell culture was made, in a tube with 5 ml of 2x TY. The culture should have the right antibiotics for the selection of the cells in use. The culture grew overnight, on shaker at 30 ºC. After this time, it was centrifugated at room temperature, for 2 minutes at 14000 rpm, to collect the cells. The cells were frozen at -18 ºC for at least 30 minutes.

The buffers used in the next steps (P_1, P_2 and P_3) were all from the kit “Plasmid Purification Kit” from QIAGEN.

In the next step the cells were resuspended in 200 µl of cold Buffer P_1 (resuspension buffer) supplemented with RNase A and 100 µl of lysozyme (10 µg/ml) was added. This suspension rested at 37 ºC, and the time depends on the strain, if the cells were from *E. coli,* they rested for 15 minutes and, if are cells were from *C. glutamicum* they were incubated at 37 ºC for 2 hours. Then, 400 µl of Buffer P_2 (lysis buffer) were added and mixed gently turning the tubes up and down, because vortex could degrade the DNA, and the mixture was at room temperature, approximately 5 min. Subsequently 300 µl of Buffer P_3 (neutralization buffer) was added, mixed gently and put in 4 ºC for 15 min.

After the 15 min the suspension was centrifugated for 10 min at 10 ºC at 14000 rpm, and the supernatant was collected into a new tube. To this tube 1450 µl of cold 96%ethanol , was added well mixed and put in 4 ºC for 20 minutes. Ethanol precipites DNA, so after this time the suspension was centrifugated one more time at the same conditions as in the previous step and the supernatant was discarded. The sediment (DNA) was resuspended in 75 µl of sterile acetate-MOPS (Annex I) and 150 µl of cold ethanol was added, well mixed and kept again 20 minutes at 4 ºC.

In the end, the suspension was centrifuged again at the same conditions, the supernatant was completely removed, and DNA was dissolved in 50 µl of 10 mM TRIS. This DNA solution can be used directly or stored at –18 ºC.

3.4. Transformation by electroporation

The transformation by electroporation was performed on a GENE PULSER at the following conditions:

- Voltage: 2.5 kV
- Resistance: 600 Ω
- Capacitance: 25 µF

In one tube with 170 µl of competent *C. glutamicum* cells, approximately 5 µl of plasmid DNA (1 to 5 µg) was added and rested on ice for 1 min. Then the mixture was transferred into an electroporation cuvette (previously cooled), carefully to avoid forming bubbles, inserted in the holder and pushed between the electrodes. After pushing the pulse buttons, the time of impulse is shown, and it should be between 8 and 14 milliseconds. Immediately after the impulse, 2 x TY was added to make up 1 ml, transferred into a new tube and incubated at 46 ºC for 6 minutes.

After the heat shock the tube was placed on a shaker at 30 ºC for 2 h to allow the cell regeneration and then centrifuged for 1 min at room temperature at 14000 rpm. Then, 800 µl of supernatant were removed and the cells resuspended on the rest of supernatant and transferred into a plate of 2 x TY with the respective antibiotics. The plate was incubated at 30 ºC for at least 48 h.

3.5. Confirmation of the transformants

After the transformation it was necessary to prove that both plasmids were in cells and that the plasmid pEC-XT99A carries the correct *sigH* gene. Before both these steps it was necessary to isolate the plasmids and then perform PCR and a digestion with the enzyme PstI.

It was possible to prove in the laboratory if the correct sigma factor was in in the plasmid. To confirm the presence of the correct promotor the isolated DNA was sent to a sequencing service.

3.5.1. Confirmation of plasmid structure by PCR

Two primers were used to amplify the region containing the sigma factor-encoding genes: the forward primer 1691 (specific for *sigH*) and the reverse primer 1695 (common multiple cloning site primer in this plasmid). According to the restriction map of pEC-XT99A/sigH (Figure 4), these primers amplify the region that contains the *sigH* gene, from the 12th nucleotide until 872nd nucleotide, which means that the PCR product should have 871 bp (Figure 5).

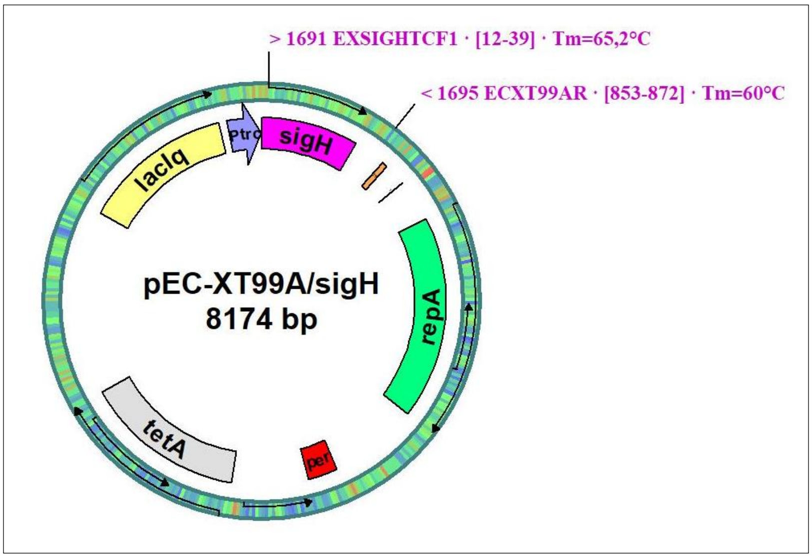

Figure 4- Map of the plasmid pEC-XT99A/sigH, only with the used primers.

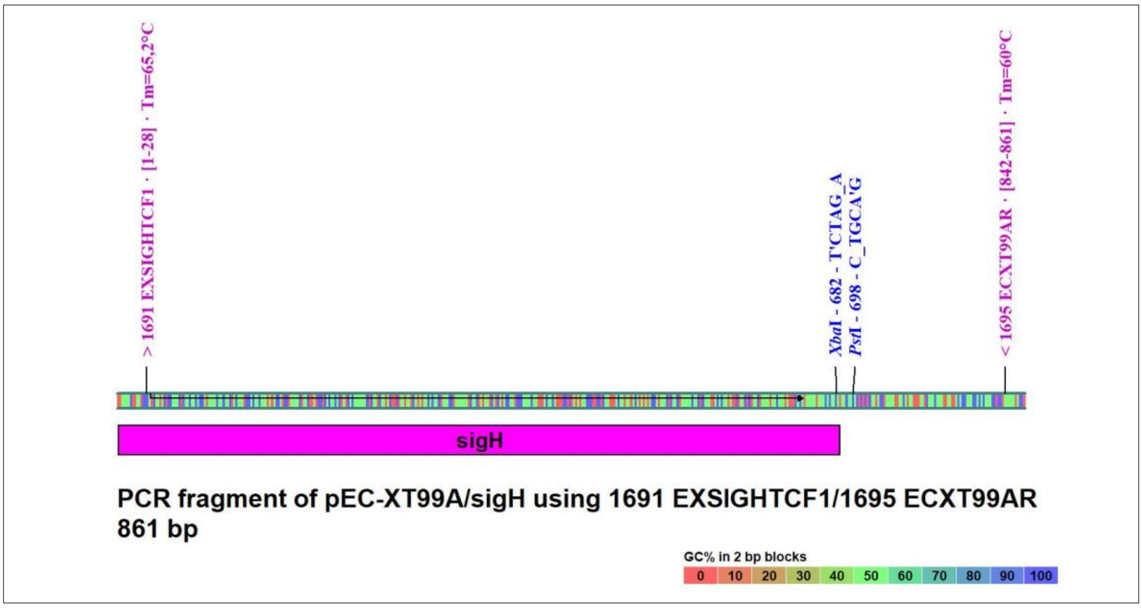

Figure 5- PCR product, using the 1691 forward primer and 1695 reverse primer.

3.5.2. Confirmation by restriction with the PstI enzyme

The next step confirming the presence of the plasmid construct with the *sigH* gene, was the digestion with the restriction enzyme PstI. The plasmid pEC-XT99A has five restriction sites for PstI (Figure 6), and digestions thus provides five fragments with different sizes. One of these fragments is too short (only 48 bp) to visualize it on a gel.

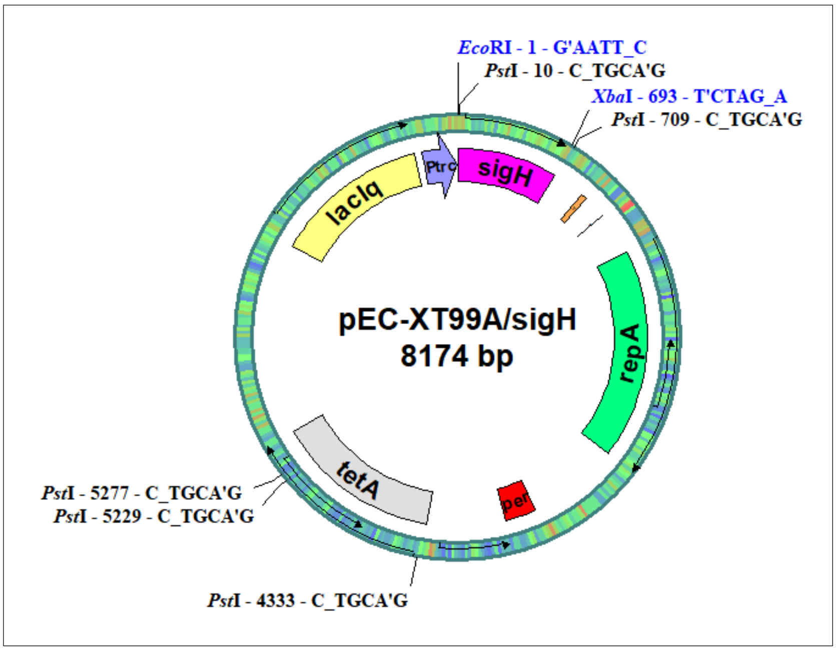

Figure 6- Restriction map of the plasmid pEC-XT99A, with the restriction sites for PstI.

After performing the restriction with PstI, the fragments were observed by agarose gel electrophoresis (2% w/v). The result should look like the results for pEC-XT99AsigH presented on figure 7.

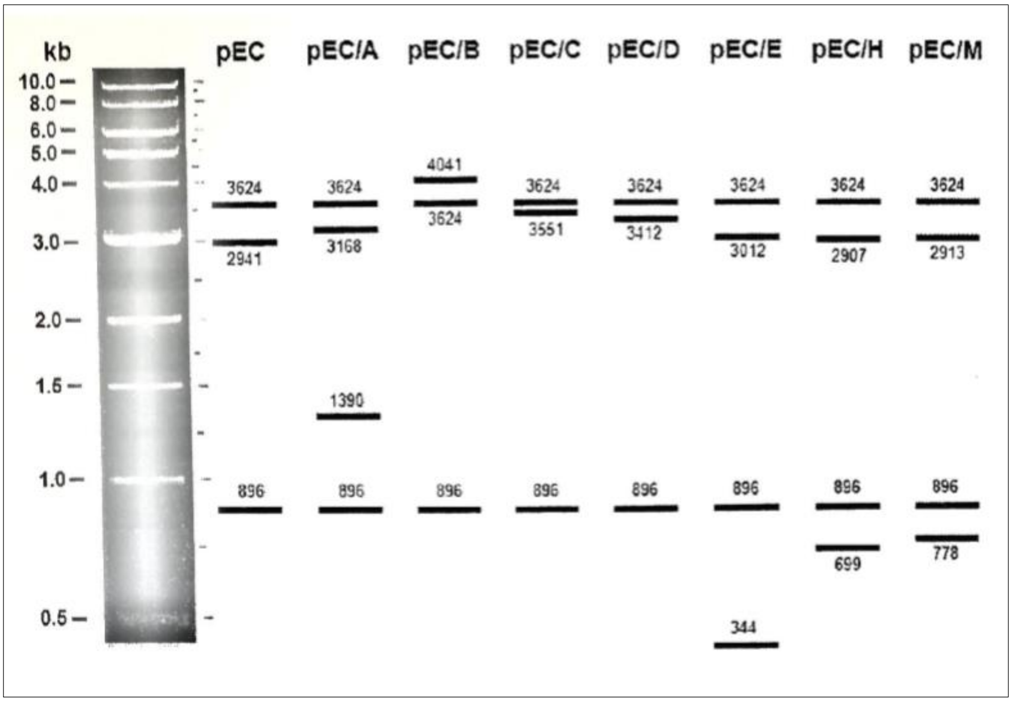

Figure 7- Theoretical restriction patterns' of pEC-XT99A with all cloned sig.genes of C. glutamicum cut by PstI.
The abbreviation pEC/A means fragments generated from the plasmid pEC-XT99A/sigA. The same is shown for other genes sigB, C, D, E, H, M.

3.6. Fluorescence assay

In this study was measured each different promotor separately, so in each fluorescence assay, six different cultures were used and each one was tested with and without IPTG induction, which is a total of twelve flasks per assay. In every experiment were tested four different cultures (pEC-XT99AsigH + pEPRI(Promoter); pEC-XT99AsigHmut170R + pEPRI(Promoter); pEC-XT99AsigE + pEPRI(Promoter); pEC-XT99AsigEmut170R + pEPRI(Promoter)) and two controls were used (pEC-XT99AsigA + pEPRI(Promoter); pEC-XT99A + pEPRI(Promoter)).

For the fluorescence experiment firstly, the cultures were prepared (Figure 8). Each fresh culture was inoculated from a Petri dish, into a 100 ml flask, containing 10 ml of 2 x TY, supplemented with antibiotics, and incubated overnight on a shaker at 30 ºC.

The next step was measuring the optical density of overnight cultures with the purpose of determining the necessary volume to obtain an O.D. of 0.6. This volume was calculated by equation 1, and transferred to inoculate. Then on a 500 ml flask with 100 ml of 2 x TY and antibiotics, that was incubated on shaker at 30 ºC until the O.D. was 1, approximately 2 hours. Once O.D.=1, the induction was made, as well as the first harvest. Into new 500 ml flasks was transferred 40 ml of each culture and 40 µl of IPTG 1M was added. All the flasks returned to the shaker at 30 ºC, and from then on, the harvests were taken at 3 h, 6 h and 24 h.

Every harvest was made exactly the same way (Figure 8). At first it was necessary measure the O.D. and according to this value, the corresponding volume of culture was transferred into a tube (consult the table in Annex II) and centrifuged at 5000 rpm for 5 minutes at 4 ºC (for smaller volumes the centrifugation was at 10000 rpm for 1 minute). The supernatant was removed, and the cells were resuspended in 1 ml of cold PBS Solution (Annex I). The centrifugation was repeated at the same conditions and then 500 µl of cold PBS Solution were added (All the samples were kept on ice during the experiment). For these experiments, a table shown in Annex III, was used to record the data, for a better organization of all information.

After all harvests, the lane volume of sample was transferred into a FASTPREP tube, already prepared with 200 µl of Matrix B (Glasperlrn Ø 0.25 – 0.5 mm) and put on MP FASTPREP instrument. Three cycles were performed on MP FASTPREP with an interval of 6 minutes between each other.

The cell lysis was performed on MP FASTPREP at the following conditions:

- Speed: 6.5 m/s
- Rotation 24 x 2
- Time: 60 s

After the lysis step it was performed a centrifugation at 14000 rpm at 4 ºC for 20 min and then, approximately 250 µl of the supernatant was transferred into a new Eppendorf tube. The centrifugation was repeated at the same conditions.

When the samples were all prepared, the fluorescence intensity was measured and the protein concentration of each one.

3.6.1. Protein concentration assay

At first it was necessary to prepare the standard solutions for the calibration curve:

- 0 mg BSA/ml
- 0.1 mg BSA/ml
- 0.25 mg BSA/ml
- 0.5 mg BSA/ml
- 0.75 mg BSA/ml
- 1 mg BSA/ml

Then, 20 µl of each standard solution was put into a cuvette, to which 980 µl of Bradford reagent was added (Annex I).

To prepare the cuvettes with samples, 10 µl of sample was transferred into the cuvette and 10 µl of water and 980 µl of Bradford reagent were added. The cuvettes had to rest for 20 min and then the protein concentration was automatically calculated by the spectrophotometer and recorded in a table (Consult table 3 on Annex II).

3.6.2. Fluorescence intensity measurement

In a Nunclon Delta Surface plate (Thermo scientific) it was put 200 µl of each sample. Then the fluorescence intensity was measured by the “Safire 2” device, with the following conditions:

- Measurement mode: Fluorescence Intensity/ Endpoint
- Plate: NUN96ft.pdf
- Measure: plate
- Fixed wavelength: GFPuv
- Excitation wavelength: 397 nm
- Emission wavelength: 509 nm
- Excitation bandwidth: 5.0 nm
- Emission bandwidth: 20.0 nm
- Gain (Manual): 80
- Lag time: 0
- Number of reads: 4
- Read mode: bottom
- Integration time: 40µs
- Time between move and flash: 10 ms
- Target temperature: 25 ºC
- Current temperature: 25 ºC
- Shaking before measuring
- Shake duration: 5 s
- Mode: Orbital
- Intensity: Medium
- Shake settle time 10 s

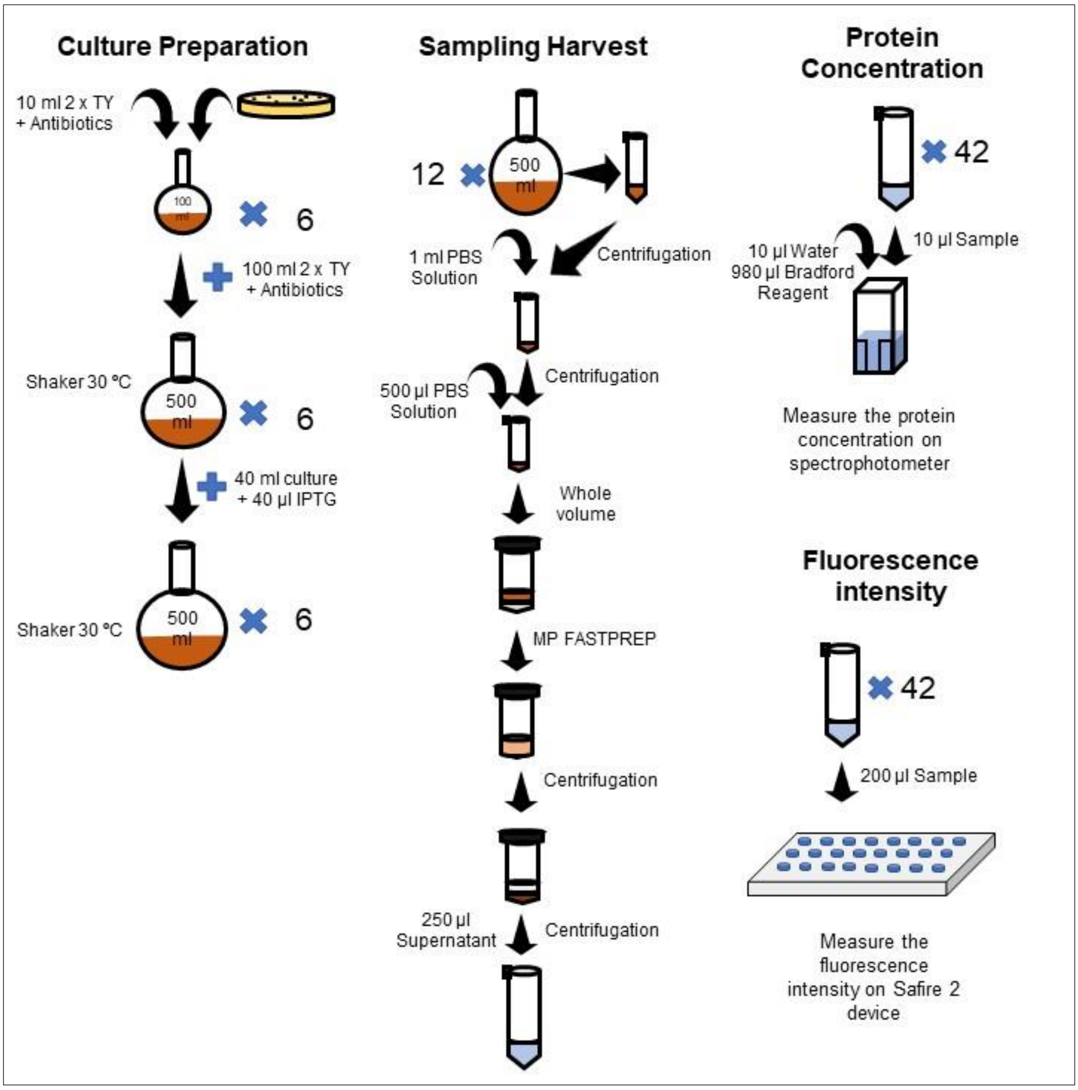

Figure 8- Schematic diagram of fluorescence assay.

For culture preparation the antibiotics are used in their respective concentration (kanamycin;30 µg/ml) Tetracycline;10 µg/ml). The volume of culture that should be transferred is calculated according to the O.D.; The harvesting step should be performed at the beginning (time 0) and after 3, 6 and 24 h; The last steps of fluorescence assay are the measurements of protein concentration and fluorescence intensity.

4. RESULTS AND DISCUSSION

4.1. Mutation of the *sigH* gene encoding sigma factor SigH

Sigma factors SigH and SigE of *C. glutamicum* exhibit a similar structure and recognize similar promoters. To prove which amino acids are those which allow different binding of the respective sigma factors to specific promoters, the mutation in the positions Arg185 and Met170 within SigH and SigE, respectively, were proposed on the basis of *in silico* homology modelling (I. Barvik, Institute of Physics, Cherles university, Prague).

The first task of this study was to create the desired mutation in the DNA sequence of the *sigH* gene encoding sigma factor H. The mutation on sigma E DNA sequence was already available in the laboratory.

For this step the “Q5 Site-Directed Mutagenesis Kit” was used to create the mutation in the *sigH* gene sequence that was cloned in the plasmid pEC-XT99A. The whole process was made with the kit and cloning in *E. coli.* Two primer combinations, SIGHmutM-RF + SIGHmutM-RRa and SIGHmutM-RF2 + SIGHmutM-RR2, were tested.

After transformation of *E. coli*, approx. ten colonies appeared on the agar plates with both combinations of primers. Two colonies of each plate were selected to confirm if the correct plasmid was really constructed.

- <u>colony number 1</u>- *E. coli* / pEC-XT99AmutsigH-170R, primer combination: SIGHmutM-RF + SIGHmutM-RRa;

- <u>colony number 2</u>- *E. coli* / pEC-XT99AmutsigH-170R, primer combination: SIGHmutM-RF + SIGHmutM-RRa;

- <u>colony number 3</u>- *E. coli* / pEC-XT99AmutsigH-170R, primer combination: SIGHmutM-RF2 + SIGHmutM-RRa2;

- <u>colony number 4</u>- *E. coli* / pEC-XT99AmutsigH-170R, primer combination: SIGHmutM-RF2 + SIGHmutM-RRa2.

To proceed with the confirmation, plasmid DNA was isolated from the selected colonies and critical regions were sequenced to guarantee that the mutation was successfully introduced.

Both colonies of both plates presented the correct sequence, so any of them could be further used. Thus, the first important step of the study, introducing the chosen mutation in SigH (Met170$\rightarrow$Arg), was successfully completed. To proceed with the study colony number 1 was used.

4.2. Transformation of *C. glutamicum* cells with pEC-XT99AsigH-mut170R

From *E. coli /* pEC-XT99AmutsigH-170R, plasmid DNA was isolated, to use it for transformation of the wild type *C. glutamicum* cells. Before the transformation, it was necessary to make competent *C. glutamicum* cells (see section 3.2). In Table 5, growth of cultures during the preparation of competent cells is shown.

Table 5. Optical density of C. glutamicum cultures A and B during cultivation for preparation of competent cells

C. glutamicum WT	t (min)	OD_{600}
A / B	0	0,22 / 0,22
A / B	60	0,30 / 0,29
A / B	90	0,34 / 0,34
A / B	120	0,48 / 0,47
A / B	135	0,51 / 0,51

Once the competent *C. glutamicum* cells were prepared, transformation (using the plasmid pEC-XT99AmutsigH-170R) by electroporation was performed. The time of electroporation charge was 8.9 ms, which is within the acceptable range. After the incubation, twenty-two colonies appeared on the plate. Ten of these colonies were transferred onto a new plate to start the confirmation. At first only five of these ten colonies were confirmed by PCR and by digestion with the restriction enzyme PstI.

In Figure 9, the electrophoresis gel with the PCR product, using the specific forward primer for sigH 1691 and reverse primer 1695 is presented. It was possible to confirm that all the PCR products (covering the *sigH* gene) have approximately the right size (861 bp) since all bands were localized between 800 bp and 900 bp.

In figure 10, the results of the plasmid digestion with the enzyme PstI are documented. Since five clones presented a correct PCR product of the right size, only two clones were digested with PstI. Four bands with different sizes in the gel were compared with the expected sizes of fragments in the restriction map (Figure 6). It was apparent that both molecular clones had the right sizes. We concluded therefore that these two clones harbour the plasmid pEC-XT99AmutsigH-170R.

As both clones were correct, the clone number 1 was used to continue the study.

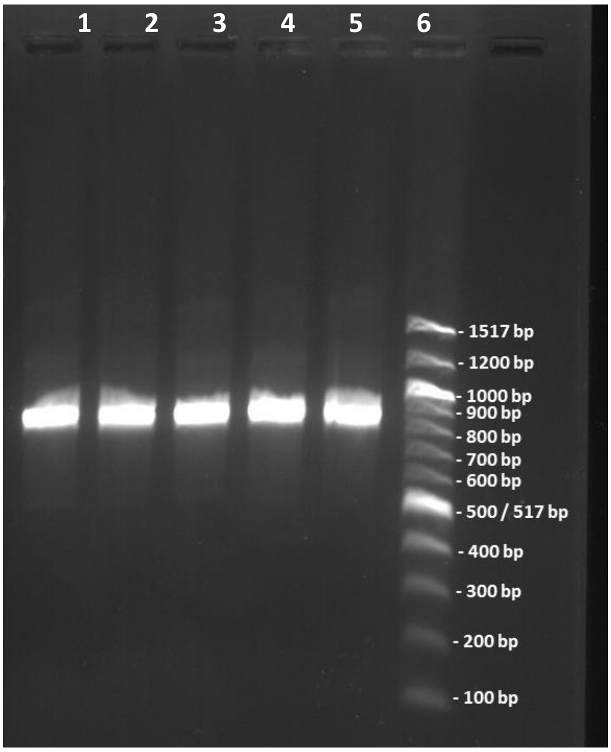

Figure 9- Electrophoresis gel with the PCR products obtained with primers 1691 and 1695. PCR products with the template plasmid pEC-XT99AmutsigH-170R isolated from five tested colonies of C. glutamicum (lanes 1 to lane 5) show approximately the expected size of 860 bp, as compared with the ladder (lane 6).

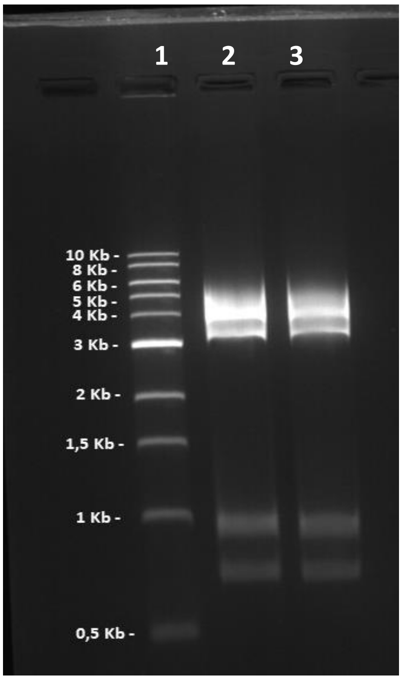

Figure 10- Electrophoresis gel with the results of digestion of XT99AmutsigH-170R with the enzyme PstI.

Comparing the samples (lane 2 and 3) with the ladder (lane 1) it is possible to see two bands between 3 kb and 4 kb which correspond to 2907 bp fragment and 3624 bp fragment and two other bands between 0.5 kb and 1 kb, which corresponds to 896 bp fragment and 699 fragment.

4.3. Transformation of *C. g.* pEC-XT99AsigH-mut170R with pEPR1 constructs

After preparing *C. g.* pEC-XT99AsigH-mut170R competent cells, transformation with the pEPR1 constructs was performed. The plasmid constructs were already prepared and confirmed with each promoter in the list.

During the preparation of competent cells, the cultures grew according with Table 6.

The transformation was repeatedly performed until the strains were all ready.

In the first transformation, growth of strains *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PcseA-mut (seven colonies) and *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PrshA (one colony) was observed. All the colonies were transferred onto a new plate.

In this step, presence of both plasmids and a correct sigma factor cloned in pEC-XT99AsigH-mut170R in the cells were tested. The plasmids were isolated from the cells and PCR and the digestion with PstI were performed.

Table 6- Optical density of cultures during C. g. pEC-XT99AsigH-mut170R competent cells preparation.

C. g. pEC-XT99AsigH-mut170R	t (min)	O.D.
A/B	0	0,24 / 0,25
A/B	60	0,32 / 0,33
A/B	90	0,37 / 0,39
A/B	120	0,47 / 0,49
A/B	135	0,51 / 0,55

For PCR, the forward primer 1691 and the reverse primer 1695 were used. These primers should create a 861-bp product, corresponding to sigma factor H. After PCR, electrophoresis with the products was carried out. According to Figure 11 the products in lanes 1, 2, 4, 5, 7 and 8, have the right size, so these plasmids were tested by digestion with the restriction enzyme PstI. The lanes 1, 2, 3, 4, 5, 6 and 7 correspond to *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PcseA-mut and in lane 8 is the PCR product of *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PrshA.

For check by plasmid digestion, only 3 colonies of *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PcseA-mut and 1 colony of *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PrshA that presented expected PCR products were used. In Figure 12, the gel obtained after the electrophoresis is presented, and it is possible to verify that only the first lane had fragments with right sizes. It is also visible a band with a bigger size which corresponds to pEPR1 plasmid. The lanes 1, 2, and 3 have fragments corresponding to *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PcseA-mut and on lane 4 are the fragments of *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PrshA.

We concluded that the required strain *C. g.* /pEC-XT99AsigH-mut170R+pEPRI-PcseA-mut was obtained from the first transformation.

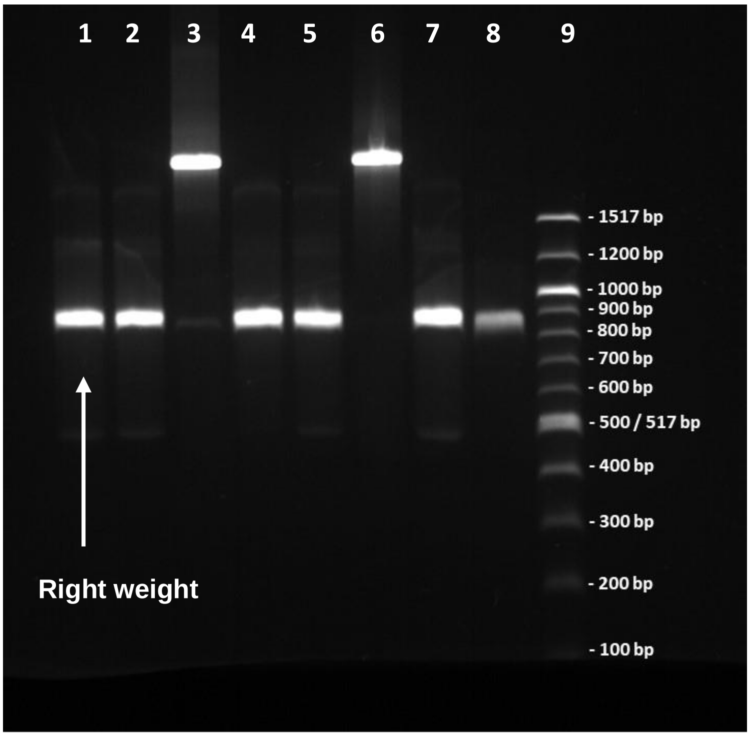

Figure 11- Electrophoresis gel containing the PCR product obtained with primers 1691 and 1695, from C. g. pEC-XT99AsigH-mut170R/pEPRI-Promoter.

From lane 1 to lane 7 are the PCR products of the seven tested colonies of C. g. pEC-XT99AsigH-mut170R/pEPRI-PcseA- mut, and on lane 8 is the PCR product of the single colony of C. g. pEC-XT99AsigH-mut170R/pEPRI-PrshA. It is possible to observe that the products presented on the lanes 1, 2, 4, 5, 7 and 8 have the right size (861 bp), comparing with the ladder (lane 9), which means that these six colonies might carry the right sigma factor.

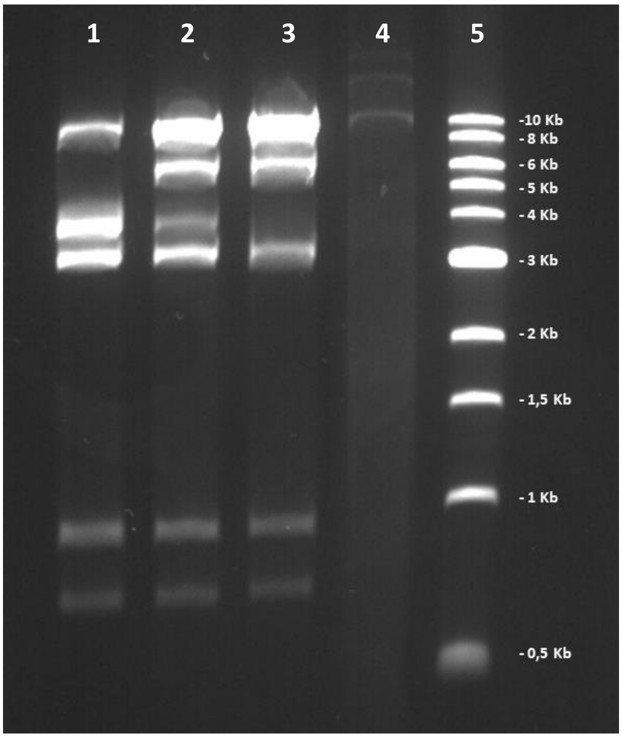

Figure 12- Electrophoresis gel with the fragments obtained after digestion of mut170R/pEPRI-PcseA-mut; with the enzyme PstI.

On lanes 1, 2 and 3 are the three tested colonies of C. g. pEC-XT99AsigH-mut170R/pEPRI-PcseA-mut; on lane 4 is the only colony tested of C. g. pEC-XT99AsigH-mut170R/pEPRI-PrshA. Comparing the fragments with the ladder (lane 5) it was possible to conclude that only the clone 1 shows fragments with right sizes. The band which presents a size between 8 Kb and 10 Kb corresponds to plasmid pEPR1.

The next transformations were performed analogously using pEPR1 with the other seven promoters. After this step all the plates presented more than fifty colonies, so ten colonies were transferred of each strain into a new plate and first only one colony of each strain was analyzed.

Figure 13 presents the electrophoresis gels with the PCR products of each strain. The first gel (Figure 13A) contains the PCR products of the strains *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PrshA-mut (lane 1), *C. g.* pEC-XT99AsigH-mut170R/pEPRI-Pcg3309 (lane 2) and *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PcseA (lane 3) and all strains present the right size of PCR products for sigma factor H.

The second gel (Figure 13B) contains the PCR products of the strains *C. g.* pEC-XT99AsigH-mut170R/pEPRI-Ptrx B1 (first lanes), *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PtrxB1-mut (second lane), *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PrshA (third lane) and *C. g.* pEC-XT99AsigH-mut170R/pEPRI-Pcg3309-mut (fourth lane). In contrast to the first gel not all strains presented the right PCR product size. As it is possible to observe, the strain *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PtrxB1 showed no PCR product, which means that in this case, the strain does not carry the right sigma factor.

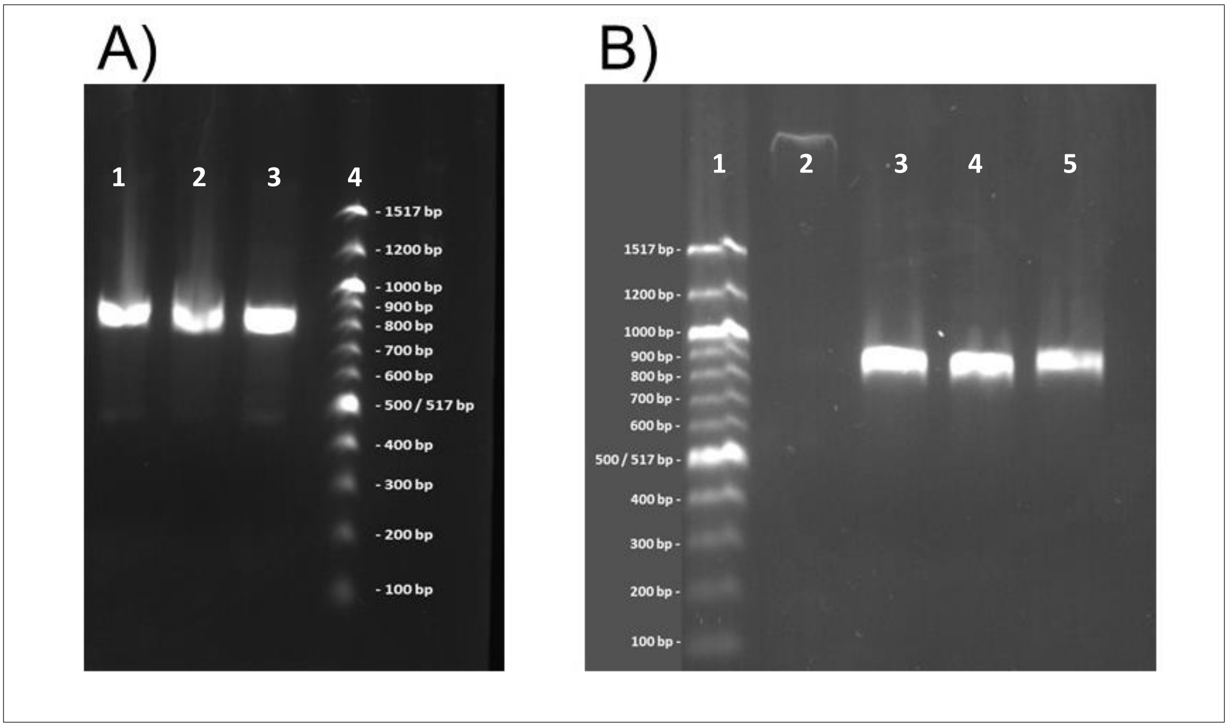

Figure 13- Electrophoresis gel with PCR products obtained with primers 1691 and 1695, from C. g. pEC-XT99AsigH-mut170R/pEPRI-Promoter.

A) Lane 1- C. g. pEC-XT99AsigH-mut170R/pEPRI-PrshA-mut; Lane 2- C. g. pEC-XT99AsigH-mut170R/pEPRI-Pcg3309; Lane 3- C. g. pEC-XT99AsigH-mut170R/pEPRI-PcseA; B) Lane 2- C. g. pEC-XT99AsigH-mut170R/pEPRI-PtrxB1; Lane 3- C. g. pEC-XT99AsigH-mut170R/pEPRI-PtrxB1-mut; Lane 4- C. g. pEC-XT99AsigH-mut170R/pEPRI-PrshA; Lane 5- C. g. pEC-XT99AsigH-mut170R/pEPRI-Pcg3309-mut. With the exception of C. g. pEC-XT99AsigH-mut170R/pEPRI-PtrxB1, all the strains presented a PCR product with a size between 800 bp and 900 bp, which means that these strains could contain the right sigma factor gene encoding.

Since the colony potentially with *C. g.* pEC-XT99AsigH-mut170R/pEPRI-PtrxB1 was not correct, it was necessary to verify if some required colonies are on the original agar plate. Thus four more colonies of this strain were confirmed (colony 2 to 5).

In Figure 14A the gel of electrophoresis with the PCR products of all colonies (lanes 2 to 5) performed with same primers (1691 and 1695) is presented, and compared with the DNA ladder (lane 1). It is possible to see that all of them presented a product with the right size, which proved that they contain the *sigH* gene.

The result of digestion with the restriction enzyme PstI is presented in Figure 14B, where it is possible to confirm with certainty that only lanes 2 and 4 presented right sizes of fragments, hence only these two have the mutated *sigH*.

Since both colony 3 and colony 5 were correct, colony 3 was chosen to continue the study.

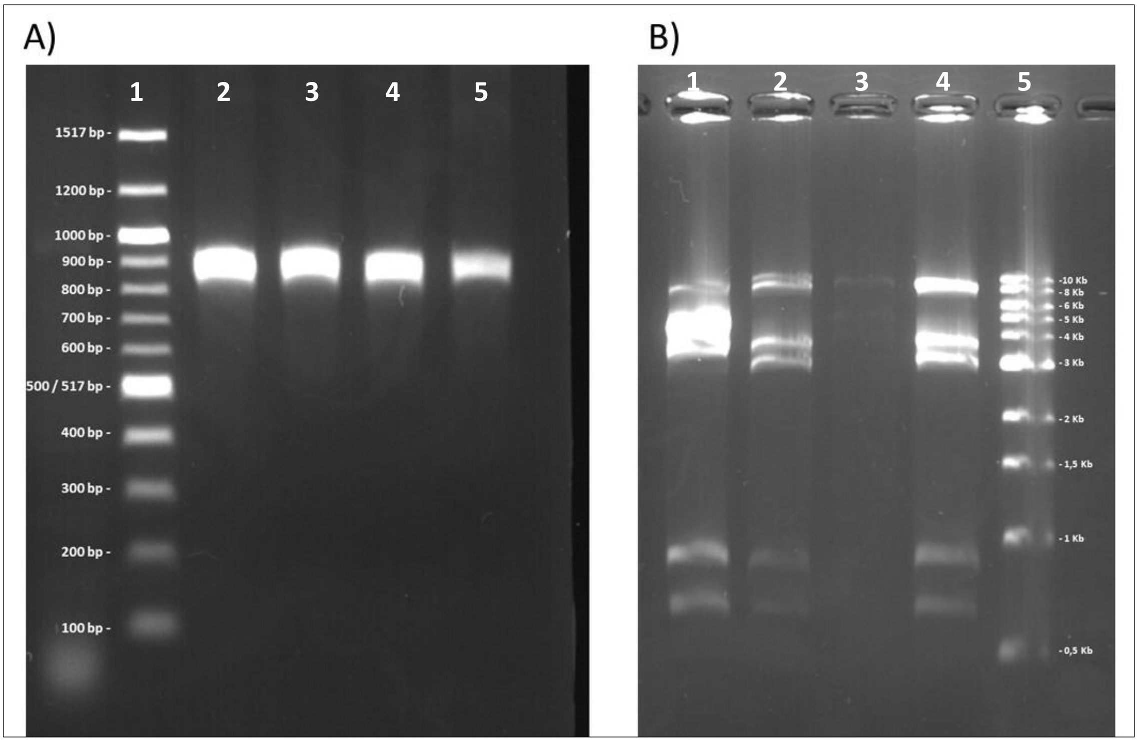

Figure 14- Confirmation of colonies 2 to 5 of C. g. pEC-XT99AsigH-mut170R/pEPRI-PtrxB1, by PCR and digestion with PstI.

A) Lanes 2 to 5 – colonies 2 to 5, all samples presented the right size of PCR product (861 bp); B) Lanes 1 to 4- colonies 2 to 5, only the colonies 3 and 5 presented the right size of fragments after the digestion with the restriction enzyme.

After preparing all strains they were stored in glycerol at –80 ºC.

As soon as all required strains were ready it was possible to start to measure promoter activities by means of the measurement of Gfpuv reporter fluorescence.

Determination of promoter activity

4.4. Determination of promoter activity

To determine promoter activity, in total 16 fluorescence measurements were performed, in replicate for each promoter.

The following groups of strains, with one of the eight different promoters under study, were assayed in this work:

- *C. g.* pEC-XT99A /pEPR1-P_promoter (no sigma factor)
- *C. g.* pEC-XT99AsigA /pEPR1- P_promoter (SigA as a control)
- *C. g.* pEC-XT99AsigH /pEPR1- P_promoter
- *C. g.* pEC-XT99AsigH-mut170R/pEPRI- P_promoter
- *C. g.* pEC-XT99AsigE /pEPRI- P_promoter
- *C. g.* pEC-XT99AsigE-mut185M/pEPRI- P_promoter

The first two strains were used as controls.

All the fluorescence assays were made twice and the graphs that present the promoter activity show the average and the standard deviation of both measurements.

The promoters used: PtrxB1, PtrxB1-mut, PrshA, PrshA-mut Pcg3309, Pcg3309-mut, , PcseA, PcseA-mut (for sequences, see Table 2)

4.4.1. Activity of promoter PtrxB1 (SigH-dependent)

The *trxB1* gene encodes a disulfide oxido-reductase that catalyzes a wide spectrum of redox reactions in the cell and is involved in oxidative stress response. Wild type form of the promoter PtrxB1 has already been studied and was found to be controlled by stress sigma factor SigH (Busche et al, 2012). The most expected result for this promoter was therefore that the highest activity should be in *pEC-XT99AsigH*-overexpressing *C. glutamicum* strain.

The graph presented in Figure 15 shows the promoter activity in each strain, and it confirmed that this promoter is strongly SigH-dependent. A weaker activity was detected with the mutant sigma SigHmut170R. According to the expectation, SigE did not provide any activity with PtrxB1. Surprisingly, neither was any activity detected with the mutant

SigE-mut185M, a variant of SigEturned more into a sigH-like sigma factor, by carrying methionine at position 185.

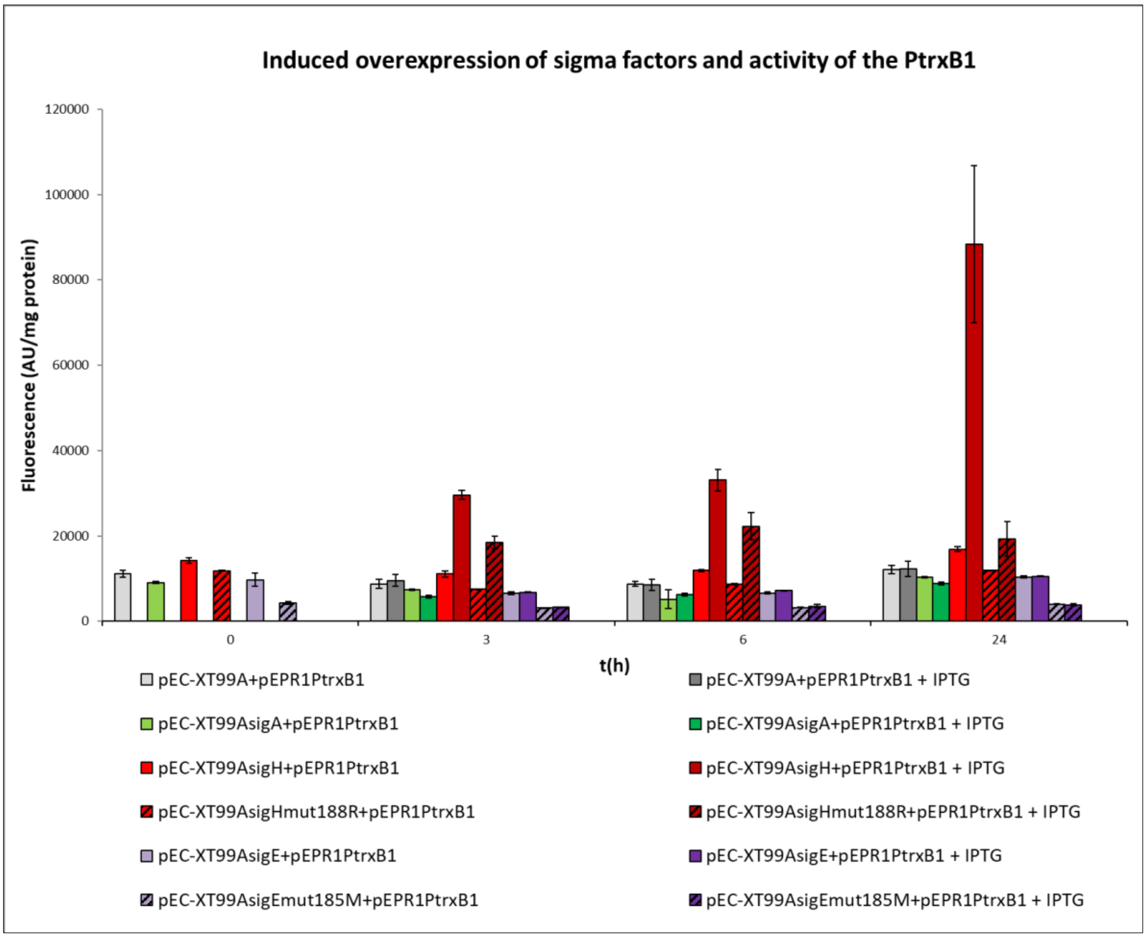

Figure 15- PtrxB1 promoter activity in C. glutamicum strains with different sigma factors.

The promoter activity was measured by Gfpuv fluorescence intensity of cell extracts presented in the graph by different colours corresponding to each sigma factor. All C. glutamicum strains carried the plasmid pEC-XT99A containing a sigma factor encoding gene and the promoter PtrxB1 on the promoter-test vector pEPR1 that carried the Gfpuv gene. All strains were tested with (striped) and without (non-striped) the induction of sig gene expression by IPTG. The strains carrying no sigma-encoding gene and SigA-*encoding gene were used as controls. The standard deviations of two measurements are depicted by error bars.*

As it was expected, the activity of PtrxB1was high with *C. g. pEC-XT99AsigH* which confirmed that this promoter is strongly dependent on SigH. Surprisingly, PtrxB1 tested in the strain *C. g. pEC-XT99AsigHmut170R* showed also some activity, which means that this SigH mutant can still recognise this promoter sequence although with lower efficiency than sigma H without any mutation. According to the expectations, no activity was observed with SigE. Moreover, the mutation which should make SigE more SigH-like also did not result in any promotor activity. The conclusion is that PtrxB1 is highly specific for SigH and some other parts of SigH protein are probably involved in the interactions with the promoter. It seems from this result, that SigH is less specific in recognition of promoters and so robust, that even the key AA mutation did not eliminate its activity with PtrxB1 completely.

4.4.2. Activity of promoter PtrxB1-mut

The promoter PtrxB1-mut had never been studied before, but since it was mutated to be more SigE-dependent we expected its activity with *C. g. pEC-XT99AsigE* strain. The activity with *sigH-mut170*, which was mutated to be more *sigE*-like, was also expected.

The graph shown in Figure 16 confirmed that the experimental results correspond to the expected ones. This promoter presents the highest activity with *C. g. pEC-XT99AsigHmut170R* and is also active with *C. g. pEC-XT99AsigE*.

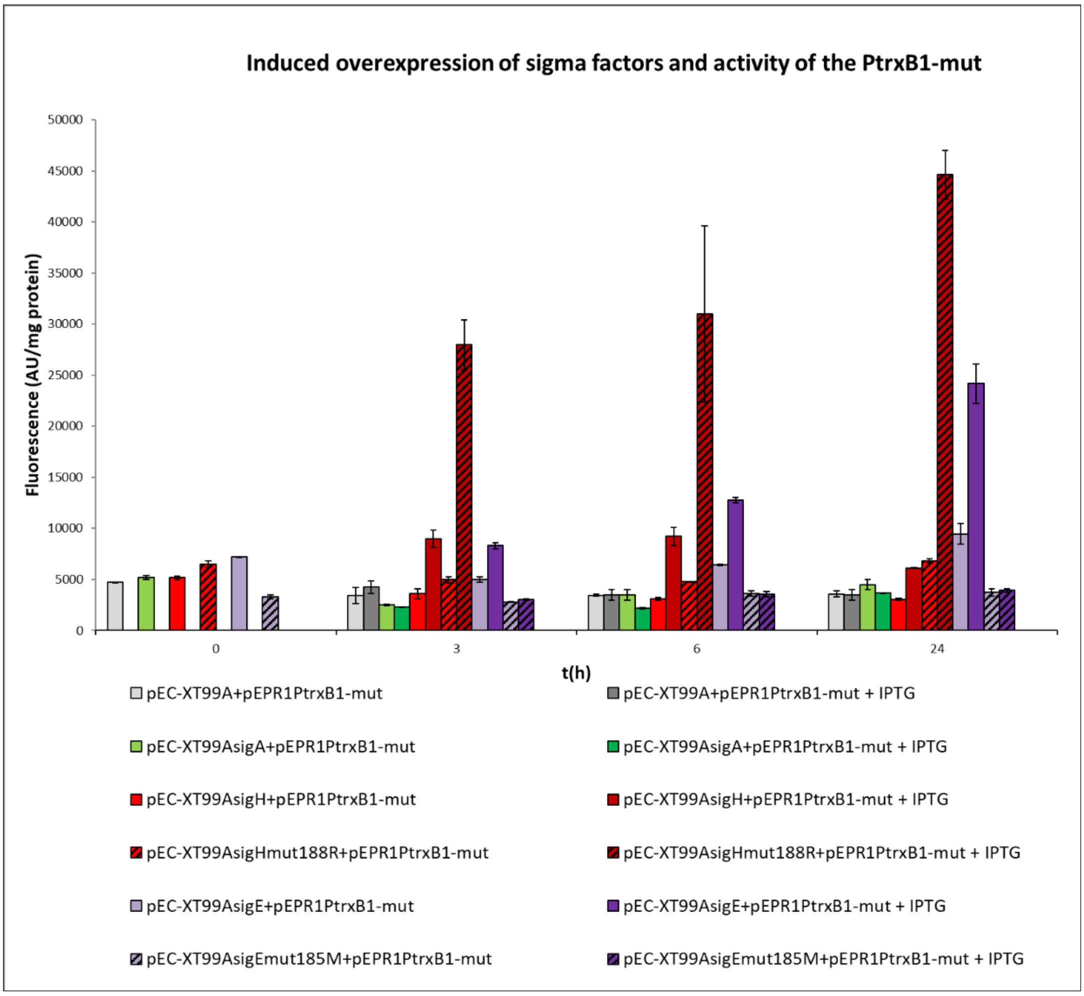

Figure 16- PtrxB1-mut promoter activity in C. glutamicum strains with different sigma factors.

The promoter activity was measured by Gfpuv fluorescence intensity of cell extracts presented in the graph by different colours corresponding to each sigma factor. All C. glutamicum strains carried the plasmid pEC-XT99A containing a sigma factor encoding gene and the promoter PtrxB1-mut on the promoter-test vector pEPR1 that carried the Gfpuv gene. All strains were tested with (striped) and without (non-striped) the induction of sig gene expression by IPTG. The strains carrying no sigma-encoding gene and SigA-*encoding gene were used as controls. The standard deviations of two measurements are depicted by error bars.*

According to the graph (Figure 16) it was possible to confirm the expected result. As the activity of this promoter is higher for *C. g. pEC-XT99AsigHmut170R,* this means that the mutation that was performed on sigma H-encoding gene had a significant influence on the recognition of the promoter sequence and it is also possible to prove that the region – 31 of the promoter has a strong influence on the dependency of promoter to a given sigma factor. This theory is reinforced by the activity of the promoter with *C. g. pEC-XT99AsigE,* which was slightly higher than the activity of PtrxB1 with the non-mutated sigma H (Figure 15). The activity of the PtrxB1-mut (which was mutated to be SigE-dependent), with SigH confirms the lower specificity of SigH.

4.4.3. Activity of promoter PrshA (SigH - dependent)

The *rshA* gene encodes anti-sigma H and it is therefore understandable that it is SigH-dependent. When *sigH* is expressed during the heat or oxidative stress, anti-sigma H is concomitantly produced. This regulation should probably ensure that enough anti-sigma molecules are available to inhibit SigH activity as soon as the stress conditions are over. This promoter PrshA in its wild type form has already been studied and was found to be strongly SigH-dependent. With this information, the most expected result was that the highest activity of this promoter should be in the *C. g. pEC-XT99AsigH* strain.

After performing the fluorescence assay with this promoter, the graph presented on Figure 17 was obtained. In this graph it is possible to see that PrshA showed activity in *C. g. pEC-XT99AsigH*, however independenly on IPTG induction, since the values are almost the same with and without IPTG induction (hence *sigH* overexpression). PrshA was also active in the strain *C. g. pEC-XT99AsigHmut170R*. The PrshA promoter was not active in

As the results were not as expected, and contradicted previous data, it was necessary to confirm both strains (*C. g. pEC-XT99AsigH* and *C. g. pEC-XT99AsigHmut170R*), to be sure that the strains have the right promoter and right sigma factor.

Since the promoters could have been confused, during the transformation step, it was considered necessary to analyze not only these two strains, but also the strains *C. g. pEC-XT99AsigH / pEPRI_PrshA-mut* and *C. g. pEC-XT99AsigHmut170R / pEPRI_PrshA-mut*, to detect the promoter carried by pEPR1.

To test the strains at first it was necessary to isolate de plasmids from them, and to determine the DNA concentration from each one, which were the following quantities:

- *C. g. pEC-XT99AsigH / pEPRI_PrshA* = 212 ng / µl
- *C. g. pEC-XT99AsigHmut170R / pEPRI_PrshA* = 220 ng / µl
- *C. g. pEC-XT99AsigH / pEPRI_PrshA-mut* = 320 ng / µl
- *C. g. pEC-XT99AsigHmut170R / pEPRI_PrshA-mut* = 371 ng / µl

Minimum quantity of DNA for sequencing is 1 µg, so from each strain, 5 µl of DNA was used, and the respective primers for each sequence.

After sequencing the plasmid DNA extracted from four strains it was confirmed that one of them was not right. It was shown that the strain *C. g. pEC-XT99AsigH / pEPRI_PrshA was in fact the strain C. g. pEC-XT99AsigM / pEPRI_PcseA*.All other strain were correct. Since this was found at the end of the stay in the Laboratory, the correct strain and repeating the fluorescence measurement will be done by colleagues in the laboratory. The value used in Fig. 17 for this strain was taken from previous results obtained by Hana Dostálová (Institute of Microbiology, Prague) with the correct strain.

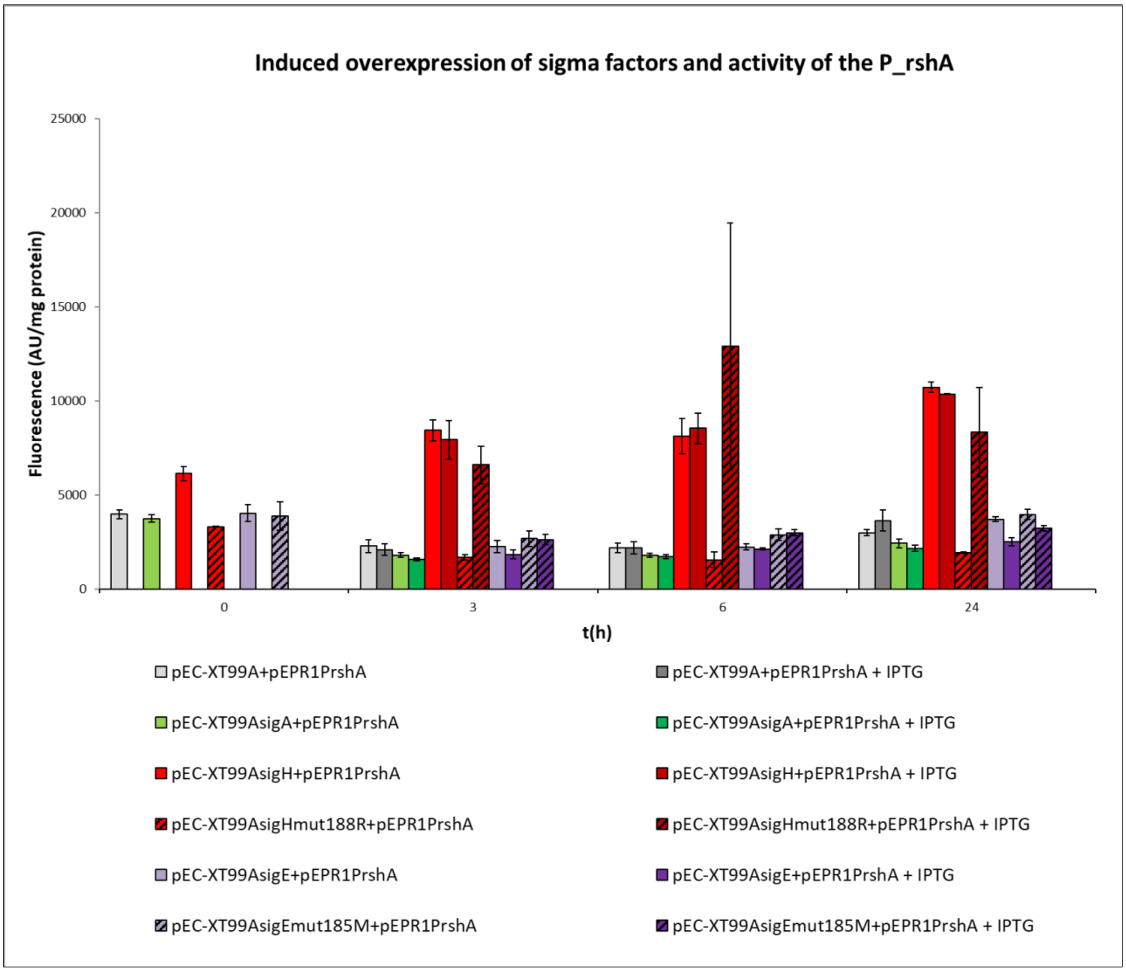

Figure 17- PrshA promoter activity in C. glutamicum strains with different sigma factors.

The promoter activity was measured by Gfpuv fluorescence intensity of cell extracts presented in the graph by different colours corresponding to each sigma factor. All C. glutamicum strains carried the plasmid pEC-XT99A containing a sigma factor encoding gene and the promoter PrshA on the promoter-test vector pEPR1 that carried the Gfpuv gene. All strains were tested with (striped) and without (non-striped) the induction of sig gene expression by IPTG. The strains carrying no sigma-encoding gene and SigA-*encoding gene were used as controls. The standard deviations of two measurements are depicted by error bars. (Note: The results with the correct strain C. g. pEC-XT99AsigH / pEPR1_PrshA were obtained by Hana Dostálová in an independent assay)*

Unusual result of the promoter activity measurement with the strain *C. g. pEC-XT99AsigH / pEPR1_PrshA* prompted us to check the strain. Fortunately, the incorrect setup of the strain was revealed by sequencing and can be thus in further work corrected. This confusion proved, that in the work with recombinant molecules of DNA, we must check the sequences of the new molecules carefully always when we doubt about the structure and function of these DNA constructs. It was several times observed in the Laboratory, that there was a recombination of a sigma factor on the plasmid and on the chromosome and consequently, their exchange.

The activity of SigH-dependent promoter PrshA was high, as expected (H. Dostálová). Mutation of SigH (the *sigHmut170R* gene) decreased the activity of SigH but only partially. This again confirmed the universal and robust character of SigH. SigE was not active with the PrshA and the mutation *C. g. pEC-XT99AsigE185M* to make the SigE more SigH-like did not influence it.

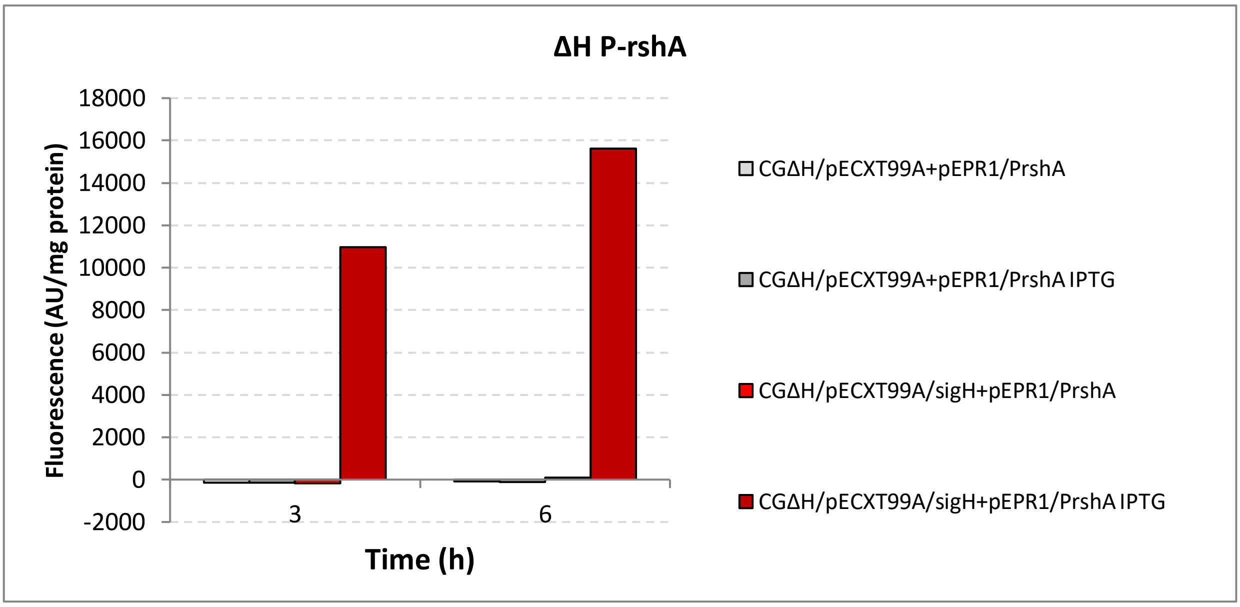

Figure 18 - PrshA promoter activity in C. glutamicum strains with sigma factor H and no sigma factor.

4.4.4. Activity of promoter PrshA-mut

The promoter PrshA-mut was mutated to be more sigma E depended so it was expected that the strain which should present the highest activity was the *C. g. pEC-XT99AsigEmut185R,* but once the previous promotor (PrshA) presented more activity than it was expected on *C. g. pEC-XT99AsigHmut170R*, this could indicate that this mutated region in this promotor could not affect it's sigma factor dependence.

Consulting the graph which is presented on figure 19 (not correct numbers of all figures!) it is possible to observe that this promotor has high activity in *C. g. pEC-XT99AsigH* and some activity in *C. g. pEC-XT99AsigE,* which proves the theory that this region does not affect the dependence of this promotor towards the sigma factors.

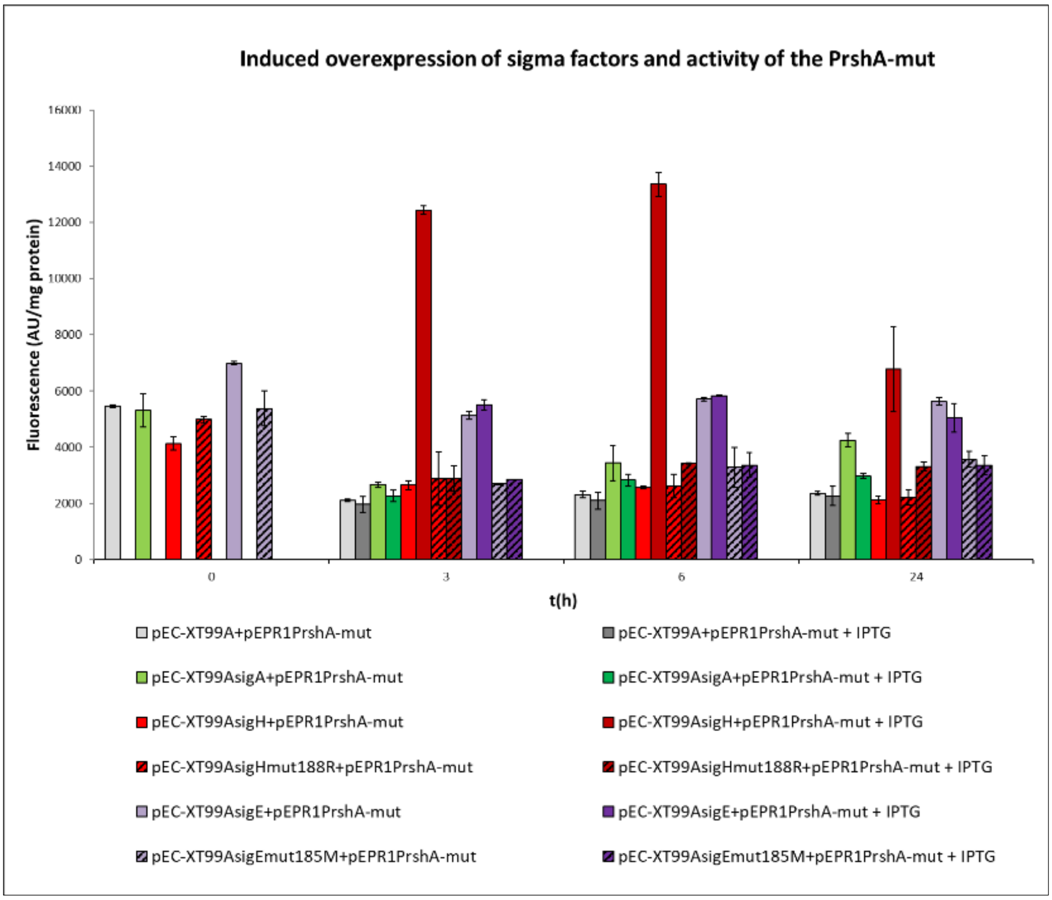

Figure 19- PrshA-mut promoter activity in C. glutamicum strains with different sigma factors.

The promoter activity was measured by Gfpuv fluorescence intensity of cell extracts presented in the graph by different colours corresponding to each sigma factor. All C. glutamicum strains carried the plasmid pEC-XT99A containing a sigma factor encoding gene and the promoter PrshA-mut on the promoter-test vector pEPR1 that carried the Gfpuv gene. All strains were tested with (striped) and without (non-striped) the induction of sig gene expression by IPTG. The strains carrying no sigma-encoding gene and SigA-*encoding gene were used as controls. The standard deviations of two measurements are depicted by error bars.*

After analysing the graph presented on figure 19 it was possible to observe that the activity of this promoter is higher for *C. g. pEC-XT99AsigH* which means that the mutation that was performed on the sequence of this promoter had no influence on the recognition by the sigma factor. These data further prove that the region – 31 of this promoter has a strong influence on the sigma factor-dependence of the promoter.

4.4.5. Activity of promoter Pcg3309 (SigE - dependent)

Previous studies performed with this promoter, in his wild type form, demonstrated that it is dependent on sigma factor E, so the most expected results would be the highest activity level being observed on *C. g. pEC-XT99AsigE*.

In figure 20 is the graph with the promoter activity in each strain, and it is possible to confirm that this promoter is sigma E dependent although it also presents a very low activity on sigma H strains.

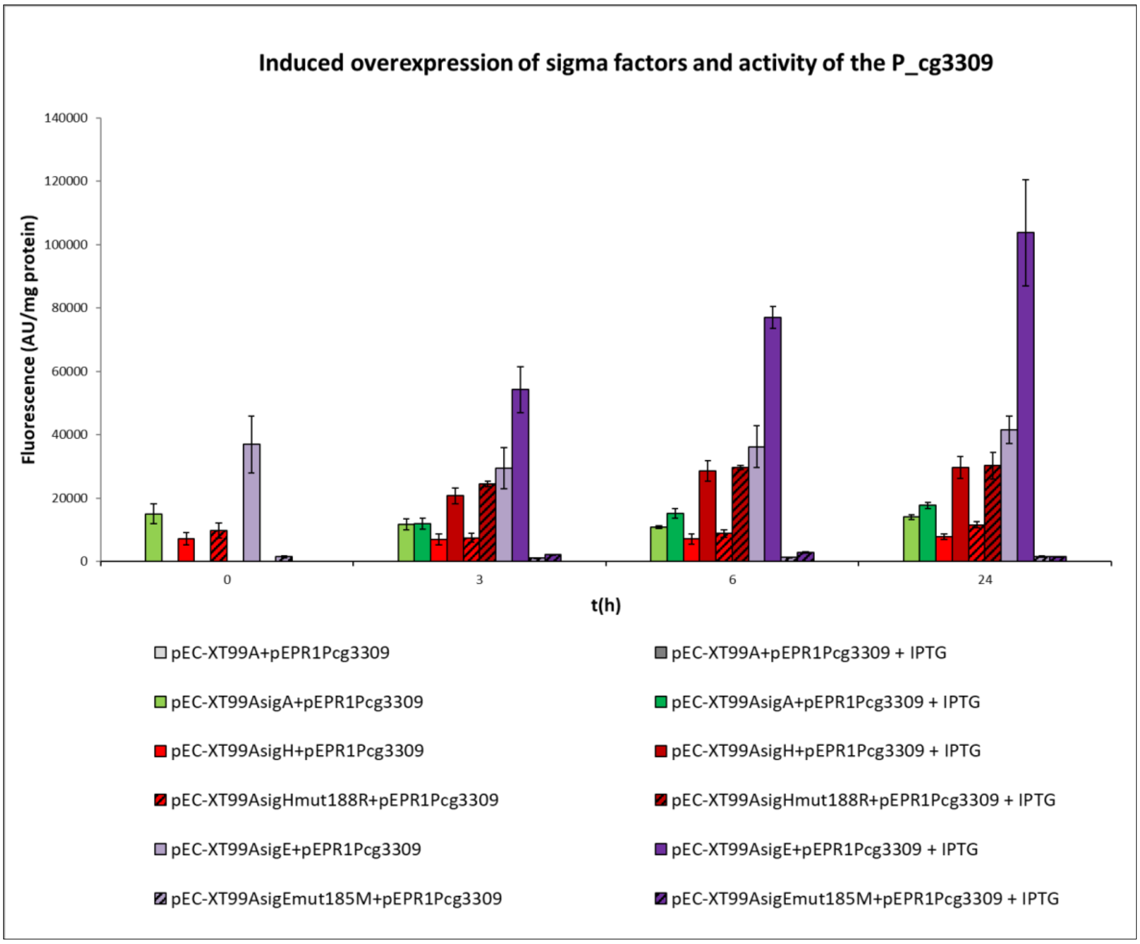

Figure 20- Pcg3309 promoter activity in C. glutamicum strains with different sigma factors.

The promoter activity was measured by Gfpuv fluorescence intensity of cell extracts presented in the graph by different colours corresponding to each sigma factor. All C. glutamicum strains carried the plasmid pEC-XT99A containing a sigma factor encoding gene and the promoter Pcg3309 on the promoter-test vector pEPR1 that carried the Gfpuv gene. All strains were tested with (striped) and without (non-striped) the induction of sig gene expression by IPTG. The strains carrying no sigma-encoding gene and SigA-*encoding gene were used as controls. The standard deviations of two measurements are depicted by error bars.*

As it was expected, this promoter presents a high activity on strain *C. g. pEC-XT99AsigE,* which means that it is dependent on sigma E. But, it is also possible to verify that for sigma H strains, this promoter also shows some activity, which indicates that the sigma factor H also recognizes this promoter sequence although not as efficiently as sigma factor E.

4.4.6. Activity of promoter Pcg3309-mut

The mutation on Pcg3309-mut promoter sequence was made with the purpose of making this promoter more dependent on sigma factor H, so the most expected result was that the strain *C. g. pEC-XT99AsigEmut185R* presents the highest values of activity, once this strain, sigma factor E was also mutated to be more like sigma H.

Consulting the graph presented on figure 21, where the activity values for this promoter are presented, it is possible to observe that the highest activity was seen in *C. g. pEC-XT99AsigEmut185R* strain, as predicted, and that some activity was also observed in *C. g. pEC-XT99AsigH,* which corresponds to the expected result, since this strain carries the sigma factor H.

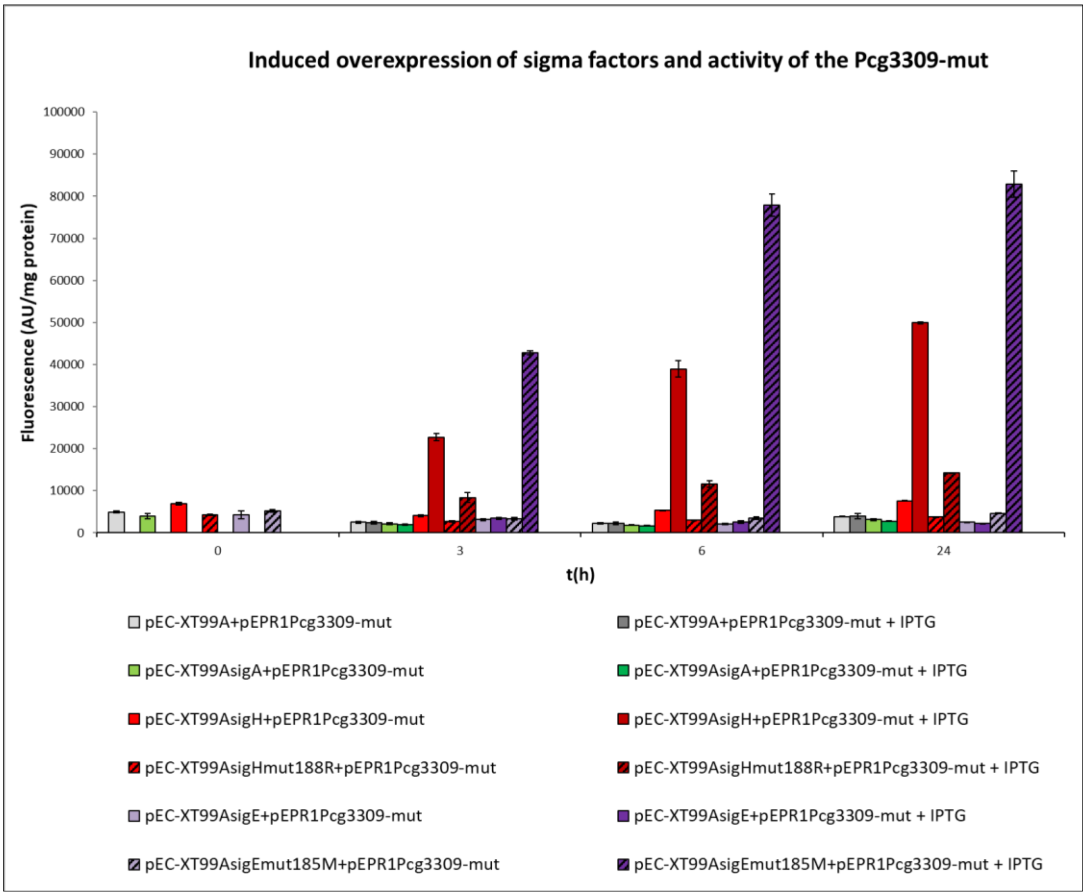

Figure 21- Pcg3309-mut promoter activity in C. glutamicum strains with different sigma factors.

The promoter activity was measured by Gfpuv fluorescence intensity of cell extracts presented in the graph by different colours corresponding to each sigma factor. All C. glutamicum strains carried the plasmid pEC-XT99A containing a sigma factor encoding gene and the promoter Pcg3309mut on the promoter-test vector pEPR1 that carried the Gfpuv gene. All strains were tested with (striped) and without (non-striped) the induction of sig gene expression by IPTG. The strains carrying no sigma-encoding gene and SigA-*encoding gene were used as controls. The standard deviations of two measurements are depicted by error bars.*

The most expected result was that this promoter would present a higher activity on strain *C. g. pEC-XT99AsigEmut185R* than in other strains. Analysing the graph presented on figure 20 it is possible to see that the expected results corresponds with the experimental ones, which means that the mutation performed on sigma H gene-encoding had a meaningful influence on the recognition of promoter sequence. This experiment gave results coherent with what was expected. The mutation introduced into the promoter Pcg3309 made it more dependent on sigma factor H, being also predominantly dependent on the sigma factor H-like mutant variant of sigma E. These results prove that the region – 31 of the promoter has a strong influence on sigma factor recognition and dependency of promoter. This theory is reinforced by the activity of this promoter on *C. g. pEC-XT99AsigH,* which was slightly higher when compared to the activity of the non-mutated variant of Pcg3309 in the same strain (Figure 20).

4.4.7. Activity of promoter PcseA (SigE - dependent)

The promoter PcseA had been studied in previous researches and it was concluded that it is mostly dependent on sigma factor E while also presenting some activity with sigma factor H. Therefore, the most expected result in this study would be that the high activity level should be observed on *C. g. pEC-XT99AsigE*. Since in the *C. g. pEC-XT99AsigHmut170R*, the sigma H-encoding gen was mutated to be more like sigma factor E it was also expected that this strain carrying this plasmid would also present high values of promoter activity.

Figure 22 has the graph with the promoter activity per milligram of protein, measured in each strain, where it is possible to observe that this promoter is sigma E dependent although it also presents a very low activity on sigma H strain. The strain *C. g. pEC-XT99AsigHmut170R* presents the highest activity value, for this promoter, on the first six hours which decreases after this time, similarly to the strain *C. g. pEC-XT99AsigH* which also decreases it's activity after six hours.

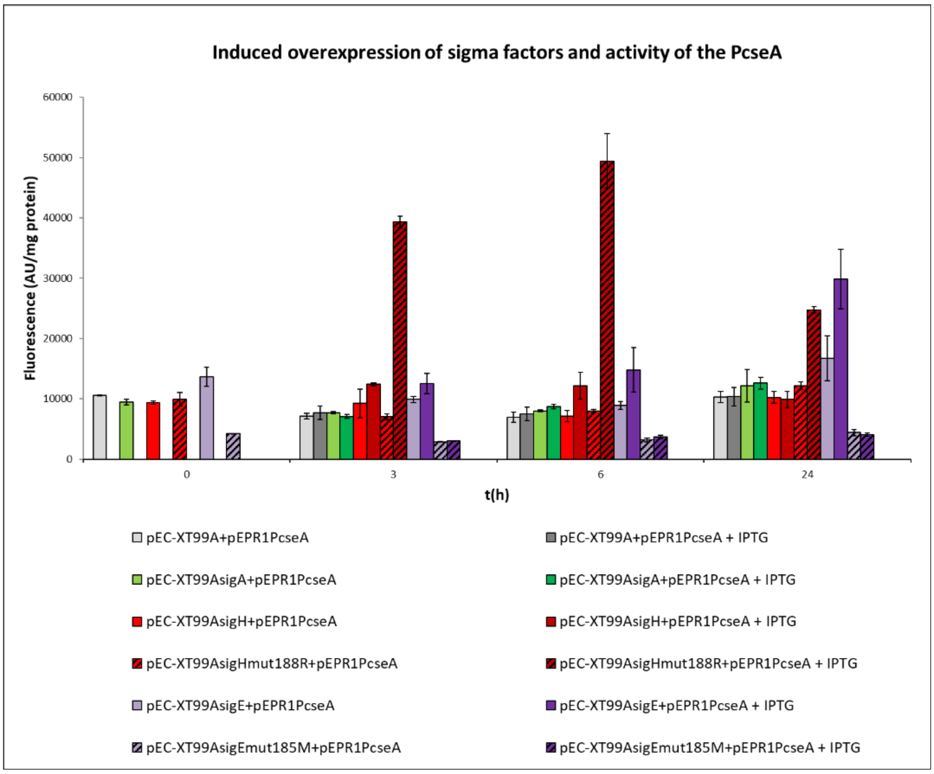

Figure 22- PcseA promoter activity in C. glutamicum strains with different sigma factors.

The promoter activity was measured by Gfpuv fluorescence intensity of cell extracts presented in the graph by different colours corresponding to each sigma factor. All C. glutamicum strains carried the plasmid pEC-XT99A containing a sigma factor encoding gene and the promoter PcseA on the promoter-test vector pEPR1 that carried the Gfpuv gene. All strains were tested with (striped) and without (non-striped) the induction of sig gene expression by IPTG. The strains carrying no sigma-encoding gene and SigA-*encoding gene were used as controls. The standard deviations of two measurements are depicted by error bars.*

As it is possible to prove after analysing the figure 22, the strain *C. g. pEC-XT99AsigHmut170R* is the one that presents the highest activity values after 6 hours, but then this value widely decreases, this trend is also verified on strain *C. g. pEC-XT99AsigH*. For *C. g. pEC-XT99AsigE* the activity is also high but is always increasing, which confirms the previous studies which stated that this promoter is dependent on sigma E.

These results show that the mutation performed on sigma H encoding gene had a meaningful influence on the recognition of promoter sequence and it is also possible to prove that the region – 31 of the promoter had a strong influence on dependence of promoter.

4.4.8. Activity of promoter PcseA-mut

A mutation was made on PcseA-mut promoter to make this promoter more dependent on sigma H, according with the information obtained on the previous assay (the activity of PcseA promotor) it was expected that the strain *C. g. pEC-XT99AsigH* was the one that presents the highest activity.

Figure 23 presents the final graph obtained after performing the promoter activity fluorescence assays. It is possible to verify that the activity on *C. g. pEC-XT99AsigH* is higher that on the other strains but is also possible to verify a considerable activity on *C. g. pEC-XT99AsigEmut185M* strain, these results are in accordance with what was expected.

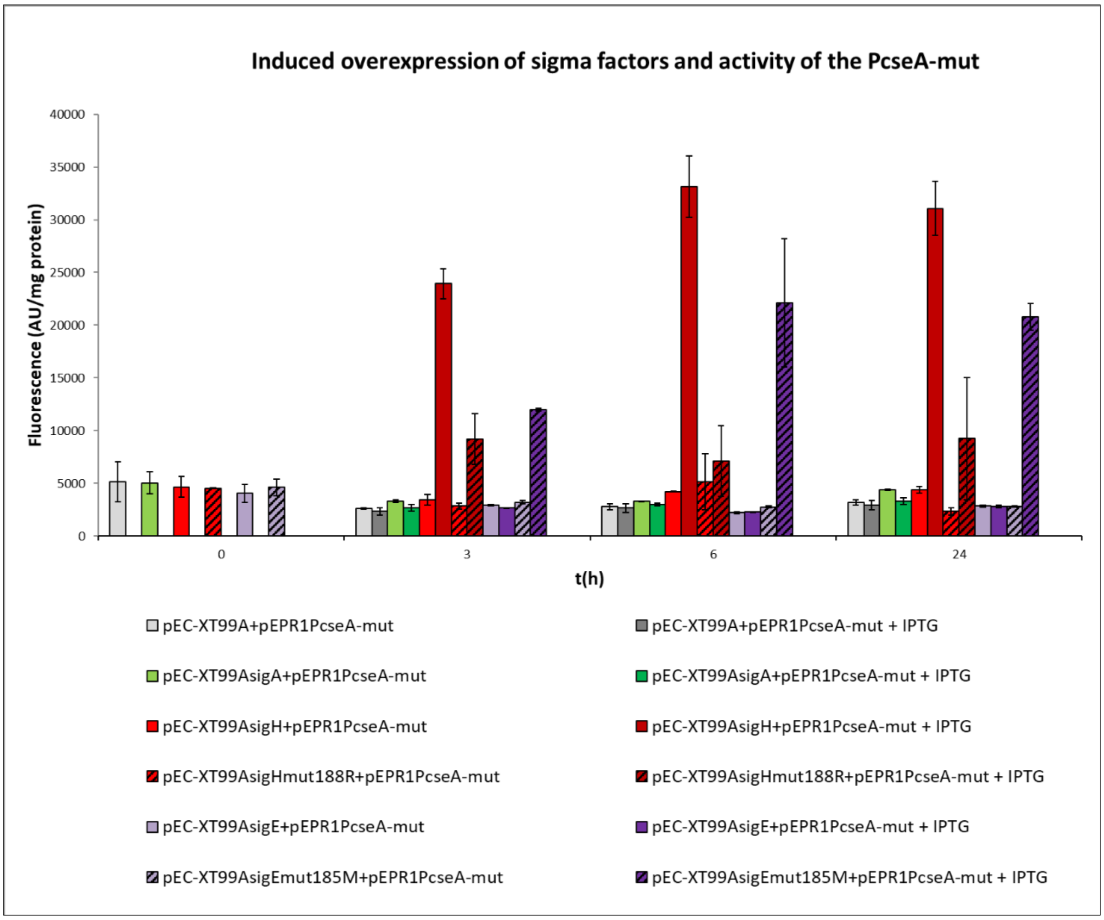

Figure 23- PcseA-mut promoter activity in C. glutamicum strains with different sigma factors.

The promoter activity was measured by Gfpuv fluorescence intensity of cell extracts presented in the graph by different colours corresponding to each sigma factor. All C. glutamicum strains carried the plasmid pEC-XT99A containing a sigma factor encoding gene and the promoter PcseA-mut on the promoter-test vector pEPR1 that carried the Gfpuv gene. All strains were tested with (striped) and without (non-striped) the induction of sig gene expression by IPTG. The strains carrying no sigma-encoding gene and SigA*-encoding gene were used as controls. The standard deviations of two measurements are depicted by error bars.*

According with the graph presented on figure 23, the fact that the activity of this promoter is higher for C. g. pEC-XT99AsigH, means that the mutation that were performed on the sequence of this promoter influenced the recognition by the sigma factor, hence demonstrating that the region – 31 of the promoter has a strong influence on sigma factor dependence of promoter. This theory is reinforced by the activity of this promoter on C. g. pEC-XT99AsigEmut185M, which was slightly higher in comparison to the activity of non-mutated PceA in the same strain.

5. CONCLUSION

The aim of this thesis was to analyze relationship between the function of two important stress sigma factors in *Corynebacterium glutamicum*, SigH and SigE and to define important specific sequences in the promoters recognized by these sigma factors. Homology modeling showed that the two most important amino acids for recognition of promoters by these sigma factors are Met170 within SigH and Arg185 within SigE. To prove that these amino acids are those which allow different binding of the respective sigma factors to specific promoters *in vivo*, the mutation in the positions Arg185 and Met170 were done in such a way, that the amino acids were exchanged in SigH-mut170R and SigE-mut185M (Table 3). Two SigH-dependent and two SigE-dependent promoters were used in the study. The homology modeling suggested that in addition to the -35 region GGAA, which is present in both classes of promoters, the 5 nucleotides downstream of the -35 region affect the specificity of the promoters. These 5-nt sequences were exchanged in the promoters PtrxB1, PrshA (both SigH-dependent), PcseA and Pcg3309 (both SigE-dependent) (Table 2). The activity of all combinations of sigma factors and promoters were used for measuring promoter activity using a two-plasmid system and fluorescence assay of promoter activity (Section 3.6).

According to the results, it is apparent that the each promoter functioned in a different way and their dependence on SigH and SigE showed also different features. However, in general, it was confirmed, that the amino acids Met170 within SigH and Arg185 within SigE play a key role in recognition of the -35 region.

The SigH-dependent promoters (PtrxB1 and PrshA) were highly active with SigH and still some activity was found with SigH-mut170R. This showed that Met in SigH is not strictly required. These promoters showed no activity with SigE, neither with SigE-mut185M which should be somewhat SigH-like. Apparently, this mutation did not improve the recognition of strictly SigH-dependent promoters.

Mutant promoters Pcg3309-mut and PcseA-mut which were closer to SigH-dependent promoters were also highly active with SigH and also with SigH-mut170R, whereas low activity was seen for SigE. Apparently Arg185 is highly important for recognition by SigE.

Naturally SigE-dependent promoters Pcg3309 and PcseA were active with SigE but their activity completely vanished with the mutant SigE-mut185M. Again, position Arg185 was shown as strictly required for SigE to recognize respective promoters. This was also confirmed by mutants PtrxB1-mut and PrshA-mut, which were closer to SigE-dependent promoters and really provided activity with SigE, in contrast to wild type promoters PtrxB1 and PrshA, which were not active with SigE at all.

Finally, the promoters Pcg3309-mut and PcseA-mut, which were made SigH-dependent by the mutation, were also active only with SigE-mut185M.

We can conclude that the SigE-dependent promoters strictly require sigma factor with Arg185 and the region downstream of -35 with consensus CTTTT is also very important for them. SigE is thus very specific. In contrast, SigH is much less specific and tolerates mutations both in the sigma protein and in the promoters. This is a reason why strictly SigH-dependent promoters exist, whereas all SigE-dependent promoters are in all cases partially SigH-dependent. Another conclusion is that homology modeling can help us in the studies of sigma-promoter interactions.

All results are also in agreement with the suggestion that SigH is a kind of global regulator.

6. CONCLUSÃO

O principal objetivo desta tese foi analisar a relação entre a função de dois fatores sigma associados a stress, importantes em *Corynebacterium glutamicum*, SigH e SigE, para definir sequências específicas importantes, de promotores reconhecidos por estes fatores sigma. A modelação por homologia mostrou que os dois aminoácidos mais importantes para reconhecimento de promotores por esses fatores sigma são a Met170 no caso do SigH e a Arg185 para o SigE. Para provar que estes aminoácidos são os que permitem as diferentes ligações dos fatores sigma aos seus respetivos promotores específicos *in vivo*, a mutação nas posições da Arg185 e da Met170 foi realizada, trocando estes aminoácidos, criando as estirpes SigH-mut170R e SigE-mut185M (Tabela 3). Neste estudo foram utilizados dois promotores SigH-dependentes e dois SigE-dependentes. A modelação por homologia sugeriu que para além da região -35 GGAA, que está presente em ambas as classes de promotores, os 5 nucleótidos a jusante da região -35 afetam a especificidade dos promotores. Estas 5 sequências de nucleótidos foram trocadas nos promotores PrshA e PtrxB (ambos dependentes de SigH), e em PcseA e Pcg3309 (ambos dependentes de SigE) (Tabela 2). A atividade de todas as combinações de fatores sigma e promotores foi utilizada para medir a atividade do promotor, utilizando um sistema de dois plasmídeos e a fluorescência (Secção 3,6).

De acordo com os resultados, é evidente que cada promotor funciona de uma forma diferente e que a sua dependência em SigE e SigH também apresenta características diferentes. No entanto, de um modo geral, confirmou-se que os aminoácidos Met170 em SigH e Arg185 em SigE, desempenham um papel chave no reconhecimento da região de -35.

Os promotores SigH-dependentes (PtrxB1 e PrshA) foram altamente ativos com o SigH e se observou alguma atividade com SigH-mut170R. Isto mostrou a Met em SigH não é estritamente necessária. Esses promotores não apresentaram atividade quer em SigE, quer em SigE-mut185M, alterado para ser mais parecido com SigH. Aparentemente esta mutação não melhora o reconhecimento dos promotores dependentes de SigH.

Os promotores mutantes, Pcg3309-mut e PcseA-mut, que estavam mais perto dos promotores dependentes de SigH, foram também altamente ativos com com o SigH-mut170R, considerando que a baixa atividade foi apresentada em SigE. Aparentemente a Arg185 é muito importante para o reconhecimento por SigE.

Os promotores naturalmente dependentes de SigE, Pcg3309 e PcseA apresentaram atividade com o SigE, mas a sua atividade desapareceu completamente com o mutante SigE-mut185M. Mais uma vez, a posição Arg185 mostrou ser extremamente relevante para reconhecer os promotores associados ao SigE. Isto também foi confirmado pelos mutantes PtrxB1-mut e pelo PrshA-mut mut, que estavam mais perto dos promotores dependentes de SigE, e que realmente apresentaram atividade com SigE, ao contrário dos promotores selvagens PrshA e PtrxB1, que não mostram qualquer atividade com SigE.

Por último, os promotores Pcg3309-mut e PcseA-mut, que foram alterados de maneira a serem dependentes de SigH, também se apresentaram ativos em SigE-mut185M.

Podemos então concluir que os promotores dependentes de SigE requerem obrigatoriamente um fator sigma que contenha uma Arg185 e que a região a jusante da -35 com CTTTT, também é bastante importante, mostrando assim que o SigE é bastante específico. Em contrapartida, o SigH é muito menos específico e tolera tanto mutações na proteína sigma como nos promotores. Esta é uma razão de existirem promotores estritamente dependentes de SigH, considerando que todos os SigE-dependentes, são em todos os casos, parcialmente SigH-dependentes. Outra conclusão é que a modelação por homologia pode ajudar nos estudos das interações sigma-promotor.

Os resultados também estão de acordo com a sugestão de que o SigH é uma espécie de regulador global.

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APENDIX I

Solutions (all the quantities presented are for the final volume of 1L):

All the solutions should be transfered to flasks with the double of the volume of solution. After the preparation the solutions have to be sterilized and stored in the cooled room.

Complex medium 2x TY:

- Trypton, m = 16g
- Yeast extrat, m = 10g
- NaCl, m = 5g

This medium could be used liquid (suspentions) or solid (plates). So, if it will be used in plates it's necessary to add agar. The agar should be added directly in the flask.

- Agar, m = 15g

GT:

- Glycerol, 10% which corresponds to 100 mL (ρ=1.26 kg/L), m = 126g
- Tris-HCl, 8mM ($M_{Tris\text{-}HCl}$= 121.14 g/mol), m = 969mg

This solution must have a pH= 7.4, and the value is fitted with HCl.

G:

- Glycerol, 10% which corresponds to 100 mL (ρ=1.26 kg/L), m = 126g

PBS buffer:

- NaCl, m = 8g
- KCl, m = 0.2g
- Na_2HPO_4, m = 1.44g (instead of this, another chemical compound can be used, $Na_2HPO_4 . 12H_2O$, and in this case the quantity is m= 3.6g)
- KH_2HPO_4, m = 0.24g

This solution must have a pH= 8.0, and the value is fitted with NaOH.

Acetate-MOPS:

- $C_2H_3NaO_2$ 0.1 M, m = 1.36 g
- MOPS 0.05 M, m = 1.047 g

This solution must have a pH= 8.0, and the value is fitted with NaOH.

APENDIX II

Table 7- Values of optical density and corresponding volume of culture to collect.

O. D.	Volume (ml)	O.D.	Volume (ml)	O.D.	Volume (ml)
0,5	24	4,3	2,790697674	8,1	1,481481481
0,6	20	4,4	2,727272727	8,2	1,463414634
0,7	17,14285714	4,5	2,666666667	8,3	1,445783133
0,8	15	4,6	2,608695652	8,4	1,428571429
0,9	13,33333333	4,7	2,553191489	8,5	1,411764706
1	12	4,8	2,5	8,6	1,395348837
1,1	10,90909091	4,9	2,448979592	8,7	1,379310345
1,2	10	5	2,4	8,8	1,363636364
1,3	9,230769231	5,1	2,352941176	8,9	1,348314607
1,4	8,571428571	5,2	2,307692308	9	1,333333333
1,5	8	5,3	2,264150943	9,1	1,318681319
1,6	7,5	5,4	2,222222222	9,2	1,304347826
1,7	7,058823529	5,5	2,181818182	9,3	1,290322581
1,8	6,666666667	5,6	2,142857143	9,4	1,276595745
1,9	6,315789474	5,7	2,105263158	9,5	1,263157895
2	6	5,8	2,068965517	9,6	1,25
2,1	5,714285714	5,9	2,033898305	9,7	1,237113402
2,2	5,454545455	6	2	9,8	1,224489796
2,3	5,217391304	6,1	1,967213115	9,9	1,212121212
2,4	5	6,2	1,935483871	10	1,2
2,5	4,8	6,3	1,904761905	10,1	1,188118812
2,6	4,615384615	6,4	1,875	10,2	1,176470588
2,7	4,444444444	6,5	1,846153846	10,3	1,165048544
2,8	4,285714286	6,6	1,818181818	10,4	1,153846154
2,9	4,137931034	6,7	1,791044776	10,5	1,142857143
3	4	6,8	1,764705882	10,6	1,132075472
3,1	3,870967742	6,9	1,739130435	10,7	1,121495327
3,2	3,75	7	1,714285714	10,8	1,111111111
3,3	3,636363636	7,1	1,690140845	10,9	1,100917431
3,4	3,529411765	7,2	1,666666667	11	1,090909091
3,5	3,428571429	7,3	1,643835616	11,1	1,081081081
3,6	3,333333333	7,4	1,621621622	11,2	1,071428571
3,7	3,243243243	7,5	1,6	11,3	1,061946903
3,8	3,157894737	7,6	1,578947368	11,4	1,052631579
3,9	3,076923077	7,7	1,558441558	11,5	1,043478261
4	3	7,8	1,538461538	11,6	1,034482759
4,1	2,926829268	7,9	1,518987342	11,7	1,025641026
4,2	2,857142857	8	1,5	11,8	1,016949153

O.D.	Volume (ml)	O.D.	Volume (ml)	O.D.	Volume (ml)
11,9	1,008403361	15,7	0,76433121	19,5	0,615384615
12	1	15,8	0,759493671	19,6	0,612244898
12,1	0,991735537	15,9	0,754716981	19,7	0,609137056
12,2	0,983606557	16	0,75	19,8	0,606060606
12,3	0,975609756	16,1	0,745341615	19,9	0,603015075
12,4	0,967741935	16,2	0,740740741	20	0,6
12,5	0,96	16,3	0,736196319	20,1	0,597014925
12,6	0,952380952	16,4	0,731707317	20,2	0,594059406
12,7	0,94488189	16,5	0,727272727	20,3	0,591133005
12,8	0,9375	16,6	0,722891566	20,4	0,588235294
12,9	0,930232558	16,7	0,718562874	20,5	0,585365854
13	0,923076923	16,8	0,714285714	20,6	0,582524272
13,1	0,916030534	16,9	0,710059172	20,7	0,579710145
13,2	0,909090909	17	0,705882353	20,8	0,576923077
13,3	0,902255639	17,1	0,701754386	20,9	0,574162679
13,4	0,895522388	17,2	0,697674419	21	0,571428571
13,5	0,888888889	17,3	0,693641618	21,1	0,568720379
13,6	0,882352941	17,4	0,689655172	21,2	0,566037736
13,7	0,875912409	17,5	0,685714286	21,3	0,563380282
13,8	0,869565217	17,6	0,681818182	21,4	0,560747664
13,9	0,863309353	17,7	0,677966102	21,5	0,558139535
14	0,857142857	17,8	0,674157303	21,6	0,555555556
14,1	0,85106383	17,9	0,670391061	21,7	0,552995392
14,2	0,845070423	18	0,666666667	21,8	0,550458716
14,3	0,839160839	18,1	0,662983425	21,9	0,547945205
14,4	0,833333333	18,2	0,659340659	22	0,545454545
14,5	0,827586207	18,3	0,655737705	22,1	0,542986425
14,6	0,821917808	18,4	0,652173913	22,2	0,540540541
14,7	0,816326531	18,5	0,648648649	22,3	0,538116592
14,8	0,810810811	18,6	0,64516129	22,4	0,535714286
14,9	0,805369128	18,7	0,64171123	22,5	0,533333333
15	0,8	18,8	0,638297872	22,6	0,530973451
15,1	0,794701987	18,9	0,634920635	22,7	0,528634361
15,2	0,789473684	19	0,631578947	22,8	0,526315789
15,3	0,784313725	19,1	0,628272251	22,9	0,524017467
15,4	0,779220779	19,2	0,625	23	0,52173913
15,5	0,774193548	19,3	0,621761658	23,1	0,519480519
15,6	0,769230769	19,4	0,618556701	23,2	0,517241379

APENDIX III

Table 8- Fluorescence assay's record sheet (Values of cultures' optical density and respective volume (ml) to harvest).

Hours	**0h**		**3h**		**6h**		**24h**	
Strain	O.D.	V	O.D.	V	O.D.	V	O.D.	V
C.g. pEC-XT99A/pEPRI (___)								
C.g. pEC-XT99A/pEPRI (___) + IPTG								
C.g. pEC-XT99AsigA/pEPRI (___)								
C.g. pEC-XT99AsigA/pEPRI (___) + IPTG								
C.g. pEC-XT99AsigH/pEPRI (___)								
C.g. pEC-XT99AsigH/pEPRI (___) + IPTG								
C.g. pEC-XT99AsigHmut170R/pEPRI (___)								
C.g. pEC-XT99AsigHmut170R/pEPRI (___) + IPTG								
C.g. pEC-XT99AsigE/pEPRI (___)								
C.g. pEC-XT99AsigE/pEPRI (___) + IPTG								
C.g. pEC-XT99AsigEmut185M/pEPRI (___)								
C.g. pEC-XT99AsigEmut185M/pEPRI (___) + IPTG								

Table 9-Fluorescence assay's record sheet (Values of protein concentration in each sample).

	Protein concentration (mg/ml)			
Strains	0h	3h	6h	24h
C.g. pEC-XT99A/pEPRI (___)				
C.g. pEC-XT99A/pEPRI (___) **+ IPTG**				
C.g. pEC-XT99AsigA/pEPRI (___)				
C.g. pEC-XT99AsigA/pEPRI (___) **+ IPTG**				
C.g. pEC-XT99AsigH/pEPRI (___)				
C.g. pEC-XT99AsigH/pEPRI (___) **+ IPTG**				
C.g. pEC-XT99AsigHmut170R/pEPRI (___)				
C.g. pEC-XT99AsigHmut170R/pEPRI (___) **+ IPTG**				
C.g. pEC-XT99AsigE/pEPRI (___)				
C.g. pEC-XT99AsigE/pEPRI (___) **+ IPTG**				
C.g. pEC-XT99AsigEmut185M/pEPRI (___)				
C.g. pEC-XT99AsigEmut185M/pEPRI (___) **+ IPTG**				