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PHENOL STRESS RESPONSE 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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Resumo

O principal objetivo deste trabalho foi estudar o efeito dos fatores Sigma A, Sigma C, Sigma E, Sigma H e um controlo (sem fator sigma) no promotor de *Corynebacterium glutamicum*.

Procedeu-se à preparação dos promotores ParoF, Pcg1277, PcydA e PctaB, por associação de primers (PAROFPEPR Forward e PAROFPEPR Reverse, PCG1277PEPR Forward e PCG1277PEPR Reverse, PCYDAPEPR Forward e PCYDAPEPR Reverse, PCTABPEPR Forward e PCTABPEPR Reverse). Após este passo, seguiu-se a ligação, transformação e isolamento dos fragmentos obtidos. Seguidamente, foram preparadas as células competentes de *C. glutamicum,* em que os fatores sigma utilizados foram, Sigma A, Sigma C, Sigma E, Sigma H e uma estirpe sem fator sigma para funcionar como fator de controlo. Foi realizada a transformação em que se obtém o sistema de dois plasmídeos (pEC-XT99A e pEPR1) com os promotores construídos com a associação de primers e as estirpes com os diversos fatores sigma. Todas as transformações e preparações de células foram confirmadas por eletroforese em gel de agarose.

Iniciaram-se os ensaios de fluorescência, que foram divididos em duas partes: no primeiro ensaio o meio de cultura continha apenas antibióticos, e no segundo ensaio houve adição de fenol ao meio de cultura. Em ambas as situações, existem estirpes com e sem IPTG, com e sem fenol, e com fenol e IPTG.

No primeiro ensaio, confirmou-se que a presença dos promotores provoca uma alteração na expressão dos fatores sigma. Nomeadamente, a presença do promotor ParoF incita uma sobre expressão do Sigma H; o promotor Pcg1277 provoca uma sobre expressão do Sigma E e os promotores PcydA e PctaB, ambos incitam que o Sigma C tenha uma expressão acentuada.

No segundo ensaio, com a adição de fenol, não houve diferenças nos fatores sigma que têm uma sobre-expressão devido à presença de determinados promotores, pois embora o fenol crie um ambiente tóxico na célula, com a presença de fenol houve expressão dos fatores sigma.

PALAVRAS-CHAVE: Fatores sigma, sobre expressão, fluorescência, promotores, IPTG, fenol

Abstract

The main goal of this project was to study the effect of the sigma factors Sigma A, Sigma C, Sigma E and Sigma H, and a control (no sigma) on the promoters of *C. glutamicum*.

It was performed the preparation of the promoters ParoF, Pcg1277, PcydA and PctaB, with the re-association of primers (PAROFPEPR Forward and PAROFPEPR Reverse, PCG1277PEPR Forward and PCG1277PEPR Reverse, PCYDAPEPR Forward and PCYDAPEPR Reverse, PCTABPEPR Forward and PCTABPEPR Reverse). After this step, ligation, transformation and isolation of the fragments were made. Then, competent cells of *Corynebacterium glutamicum* were prepared and the sigma factors used were Sigma A, Sigma C, Sigma E, Sigma H and a control sigma to use as a control. The two-plasmid strain was obtained by transformation by electroporation, and all the steps that include transformation and preparation were confirmed by PCR and then electrophoresis in agarose gel.

The fluorescence assays were initiated, and they were divided in two concepts: in the first assay the medium had only the antibiotics and the second assay contained phenol. In both situations, there were strains with and without IPTG, with and without phenol, and strains with both. IPTG functions by binding to the lacI repressor and altering its conformation, which prevents the repression of the β-galactosidase coding gene *lacZ*.

In the first set of assays, it was confirmed that the presence of the promoters stimulates the overexpression of the sigma factors. Namely, ParoF makes Sigma H have a higher expression, Pcg1277 does the same to Sigma E and both PcydA and PctaB have the same effect on Sigma C.

In the second set of assays, with the addition of phenol, there were no changes in terms of which sigma has a higher overexpression to certain promoters. However, phenol is a toxic element to the cell, but there still was some expression in the strains that had phenol

Keywords: Sigma factors, overexpression, fluorescence, promoters, IPTG, phenol

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1. Introduction

Research in the Institute of Microbiology of the Czech Academy of Sciences (CAS) is focused in the molecular mechanisms and regulatory systems of several microorganisms. Along with these studies, it also includes the study of mammalian cells with the purpose of getting more information and exploitation in the medicine industry. In the Institute of Microbiology, there is the Laboratory of Molecular Genetics, which is focused on the studies of bacterial strains, regulation of bacterial gene expression, biosynthesis of new enzymes, and genetic analysis of bacteria degrading toxic compounds [1].

The work presented in this project was carried out in the Laboratory of Molecular Genetics, Institute of Microbiology of the CAS, and had as target-organism, the species of bacteria, *Corynebacterium glutamicum* [2].

C. glutamicum contains seven sigma factors that have all been studied and reported to respond in different ways to several types of stresses [1]. These stresses include: heat shock, oxidative stress, cell surface stress, transition phase, general stress response and also, phenol stress. The type of stress that will be studied and analysed in this work will be the phenol stress. Using previous transcriptome analysis of phenol-grown *C. glutamicum* cells (Laboratory of Molecular Genetics, Institute of Microbiology, Prague) two promoters, namely ParoF and Pcg1277, which are assumed to be recognized by sigma factors SigH and/or SigE and two other promoters, PcydA and PctaB, that are believed to respond to SigC, were chosen for analysis [3].

The main goal of this project was to study the effect of the sigma factors Sigma A, Sigma C, Sigma E and Sigma H, and a control (no sigma) on the promoters of *C. glutamicum*. This experiment has been carried out by using a two-plasmid system containing the vectors pEC-XT99A and pEPR1. The pEC-XT99A vector contains a gene encoding one of the sigma factors, and pEPR1 contains the promoters that were cloned in *E.coli* [2], [3]. The same experiment will be repeated but with the addition of phenol to cause stress in the cells and see how they respond.

The study is based on the results provided by other external laboratory that is collaborating with the Czech Academy of Sciences, namely the Department of Molecular Genetics of Bacteria.

2. Literature Review

2.1 Transcription

The expression of bacterial genes is an important concept because its control is related to the beginning of the transcription that is performed by the action of a RNA polymerase (RNAP) holoenzyme. This holoenzyme is structured by a five subunit (α_2, β, β', ω) core RNAP enzyme and a dissociable sigma factor, that has the function of recognizing certain promoter DNA sequences. So, the sigma factors have the primary function of regulating the transcription, which can alter the expression of large gene groups, when it is faced with environmental, intracellular and extracellular conditions changes [3].

Bacteria naturally holds a primary sigma factor that is responsible for the transcription of most genes, housekeeping genes, mainly those who maintain a certain activity during the exponential growth phase. Adding to this, the bacterial genomes code also for non-essential alternative sigma factors, which identifies the promoters of such genes that are suddenly active as a response to numerous stress conditions, mainly in the stationary phase or during starvation for various nutrients. The promoter sequences are recognized mostly by identifying the transcriptional start points of the genes [4].

2.2 Corynebacterium glutamicum

C. glutamicum (Figure 1) is a Gram-positive, non-sporulating and non-pathogenic bacterium that belongs to the order Actinomycetales. *C. glutamicum* possesses numerous characteristics that make this bacterium useful in biotechnology: it grows relatively fast, does not have a lot of growth requirements, does not produce extracellular proteases, and has a stable genome [2].

C. glutamicum's genome encodes seven sigma factors, of RNA polymerase, that are: SigA, which is the primary sigma factor; SigB, that represents a primary-like sigma factor, and other 5 alternative sigma factors, SigC, SigD, SigE, SigH and SigM. These sigma factors are important regulatory elements that can control different classes of promoters and can enable the expression of some groups of genes, called regulons. This behaviour of the bacterial cells is related to the changes that they must make to adapt to certain conditions, whether it's environmental or nutritional [2].

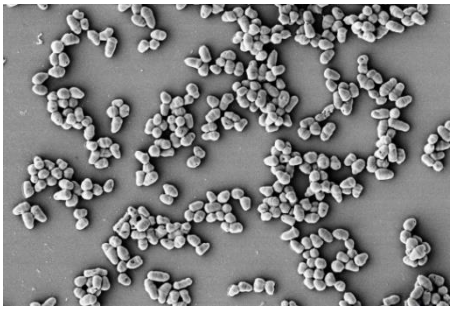


Figure 1- Microscopic image of *Corynbacteriumglutamicum*, observed under a scanning electronic microscope. (Magnification: 10.00 K X) (source: *Scanning electron microscopy was performed in CeNSE, IISc, Bangalore)*

2.3 Sigma factors of RNA polymerase

Sigma factors are found in prokaryotic cells and are described as a single subunit of RNA polymerase, and their main function is to arrange the process of transcription initiation (Figure 2). Sigma factors binds to the multisubunit core RNA polymerase ($\alpha_2\beta\beta'$), creating RNA polymeraseholoenzyme ($\alpha_2\beta\beta'\sigma$), which performs transcription initiation. Holoenzyme recognizes the two conserved hexamer sequences that constitute a prokaryotic promoter, exposes the single-stranded DNA template necessary for transcription initiation, and begins synthesizing the RNA chain. When the nascent RNA is five to ten nucleotides long, sigma is released, terminating the initiation phase of transcription [3].

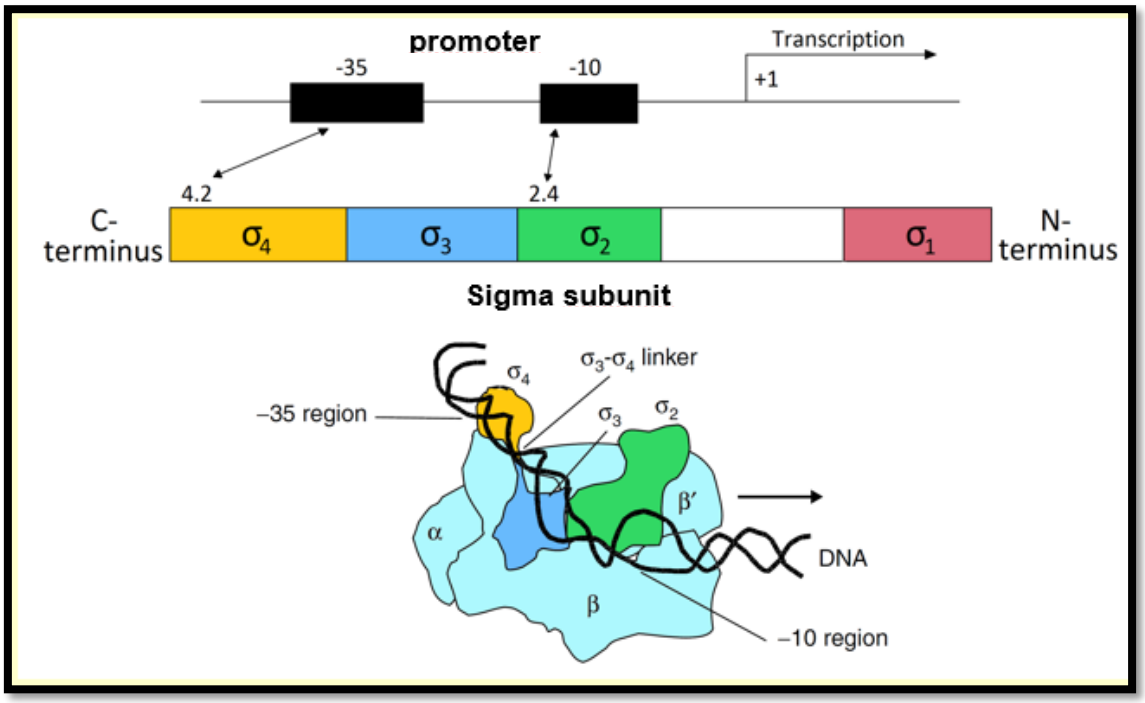


Figure 2- RNAP+sigma-promoter binding in the -35 and -10 regions before the transcription. (source: documents provided by mentor in the Institute)

Regularly, the sigma factor expression or activity is dependent on specific growth phase transitions of the organism. If transcription of genes involved in growth is

necessary, the sigma factors will be translated to allow for transcription initiation to occur (Figure 3). However, if transcription of genes is not required, sigma factors will not be active. In specific instances when transcriptional activity needs to be inhibited, there are anti-sigma factors which perform this function. The anti-sigma factors will bind to the RNA polymerase and prevent its binding to sigma factors present at the promoter site. The anti-sigma factors are responsible for regulating inhibition of transcriptional activity in organisms that require sigma factor for proper transcription initiation [4].

2.3.1 Sigma A

C. glutamicum σ^A is an essential primary σ factor that directs the transcription of the majority of genes expressed during exponential growth which are termed "housekeeping" or "vegetative" [4]. The promoters are usually considered housekeeping (σ^A dependent) if their −35 and −10 promoter sequences match the generally accepted consensus of housekeeping promoters. The σ^A gene expression is high throughout the exponential growth phase of C. glutamicum cultures and decreases with the onset of the transition phase between the exponential and stationary growth phases [5].

2.3.2 Sigma C

Regarding the *C. glutamicum* σ^C, there are no reported results that entirely confirm what behaviour this sigma factor has if it is subjected to a certain type of stress, or some nutrient addition. Although there are no official reports of it, it is known that this sigma factor supposedly responds to phenol stress in two known promoters (Pcyd A and PctaB) [5, 6].

2.3.3 Sigma E

C. glutamicum σ^E is known to respond to cell surface and heat stress and probably also in other processes regulating cell adaptation to non-optimum growth conditions in *C. glutamicum.* Expression of σ^E is rather low during exponential growth, whereas it increases after cell surface stress and heat shock. In addition to transcriptional regulation, the activity of σ^E is controlled by its cognate anti-sigma factor CseE coded by the downstream cseE gene that is co-transcribed in the σ^E-cseE transcript. It is known also the σ^E and σ^H have a certain overlap in promoter recognition specificity [5, 6].

2.3.4 Sigma H

σ^H is the most studied *C. glutamicum* extracytoplasmic function (ECF) sigma factor, which controls a transcriptional regulatory network enabling the *C. glutamicum* cell to respond to temperature, oxidative and growth-phase induced stresses. According to various published articles and results, it is supposed that σ^H is a global regulator in the *C. glutamicum* gene regulatory network and controls responses to oxidative and heat

stress as well as the whole set of stress stimuli induced at the onset of the stationary growth phase [6].

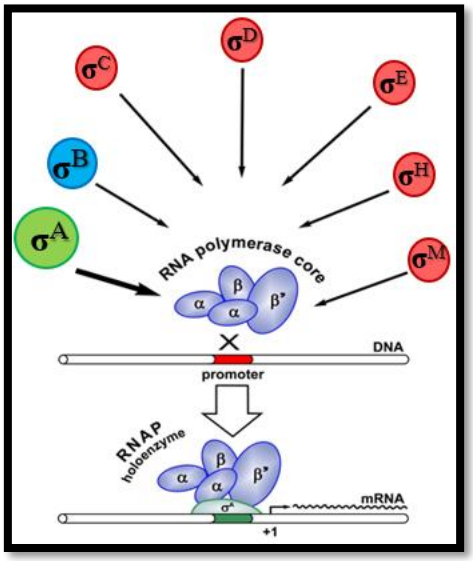


Figure 3- Action of the Sigma factors of RNA polymerase in *Corynebacterium glutamicum* in the process of transcription (source: documents provided by mentor in the Institute).

2.4 *Corynebacterium glutamicum* competent cells

The process that allows the cells to incorporate extracellular DNA called transformation, that alters the cells genetics, is not possible to perform unless the cells have the ability of competence (Figure 4), in other words, it is necessary to change the cells into competent cells. Competence can be obtained naturally, a bacteria can have a genetically specified ability that occurs under natural conditions, or artificially, in the laboratory where the cells are treated in several procedures to make them transiently permeable to foreign DNA [3,5].

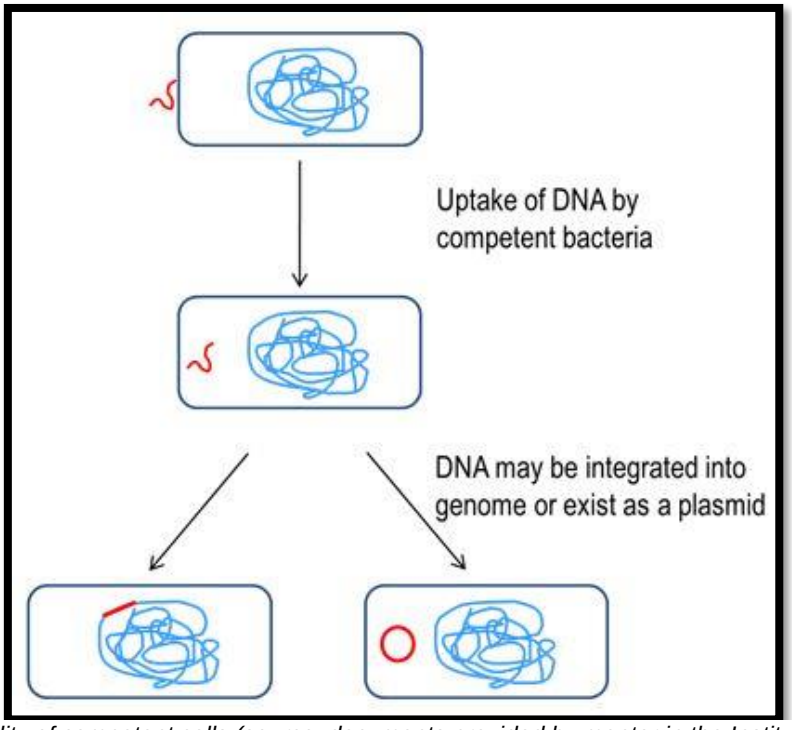


Figure 4- Ability of competent cells (source: documents provided by mentor in the Institute)

2.5 Transformation by electroporation

Transformation is used in molecular biology to provoke a genetic alteration of a certain cell resorting to the incorporation of exogenous genetic material from its surroundings through the cell membrane. To perform transformation in a cell, it is necessary that the bacteria to be in a state of competence, which might occur in nature as a time-limited response to environmental conditions such as starvation and cell density and may also be induced in a laboratory [6,8].

Electroporation, or electropermeabilization, is a microbiology technique in which an electrical field is applied to cells in order to increase the permeability of the cell membrane, allowing chemicals, drugs, or DNA to be introduced into the cell (also called electrotransfer) [6,8].

In microbiology, the process of electroporation is often used to transform bacteria, yeast, or plant protoplasts by introducing new coding DNA (Figure 5). If bacteria and plasmids are mixed together, the plasmids can be transferred into the bacteria after electroporation, Electroporation works by passing thousands of volts across a distance of one to two millimetres of suspended cells in an electroporation cuvette. Afterwards, the cells have to be handled carefully until they have had a chance to divide, producing new cells that contain reproduced plasmids. This process is approximately ten times more effective than chemical transformation [5,6].

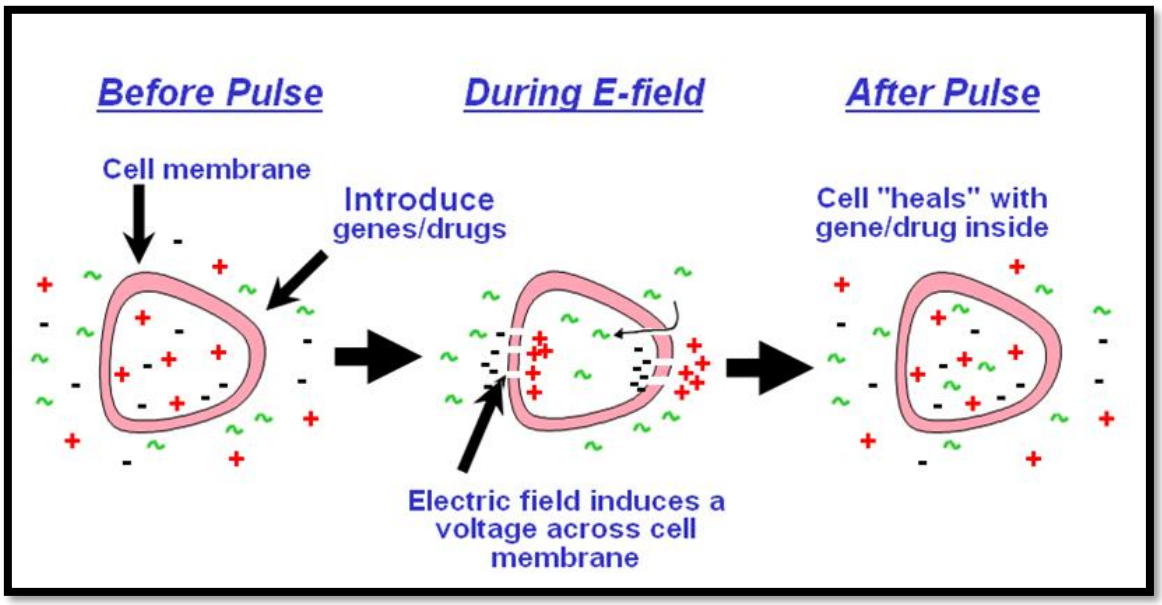


Figure 5 - Transformation by electroporation process (source: https://www.btxonline.com/technical-resources/faq.html)

2.6 Cloning

DNA cloning is the starting point for many genetic engineering approaches to biotechnology research. Large amounts of DNA are needed for genetic engineering. Multiple copies of a piece of DNA can be made either by using polymerase chain reaction (PCR) (Figure 6) or by cloning DNA in cells [7].

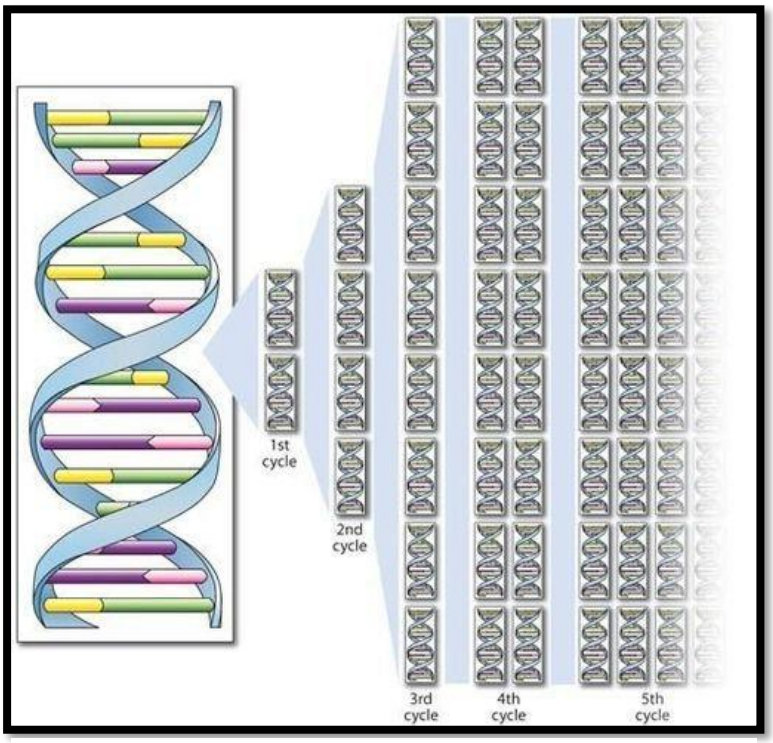


Figure 6- Result of the PCR, with multiple copies of DNA. (source: https://www.genscript.com/molecular-cloning-strategy.html)

To get multiple copies of a gene or other piece of DNA, it is performed an isolation of the DNA from its source and then insert it into a DNA vector that can replicate itself. The four main steps in DNA cloning include choosing the chosen piece of DNA that is 'cut' from the source organism using restriction enzymes. The piece of DNA is 'pasted' into a vector and the ends of the DNA are joined with the vector DNA by ligation. The vector is introduced into a host cell, often a bacterium or yeast, by transformation. The host cells copy the vector DNA along with their own DNA, creating multiple copies of the inserted DNA. The vector DNA is isolated (or separated) from the host cells' DNA and purified [3, 7].

DNA cloning is used to create a large number of copies of a gene or other piece of DNA. The cloned DNA can be used to work out the function of the gene, investigate a gene's characteristics (size, expression, tissue distribution), look at how mutations may affect a gene's function and make large concentrations of the protein coded for by the gene [7].

3. Materials and Methods

3.1 Preparation of promoter insert by re-association of primers

Firstly, the promoters inserts for subsequent cloning of obtained products into vector pEPR1 were constructed, by performing the re-association of primers. The double-stranded DNA inserts were (complete in Table 1):

- **PAROF**PEPR Forward + **PAROF**PEPR Reverse;
- **PCG1277**PEPR Forward + **PCG1277**PEPR Reverse;
- **PCYDA**PEPR Forward + **PCYDA**PEPR Reverse;
- **PCTAB**PEPR Forward + **PCTAB**PEPR Reverse.

Table 1- Set primers for re-associated promoters

2239	PAROFPEPRF	***G***AGAACCGGAGTCGAGCAGCACCTCCCCGCAAGGGTAGAGGGGC TGCTTTTTTGTTTCCTAAATTCACCC***G***
2240	PAROFPEPRR	*G**ATCC***GGGTGAATTTAGGAAACAAAAAAGCAGCCCCTCTACCCTTG CGGGGAGGTGCTGCTCGACTCCGGTTCT***CTGCA***
2241	PCG1277PEPRF	*G*GGAAATTCCACTCATGAACGCCTAGTCTACGGGAACCATTTCACC AGCGTGTACGTTGTAAATGTGAAC***G***
2242	PCG1277PEPRR	***GA****TCC*GTTCACATTTACAACGTACACGCTGGTGAAATGGTTCCCGT AGACTAGGCGTTCATGAGTGGAATTTCCC***TGCA***
2243	PCYDAPEPRF	*G*GTTAACCTTTATAAAGTTAAGCTGTGAGCGGGAACTTAGGAATAA ACTTCAACGACAACCTTTAAGAAG***G***
2244	PCYDAPEPRR	***GATCC***CTTCTTAAAGGTTGTCGTTGAAGTTTATTCCTAAGTTCCCGC TCACAGCTTAACTTTATAAAGGTTAAC***TGCA***
2245	PCTABPEPRF	***G***TGGTTTGAAAACATTAAAAAGATTGATTCGGGAACTTTTAATTAAC TTCATCCGACTAATTTGCCGTGTG
2246	PCTABPEPRR	***GATC***CACACGGCAAATTAGTCGGATGAAGTTAATTAAAAGTTCCCG AATCAATCTTTTTAATGTTTTCAAACCA***CTGC***A

The association of the primers was done by mixing into 0.5 ml PCR tubes, 2.5 µl of each pair of undiluted primers (2239+2240, 2241+2242, 2243+2244, 2245+2246), 5 µl of High-Fidelity Buffer (HIFI) 10x, 1.25 µl of 50mM $MgSO_4$ and 39 µl of H_2O. This mixture was inserted into a PTC-200 Peltier Thermal Cycler, to perform the re-association of the primers. This procedure involved 50 cycles, in which 60 seconds of it were at 95ºC (denaturation) , and after the 60 seconds, the temperature decreased 1ºC per cycle until it reached 45ºC (annealing), this step also took 60 seconds. Lastly, the mixture spent 10 minutes at 4ºC.

However, this procedure was not referred as a PCR because it was not added any polymerase to the mix, so there was not any amplification of the sequences, this

step is strictly to create cohesive ends in the inserts in order to obtain two DNA chains. This allowed the possibility of cloning.

3.2 Ligation

After this step, it was carried out the preparation and subsequent ligation of the fragment, with the pEPR1 vector, for a following transformation in *E.coli*. So, for the preparation of the fragment it was added 6 µl of each one the primers that were previously constructed (2239+2240, 2241+2242, 2243+2244, 2245+2246), 1 µl of the promoter-test pEPR1 vector, (including the promoter-less *gfpuv* reporter gene) with the restriction enzymes NsiI and Bam HI (Figure 7), 1 µl of T4 DNA Ligase Buffer (10x), 1 µl of 50% PEG (Poly-ethylene glycol) 4000 and 1 µl of T4 DNA Ligase in four 0.5 ml PCR tubes. After this step, the tubes were stored in the fridge at 18ºC for 3 hours.

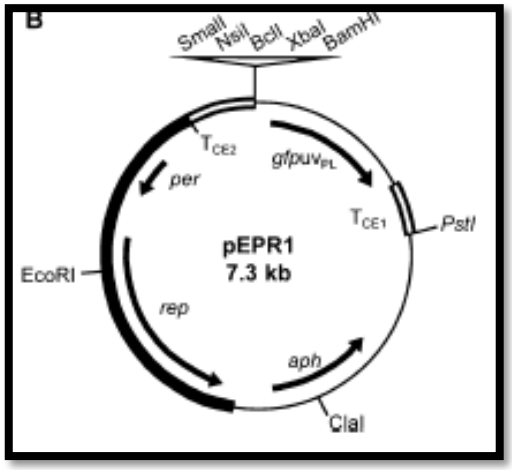


Figure 7- pEPR1 vector (source: documents provided by mentor in the Institute)

3.3 Transformation

Following the preparation and ligation of the fragment, the transformation was done by transferring 10 µl of the fragments previously obtained, to Top 10 *E.coli* tubes and let it rest for 30 minutes. Then, the tubes were submitted to a heat shock stress for 90 seconds at 43ºC and put into ice for 5 min. It was added 400 ml of 2xTY complex medium (Annex 1) and the tubes were put in a shaker at 37ºC for about 1 hour. After the time in the shaker, approximately 160 µl of the sample was inoculated into 3 plates supplemented with kanamycin (30 µg/ml), with each restriction fragment, so in total there were 12 plates, and finally the plates were put into 37ºC, for overnight growth.

After the overnight growth, the colonies that grew were transferred by the spread plate technique to other plates supplemented with kanamycin. The plates were again stored in 37ºC, for overnight growth once again.

3.4 Isolation

When it was possible to have the colonies obtained by the spread plate technique the isolation of the plasmid was performed. But before that, the colonies needed to grow in the 2xTY Complex medium. So, with a toothpick, it was collected a portion of the colonies and the toothpick was inserted into a test tube with 5 ml of medium and kanamycin, at 37ºC overnight.

The isolation was carried out following the Macherey-Nagel kit, for DNA, RNA and protein purification. First, collect the test tube and centrifuge the complete 5 ml of liquid medium in a Eppendorf tube, and when there is no medium left, add 250 µl of Buffer A1 and 6 µl of lysozyme (for the cell lysis) to the Eppendorf tube. After it is all in the tube, the mix in resuspended with a pipet until the mix is homogeneous. This mix is put into 37ºC for 15 minutes. 15 minutes after, it is added 250 µl of Buffer Lysis A2, homogenise by inverting the tube carefully 6 to 8 times, and wait 5 minutes. Then, add 300 µl of Neutralization Buffer A3, and again homogenise by inverting carefully 6 to 8 times. After all these buffers are inserted, it is performed a centrifugation at 14000 rpm, for 10 minutes, and when it is finished, 750 µl of the supernatant is collected and put into a column with a membrane of the kit. Now, the 750 µl of supernatant in the column is centrifugated at 9000 rpm for 1 min, and the product that surpassed the membrane is thrown out. It is added 600 µl of buffer A4, supplemented with ethanol (information provided by the kit), and again centrifugated at 9000 rpm, 1 min. It is removed again the product that surpassed the membrane and the tube is centrifugated alone with no addition of buffers, with the goal of drying the membrane and remove any residue of ethanol that could have been ‘stuck’. The column tube is put into another clean tube and it is added 30 µl of Buffer AE, it is centrifugated for 1 min at 9000 rpm, the column tube is discarded and the new clean tube it’s stored in the freezer.

The procedure explained before is only for one sample, but it was carried out for 16 samples, 4 of each promoter with the goal of having multiple samples of isolated plasmids.

3.5 Preparation of *Corynebacterium glutamicum* competent cells

Before constructing the two-plasmid system, *C. glutamicum* cells competent were made, with the sigma factors properly associated. Firstly, it was necessary to cultivate the cells with their respective sigma factor, and one strain with no sigma factor linked. The five types of strains studied were: *C. glutamicum* with no sigma*, C. glutamicum* σ^A*, C. glutamicum* σ^C*, C. glutamicum* σ^E*, and C. glutamicum* σ^H*.* This cells were cultivated in complex medium 2xTY, with Tetracycline (10 µg/ml). This procedure was previously performed by an analyst in the laboratory for further use. When the cells are properly grown in the medium, it is possible to start the procedure to change the cells into a state of competence. For this, it’s necessary to inoculate the cells from the plate into a 100 ml flask with 10 ml of Complex Medium 2xTY. After the inoculation, the flasks remain at 30ºC overnight in a shaker. All strains with the several sigma factors will be prepared. In

the next day, with the cells properly grown, it is inoculated 65ml of Complex Medium 2xTY into two 500ml flasks, for each *C. glutamicum* cells with their respective sigma factor, so in total it is ten 500ml flasks, with 2 flasks for each strain.

The cells from the overnight culture are inoculated into the flasks and they have to start at the Optical Density (OD) of 0.2. Then, the flasks have to be at 30ºC in a shaker until they reach the OD of approximately 0.5, which takes about 2 to 3 hours, depending on the growth. When the OD reaches 0.5, the flasks are removed from the shaker and cooled on ice for about 15 minutes. After the 15 minutes, it is removed 30 ml of cell culture from the flask and put into a 50ml Falcon tube and centrifugated at 4500 rpm, for 10 minutes at 4ºC, and the supernatant is removed. This step is repeated for the remainder cell culture in the flasks. The pellet in the Falcon tube is resuspended in 15 ml of GT cold solution (Annex 2) and then the cells with the same strain are spilled into 1 single tube. It is performed another centrifugation with the same parameters as before, the supernatant is removed, and now the pellet is resuspended in 30 ml of GT cold solution and centrifugated again. The supernatant is removed, and the pellet in resuspended in 1 ml of G cold solution (Annex 2), put into a 2ml Eppendorf tube and centrifugated at 10000 rpm, for 3 minutes at 4ºC, and the supernatant is removed. The pellet is resuspended in 1 ml of G cold solution and divided into 170µl aliquots and stored at -70ºC or used directly.

3.6 Confirmation of pEPR1 vector

As mentioned before, the previously constructed promoters were transformed into the promoter-test vector pEPR1 using the restriction enzymes *NsiI* and *BamHI*. So, it was necessary to confirm that the procedure was successfully performed, and to make that confirmation it was made a digestion with the restriction enzymes *PstI* and *NsiI*, because the original plasmid has a restriction site for the two enzymes, but when it is transformed, it only has for the PstI. With the digestion it is possible to understand, by the number of fragments, if the alteration was successfully made.

The digestion consisted in adding in a Eppendorf tube 1 µl of the plasmid pEPR1, 1 µl of Cut Smart Buffer, 1 µl of *PstI* and 7 µl of H_2O to make the volume of approximately 10 µl. This mix was put in 37ºC, for 1 hour with no agitation. The same procedure was made for the other restriction enzyme, *NsiI.* After the digestion it is made an electrophoresis in agarose gel, separately, so 4 samples of one construct were digested with *PstI* and other 4 samples were digested with NsiI, so in total it was made 4 gels with 8 samples each.

3.7 Transformation by electroporation

This step is the one when it is constructed the two-plasmid system, the pEPR1 vector with phenol-stress induced promoters and the expression vector pEC-XXT99A

with the *C. glutamicum* cells with their respective sigma factors, with the transformation by electroporation. And for the transformation it is used a Gene Pulser, with the following parameters: 2.5 kV. 25 µF and 600 Ω.

5 µl of the isolated pEPR1 plasmid was added to the Eppendorf tube containing the 170 µl of competent cells and rested on ice for 1 minute. Then, the entire quantity on the tube was transferred to an electroporation cuvette that was stored in a freezer at -20ºC. The cuvette was inserted in a proper holder that was also stored at -20ºC, and the holder was put between 2 electrodes. On the Gene Pulser device, it was pushed the pulse buttons and the voltage is passed through the electroporation cuvette. After this step, it was added 800 ml of 2xTY Complex Medium in the cuvette and the whole quantity is transferred into a new Eppendorf tube and immediately put in a bath at 46ºC to perform the heat shock to prevent restriction systems. The Eppendorf tube stayed in the bath for 6 minutes and then the cells were shaken for 2 hours at 30ºC. After that, the cells were centrifuged for 1 minute and it was removed approximately 700 to 800 µl of the supernatant and the cells were resuspended at 100 to 200 µl of lower volume. This quantity was then spread in a Petri plate with 2xTY Complex Medium plus the antibiotics, tetracycline and kanamycin, and placed at 30ºC for growth.

3.8 Fluorescence Experiment

After the transformation by electroporation, the colonies that grew on the Petri plates were used for the fluorescence experiment. The experiment was carried out by measuring each promoter, not each sigma factor. The cells on the Petri plate were inoculated into a 100 ml flask with 10 ml of 2xTY Complex Medium, with the respective antibiotic, tetracycline, and the flasks were shaken in a shaker at 30ºC overnight. The next day, OD of the cells, 40x diluted (975µl H_2O, 25 µl cells) was measured. Depending on the value of the OD, the amount of cells that were added in the next step was calculated using the equation 1:

$$ml/cells = \frac{6}{OD(40x\ diluted)} * 8 \qquad \text{Equation 1}$$

After the calculation of value of ml of cells, 80 ml of 2xTY Complex Medium were added into a 500 ml flask, with the quantity of the cells measured and the respective antibiotics, tetracycline and kanamycin. The flasks were shaken in a shaker at 30ºC until reaching an OD of 1, and this waiting time took about 1 to 2 hours, depending on the growth of the cells. When the OD reached 1, the flasks were removed briefly from the shaker, and 30 ml of the cells were transferred into a new 500 ml flask, and 30 µl of 1M Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. The induction started and a harvest of the cells, at 0 hours was done. This harvest was done without any dilution of the cells and it depended on the OD values measured, corresponding with the information in Annex 5.

The harvest was done at regular time intervals, at 0h, with no dilution of the cells, 3h, with 5x diluted cells, 6h, with 10x diluted cells, and 24h, with 20x diluted cells. For all the harvests, the subsequent procedure was the same and includes a centrifugation of the cells harvested at 5000 rpm, 5 min at 4ºC, remove supernatant, resuspend cells in 1 ml of cold PBS (Annex 3), centrifugate again with same parameters. Resuspend the cells with 0.5 ml of cold PBS and then, keep the cells on ice in a Eppendorf tube. When all the harvests are complete with all the centrifugations made, the whole 0.5 ml were transferred into a FASTPREP tube with approximately 200 µl of Matrix B (Figure 8). The FASTPREP device (Figure 9) utility was used to perform the cells lysis. Then, all the tubes were put in the FASTPREP device and the lysis was carried out (6.0 m/s, 60 sec).

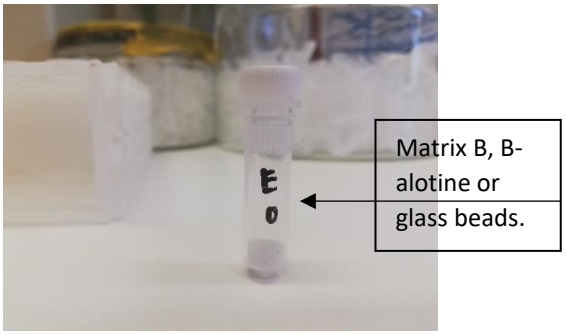


Figure 8 - FASTPREP tube with Matrix B, or B-alotine (source: Miguel Alves)

Figure 8- FASTPREP device. (source: Miguel Alves)

After the cell lysis, the tubes were removed from the FASTPREP device and centrifuged at 14000 rpm, 20 minutes at 4ºC. When the centrifugation was completed, approximately 230 µl of the supernatant (mixture without beads) was transferred into a news microcentrifuge tubes that were centrifuged again to precipitate any glass beads that could transfer from the FASTPREP tubes to the microcentrifuge tubes. Subsequently, total protein concentration was measured using the Bradford assay. The Bradford assay

was done in a spectrophotometer and the sampling was prepared with 10 µl of the supernatant, 10 µl of distilled H_2O and 980 µl of Bradford working solution (Annex 3), with this volume being mixed in a cuvette. Linked with this step, Fluorescence Intensity was measured, in a 96-well microplate (Figure 10), and 200 µl of the supernatant was added to each well and the fluorescence was measured in a computer with a software called 'Safire2'.

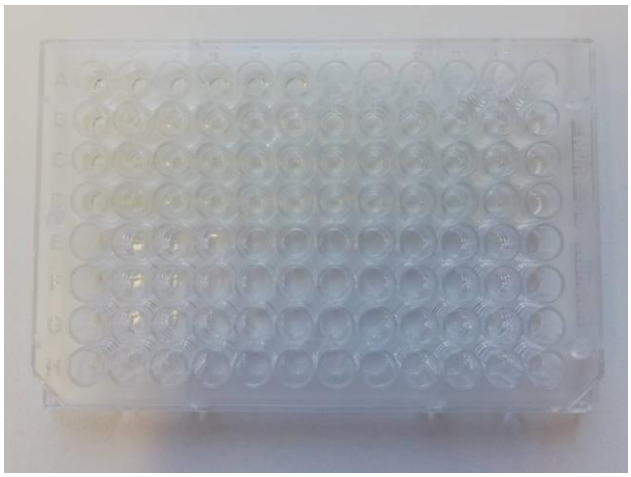


Figure 10- Microplate for fluorescence intensity measurement. (source: Miguel Alves)

When both measurements, total protein concentration with the Bradford assay, and the fluorescence were completed, all the values were introduced in an Excel sheet to get a graphic with the various responses to the promoters of the sigma factors at different hours. The final value was an arbitrary one that was mentioned as AU, and checks the response that the sigma factors had to their respective promoters, and was obtained by using equation 2:

$$Fluorescence\ (AU) = \frac{Fluorescence\ Int.}{Protein*2}$$

Equation 2

3.9 Cultivation in phenol

After the fluorescence experiment with the two-plasmid system and knowing which sigma factor responds to the different promoters, another fluorescence experiment was performed. But this time instead of having only the cells in 2xTY culture medium growing with the respective antibiotics for both plasmids, it was added phenol and glucose to start the cultivation in a different medium, that will be Minimal Medium CGXII (Annex 1).

The two-plasmid system cells were cultivated in 10 ml of Minimal Medium CGXII, with kanamycin and tetracycline, with 3.4 mM (standard concentration in the lab) phenol and 10% glucose for overnight growth. The main cultivation started at OD_{600} = 0.7, in 100

ml of Minimal Medium CGXII and when the OD_{600} reached 1, IPTG was added, and the samples harvested at 0, 3, 6 and the 24 hours of growth. The rest of the procedure is identical to the Fluorescence Experiment that was performed to the other strains without cultivation with phenol and glucose.

In this fluorescence assay, sigma factors that present a greater response to the promoters were studied, with the results from the previous measurements, that are believed to be:

- Sigma H, for the ParoF promoter;
- Sigma H/E, for the Pcg1277 promoter;
- Sigma C, for the PcydA promoter;
- Sigma C, for the PctaB promoter.

However, this sigma factors were not studied unaccompanied, since it is necessary to have some sort of comparison between the responses. So, a control (no sigma) will be studied as well and also another sigma factor if the results show some overlap between the sigma factors.

4. Results and Discussion

4.1 Product of the re-association of primers

When the re-association of primers was made, four different sets of promoter inserts were obtained, and different names were given in order to distinguish them from each other, namely, ParoF (constructed with the primers 2239 and 2240), Pcg1277 (constructed with the primers 2241 and 2242, PcydA (constructed with the primers 2243 and 2244), and finally, PctaB (constructed with the primers 2245 and 2246):

- ParoF (2239 + 2240)

5′ – GAGAACCGGAGTCGAGCAGCACCTCCCCGCAAGGGTAGAGGGGCTGCTTTTTTGTTTCCTAAATTCACCCG – 3′
3′- ACGTCTCTTGGCCTCAGCTCGTCGTGGAGGGGCGTTCCCATCTCCCCGACGAAAAAACAAAGGATTTAAGTGGGCCTAG – 5′

- Pcg1277 (2241 + 2242)

5′ –GGTTAACCTTTATAAAGTTAAGCTGTGAGCGGGAACTTAGGAATAAACTTCAACGACAACCTTTAAGAAGG– 3′
3′- ACGTCCAATTGGAAATATTTCAATTCGACACTCGCCCCTTGAATCCTTATTTGAAGTTGCTGTTGGAAATTCTTCCCTAG – 5′

- PcydA (2243 + 2244)

5′ – GTGGTTTGAAAACATTAAAAAGATTGATTCGGGAACTTTTAATTAACTTCATCCGACTAATTTGCCGTGTG – 3′
3′ – ACGTCACCAAACTTTTGTAATTTTTCTAACTAACTAAGCCCTTGAAAATTAATTGAAGTAGGCTGATTAAACGGCACACCTAG – 5′

- PctaB (2245 + 2246)

5′ – GGGAAATTCCACTCATGAACGCCTAGTCTACGGGAACCATTTCACCAGCGTGTACGTTGTAAATGTGAACG – 3′
3′- ACGTCCCTTTAAGGTGAGTACTTGCGGATCAGATGCCCTTGGTAAAGTGGTCGCACATGCAACATTTACACTTGCCTAG – 5′

4.2 Result of the ligation

After the re-association of the primers, the ligation of the same primers was performed in the promoter-test pEPR1 vector with the reporter gene, *gfpuv*, with the respective restriction enzymes, BamHI and NsiI, T4 DNA Ligase, T4 DNA Ligase buffer and 50% PEG 4000, which is a non-ionic, hydrophilic and non-toxic polymer, that helps the purification and crystal growth of proteins and nucleic acids. The ligation was made using all the four primers previously constructed, so in total, there were four total ligations, for each one of the promoters (ParoF, Pcg1277, PcydA, PctaB). The product of the ligation is shown in Figure 11, with the restriction enzymes PstI and BamHI, that

are the restriction sites of the pEPR1 vector, with a model that represents what happened in the process of ligation:

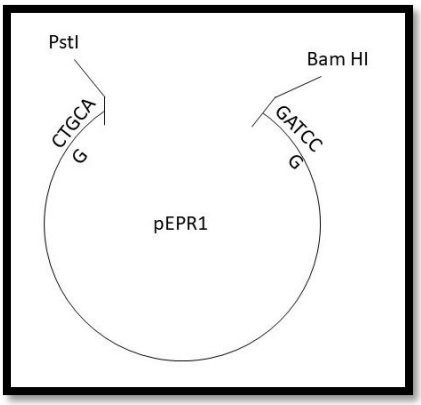


Figure 11- pEPR1 vector with the action of the restriction enzymes (source: documents provided by mentor from the Institute).

The result shown in Figure 11 is possible because of the restriction sites that both enzymes have, which are:

- Bam HI : 5' – GGATCC – 3'
  3' – CCTAGG – 5'
- PstI: 5' – CTGCAG – 3'
  3' – GACGTC – 5'

At this time, the promoters carried on the BamHI-PstI DNA fragments were obtained and cloned in the promoter-test pEPR1 vector containing the promoter-less *gfpuv* reporter gene. The four combinations of the promoters with the vector were pEPR1-ParoF, pEPR1-Pcg1277, pEPR1-PcydA, pEPR1-PctaB. After the ligation, it was performed the transformation of the vector in *E.coli*.

4.3 Transformation in *E.coli*

After the ligation, the transformation of the pEPR1 vector was performed in *E.coli*, with the Top 10 *E.coli* tubes. The transformation resorting to heat shock was carried out in order to insert a foreign plasmid or a ligation product into bacteria, in this case, *E.coli.* The procedure is simplified by a diagram that is represented in Figure 12.

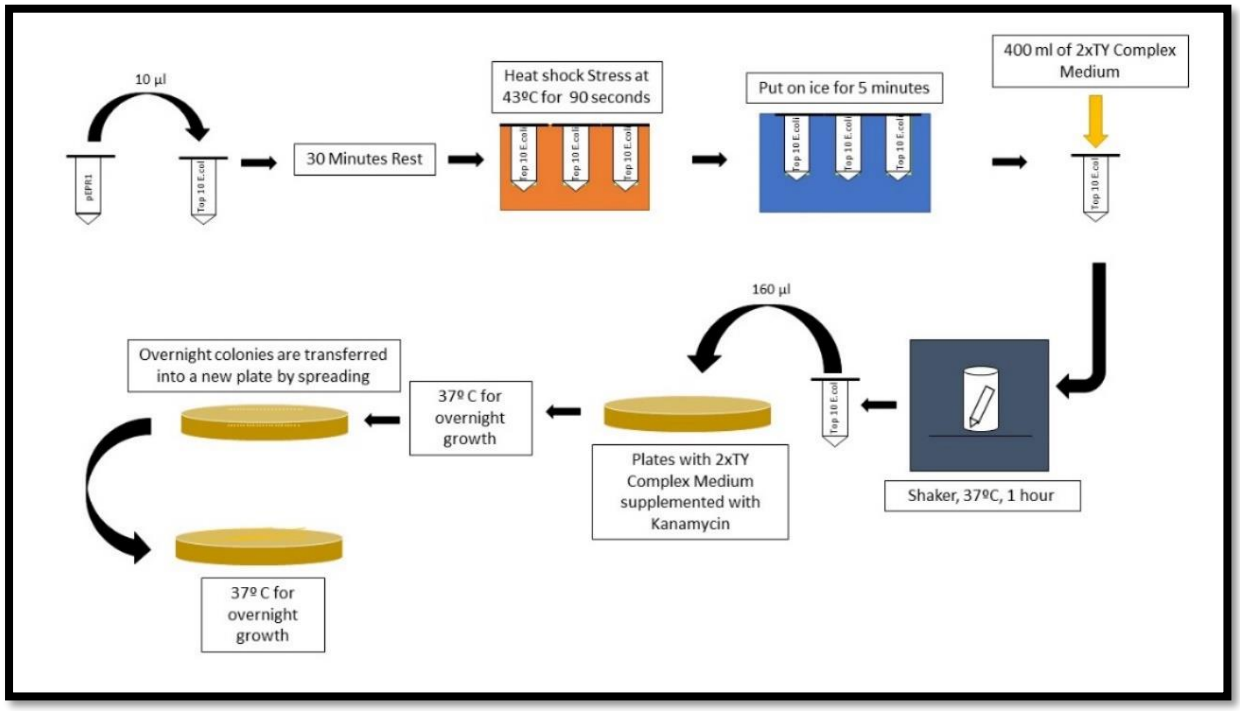


Figure 12- Transformation of the pEPR1 in E.coli. (source: Miguel Alves)

The step where the 2xTY Complex Medium was added with the purpose of regeneration of the cells, is done in a smaller shaker, detailed to agitate 100 ml flasks and lower volume laboratory equipment. The tubes were put into a small plastic cup and were shaken all at once. After the shaking period, 160 µl of the medium with transformed cells were transferred to a plate with 2xTY complex medium (Figure 12) because this amount of volume is more adequate to obtain colonies, with more volume it's harder to dry the cells in medium.

4.4 Confirmation of the transformation

Then it was performed the isolation of the plasmid, in order to confirm that the transformation was correctly completed. The isolation of the plasmid was carried out with the Macherey-Nagel DNA and RNA Purification kit that was explained previously. After the isolation, the confirmation proceeds to an electrophoresis in agarose gel, by digestion of the plasmid with both restriction enzymes *PstI* High-Fidelity and *NsiI* High-Fidelity. High-Fidelity restriction enzymes have the same specificity as native enzymes but have been engineered for significantly reduced star activity and performance in a single buffer (CutSmart Buffer). All HF-restriction enzymes come with Purple Gel Loading Dye, 6x diluted. The digestion is made with this enzymes because the original non-transformed plasmid has the restriction sites for both restriction enzymes, whereas the transformed plasmid only has a restriction site for the *PstI*, so it is possible to know if the transformation was correctly done by the number of fragments that the gel will show.

Two digestions were made, and afterwards four gels, for each promoter in which each one had eight sites, four for each restriction enzyme. The results of the digestion are shown in Figure 13, 14, 15 and 16:

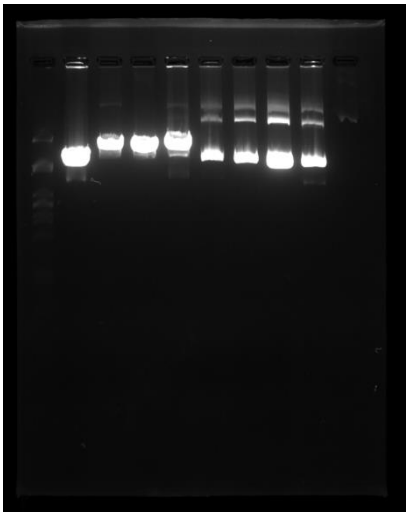


Figure 13- Electrophoresis result in agarose gel, of the ParoF promoter, with the first 4 wells representing the digestion with the restriction enzyme PstI and the other 4 wells representing the restriction enzyme NsiI. (source: Miguel Alves)

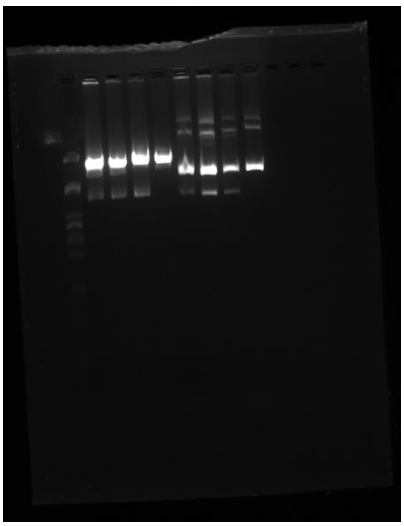


Figure 14- Electrophoresis result in agarose gel, of the Pcg1277 promoter, with the first 4 wells representing the digestion with the restriction enzyme PstI and the other 4 wells representing the restriction enzyme NsiI. (source: Miguel Alves)

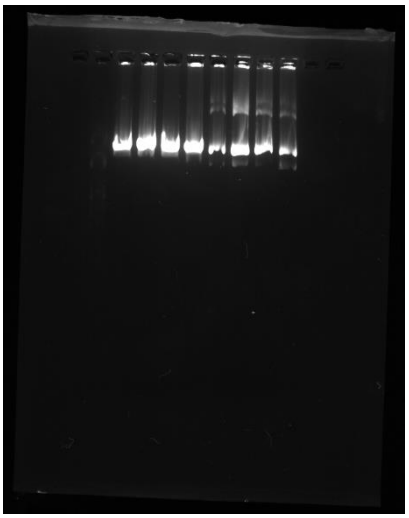


Figure 7- Electrophoresis result in agarose gel, of the PcydA promoter, with the first 4 wells representing the digestion with the restriction enzyme PstI and the other 4 wells representing the restriction enzyme NsiI. (source: Miguel Alves)

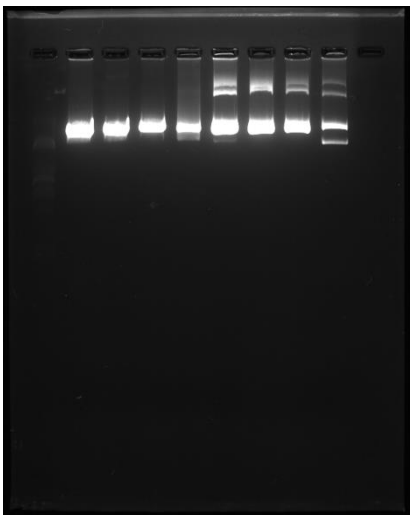


Figure 16- Electrophoresis result in agarose gel, of the PctaB promoter, with the first 4 wells representing the digestion with the restriction enzyme PstI and the other 4 wells representing the restriction enzyme NsiI. (source: Miguel Alves)

In the Figures 13, 14, 15 and 16 there are shown the several results of the restriction made with the two enzymes, PstI and NsiI. The first four wells of each gel represent the restriction with PstI, and the other four wells represent the restriction with NsiI. So, as mentioned before, to confirm that the transformation of the plasmid pEPR1 was correctly done, the results of the gel should show that on the PstI restriction wells there is only one well and on the NsiI restriction site there is still two wells.

In the Figures 13, 14, 15 and 16 it is confirmed that the transformation was performed properly for each one of the promoters. Now that the transformation is confirmed, it is possible to proceed to the next step of the experiment. In this next step, it is necessary to have the transformed pEPR1 plasmid and the competent cells previously prepared and that are stored in -80ºC for preservation.

4.5 Creation of the two-plasmid systems with transformation by electroporation

With the pEPR1 vector fully prepared and confirmed, and the *C. glutamicum* competent cells also set, it was possible to start the construction of the two-plasmid system containing both the pEPR1 vector, and the pEC-XT99A expression vector with the respective sigma factors of *C. glutamicum*. To obtain the two-plasmid system, a transformation by electroporation was performed. The strains that were obtained by the electroporation are shown in Table 2.

Table 2- Two-plasmid system constructed strains after transformation by electroporation

pEPR1/pEC-XT99A sigma factors	**ϴ**	A	C	E	H
ParoF	X	X	X	X	X
Pcg1277	X	X	X	X	X
PcydA	X	X	X	X	X
PctaB	X	X	X	X	X

The transformation was performed in a device called Gene Pulser, and the procedure is represented in Figure 17. In total, 20 different types of strains were obtained with the two-plasmid system, and 20 transformations were made.

When the electroporation cuvette was inserted into the GENE PULSER device

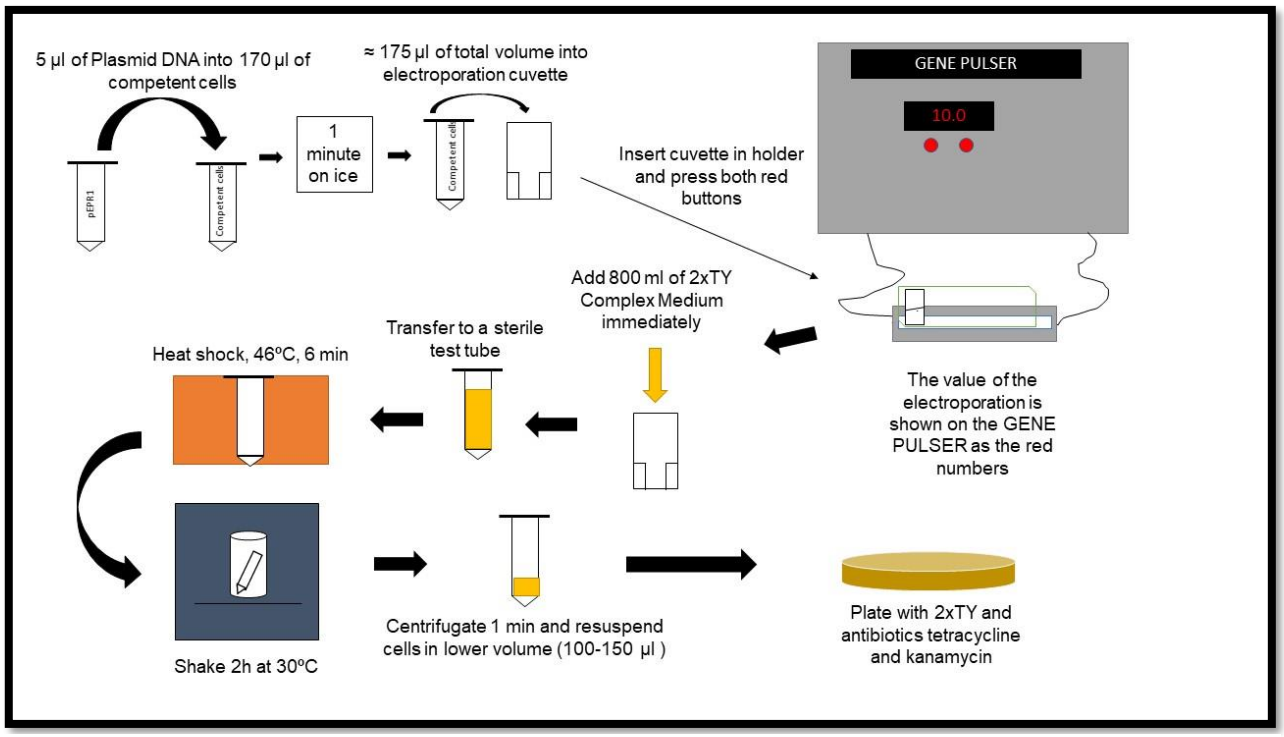


Figure 17- Diagram representing the procedure of the transformation by electroporation of the *C. glutamicum* cells in pEC-XT99A and the phenol-stress induced promoters in pEPR1. (source: Miguel Alves)

and both red buttons (Figure 17) were pressed, a value was shown. This value is the time of impulse, that should range between eight and 14 milliseconds (ms). However, sometimes because of the presence of bubbles on the cuvette or the lid of the cuvette isn't properly attached, the value is not within the recommended range. So, the transformation needs to be repeated. This problem occurred a few times during the process of the transformation of all the strains, so in the Tables 3, 4, 5, 6, and 7, are only recorded the values that were correctly measured and in the recommended range

Table 3 - Impulse values of the electroporation of the control sigma strain, with the four promoters.

C. *glutamicum* Sigma factors/pEPR1	Impulse values (ms)
No Sigma/ParoF	8.2
No Sigma/Pcg1277	10.7
No Sigma/PcydA	12.9
No Sigma/PctaB	11.4

Table 4 - Impulse values of the electroporation of Sigma A, with the four promoters.

C. glutamicum Sigma factors/pEPR1	Impulse values (ms)
Sigma A/ParoF	11.4
Sigma A/Pcg1277	11.4
Sigma A/PcydA	12.2
Sigma A/PctaB	11.8

Table 5 - Impulse values of the electroporation of Sigma C, with the four promoters.

C. glutamicum Sigma factors/pEPR1	Impulse values (ms)
Sigma C/ParoF	12.7
Sigma C/Pcg1277	12.5
Sigma C/PcydA	12.2
Sigma C/PctaB	12.6

Tabel 6 - Impulse values of the electroporation of Sigma E, with the four promoters

C. glutamicum Sigma factors/pEPR1	Impulse values (ms)
Sigma E/ParoF	10.9
Sigma E/Pcg1277	12.9
Sigma E/PcydA	11.6
Sigma E/PctaB	10.0

Tabel 7 - Impulse values of the electroporation of Sigma H, with the four promoters

C. glutamicum Sigma factors/pEPR1	Impulse values (ms)
Sigma H/ParoF	12.3
Sigma H/Pcg1277	13.4
Sigma H/PcydA	11.6
Sigma H/PctaB	11.2

As it is possible to observe in Tables 3 to 7, after some repetitions, it was able to obtain the values within the correct range for all transformations. When the transformation is complete and the plate with the cells is put into 30ºC, instead of waiting just 1 day of overnight growth, the plate stays in 30ºC for 2 days, because it is required more time to fully grow the colonies, and 1 day is not enough time. After the 2 days growth, the plates have the colonies (Figure 18) and the colonies are transferred to another plate, by spreading technique (Figure 19). This time, the plate stays just 1 day at 30ºC for overnight growth.

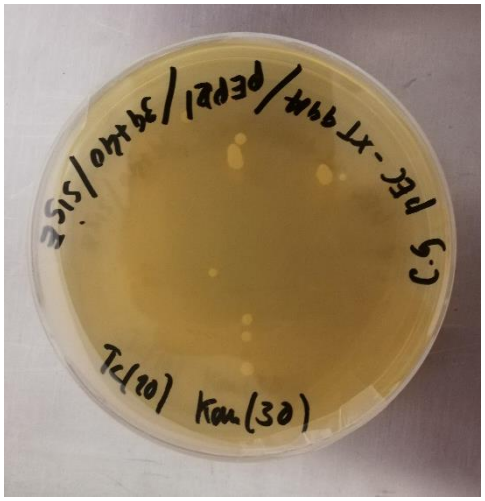


Figure 18- Colonies obtained after the incubation at 30ºC for 2 days. (source: Miguel Alves)

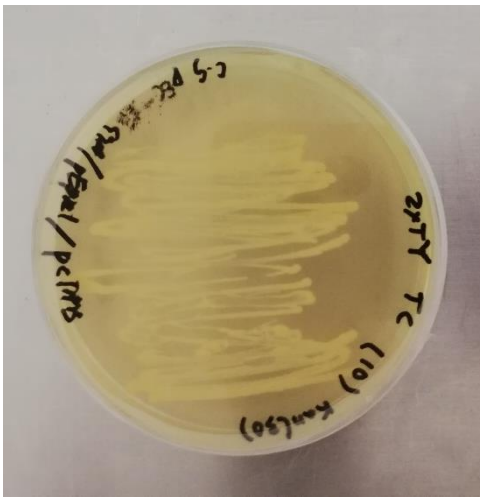


Figure 19- Colonies transferred by spreading from the plate of the previous day. (source: Miguel Alves)

As is it possible to see by Figure 19, that is the final representation of the two-plasmid system before the start of the fluorescence assay. Nevertheless, before starting the fluorescence assay it is necessary to confirm that the transformation is correctly performed, to both check the presence of the several sigma factors of *C. glutamicum* in pEC-XT99A and pEPR1 with the promoters.

4.6 Confirmation of the transformation by electroporation

Two types of confirmations regarding the transformation by electroporation were done: restriction with PstI and PCR. This procedure was essential in order to confirm that the transformation was performed correctly, and with this, avoid further mistakes in the study. However, during the confirmation by the two techniques there were some strains that weren't confirmed twice, which were: Sigma E with PcydA, Sigma E with PctaB, Sigma H with PcydA and Sigma H with PctaB. This happened because these colonies grew at a lower development rate and when it was time to confirm them, there was only time to make one confirmation, which was by PCR.

4.6.1 Confirmation by Restriction

In order to perform the restriction with PstI, firstly the product of the transformation that is shown on Figure 18, the two-plasmid system strain, needs to be isolated. The isolation is performed by the Macherey-Nagel kit for DNA and RNA purification and isolation, which procedure was already described earlier. Then, when the strain is properly isolated, it is ready for the electrophoresis in agarose gel.

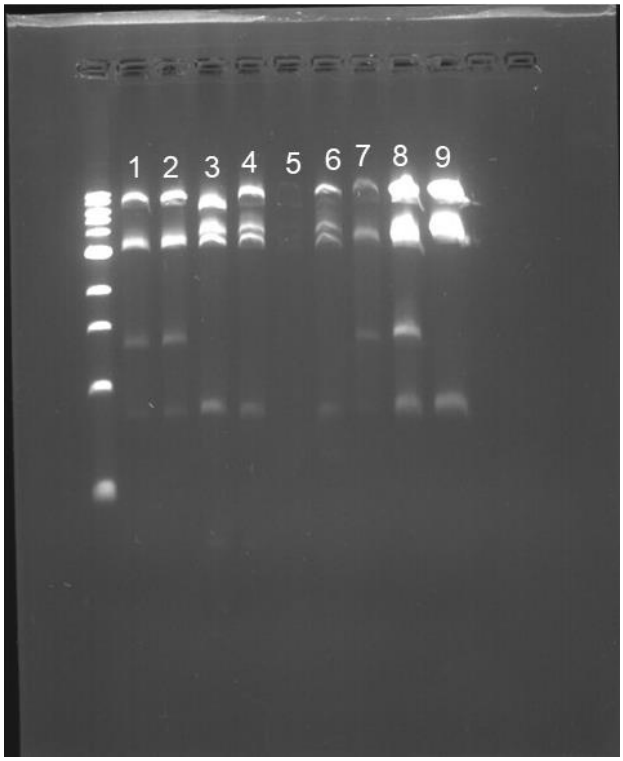


Figure 8- Agarose Gel with the results of the Restriciton with PstI of the transformated two-plasmid system. (source: Miguel Alves)

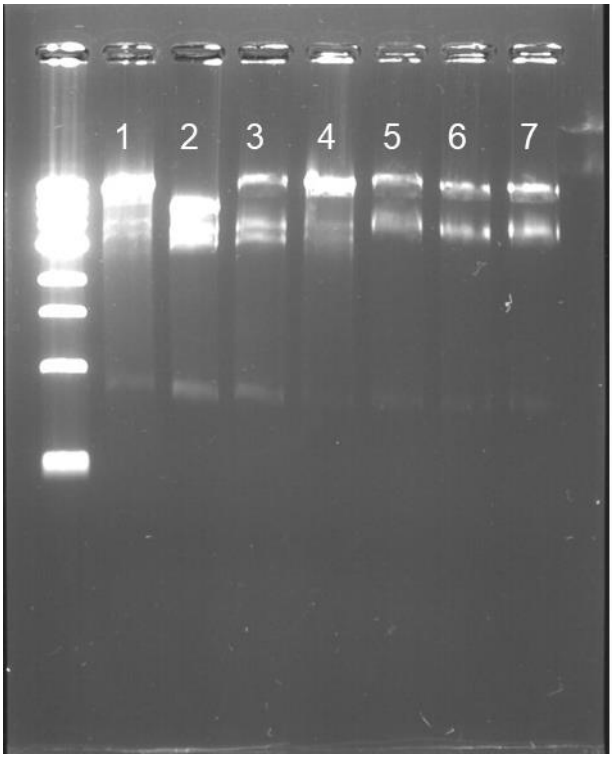


Figure 21- Agarose Gel with the results of the Restriciton with PstI of the transformated two-plasmid system (source: Miguel Alves)

The numbers in Figure 20 represent the following:

1. Control strain, no sigma, with ParoF promoter.
2. Control strain, no sigma, with Pcg1277 promoter.
3. Control strain, no sigma, with PcydA promoter.
4. Control strain, no sigma, with PctaB promoter.
5. Sigma C, with ParoF promoter.
6. Sigma C, with Pcg1277 promoter.
7. Sigma C, with PcydA promoter.

In Figure 21, the numbers represent:

1. Sigma A, with PcydA promoter
2. Sigma A, with PctaB promoter
3. Sigma E, with ParoF promoter
4. Sigma E, with Pcg1277 promoter
5. Sigma H, with ParoF promoter
6. Sigma H, with Pcg1277 promoter

7. Sigma A, with ParoF promoter
8. Sigma A, with Pcg1277 promoter
9. Sigma C, with PctaB promoter

The first set of wells in each gel represents the marker, that shows the size of fragments until 1000 bp. Since the strains were isolated and then digested with PstI, all of them should show similar results, which is to have two fragments with their size being 10kbp bp and 6 kbp. In the Figures 20 and 21 it is possible to see that all of the strains are correctly constructed, having those two fragments with their respective sizes. However, in the lane 5 of Figure 20 and lane 2 of Figure 21, the sizes are not completely viewable and clear. In the first instance, in Figure 20, lane 5 has a really soft mark that is not clear to have the same fragment sizes that the other lanes have. To check this problem, it was measured the concentration of the isolated strain of the two-plasmid system, with the result being 45 µg/µl, which is a bit lower that expected. But the results were checked with the laboratory experts and it was concluded that the transformation was correctly performed, with just the setback being that the concentration is lower than expected.

In Figure 21, the lane 2 does not have a fragment near the 10 kbp, whereas the other lanes have that fragment in that specific size. Instead that fragment is between the 8kbp and 9 kbp, which is acceptable by consulting with the laboratory experts, since although the first fragment is not 10 kbp like the other ones, the second fragment is practically the same size as the other lanes, so the restriction for the strains represented in both Figures were all correct.

4.6.2 Confirmation by PCR

Besides the confirmation with the digestion and restriction with PstI, in order to have complete certainty that the transformation by electroporation was correct, a PCR was made for all the strains. In this PCR, the mix that was made contained:

- 0.5µl primer forward;
- 0.5µl primer reverse;
- 2µl of isolated DNA (two-plasmid strain);
- 9.5µl PCR H_2O;
- 12.5µl of 2x PCR Bio Taq Mix.

Both of the primers were provided by a laboratory expert, and they were constructed by the same person. The fragment sizes that it is supposed to observe in the agarose gel for each strain is:

- 400 bp for the Control Sigma;
- 1500 bp for Sigma A;
- Between 800 and 900 bp for Sigma C;
- Between 700 and 800 bp for Sigma E and H.

In Figure 22, the lanes that are represented by several numbers, each one characterizes a type of a two-plasmid system strain, that are:

1. Sigma E with ParoF promoter.
2. Sigma E with Pcg1277 promoter.
3. Sigma A with ParoF promoter.
4. Sigma A with Pcg1277 promoter.
5. Sigma A with PcydA promoter.
6. Sigma A with PctaB promoter.
7. Control Sigma with ParoF promoter.
8. Control Sigma with Pcg1277 promoter.
9. Control Sigma with PcydA promoter.
10. Control Sigma with PctaB promoter.
11. Sigma C with ParoF promoter.
12. Sigma C with Pcg1277 promoter.
13. Sigma C with PcydA promoter.
14. Sigma C with PctaB promoter.

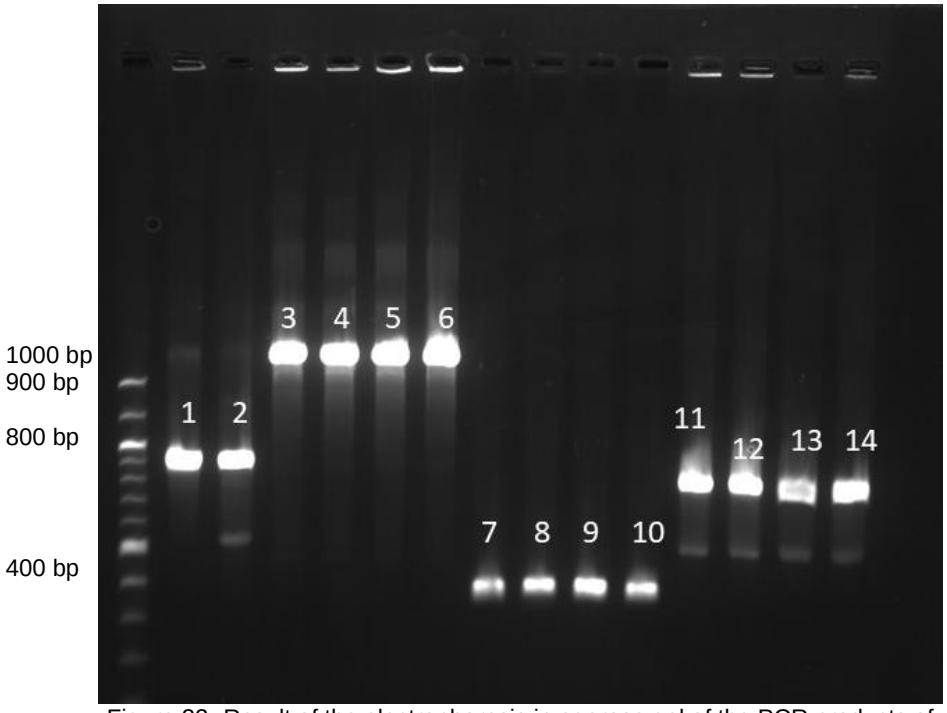


Figure 22- Result of the electrophoresis in agarose gel of the PCR products of the two-plasmid system strains (source: Miguel Alves)

Analysing the result of the electrophoresis in agarose gel of products obtained by PCR, it is possible to conclude that all the strains have the fragment size that they are supposed to have and with the second confirmation by PCR, the transformation by electroporation was successfully performed for these strains in Figure 23. In this agarose gel it was not possible to include all the strains, so the strains,

Sigma E with the PcydA promoter, Sigma E with the PctaB promoter and the Sigma H with all of the respective promoters will be presented next.

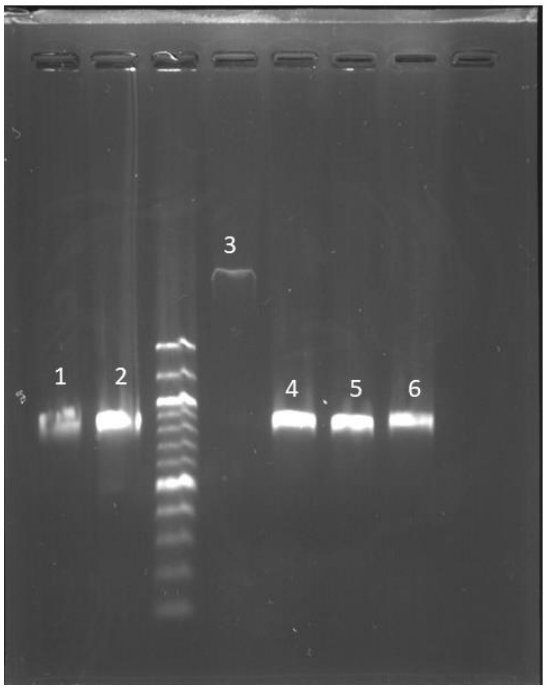


Figure 23- Electrophoresis in agarose gel with the products of PCR of the two-plasmid strain (source: Miguel Alves)

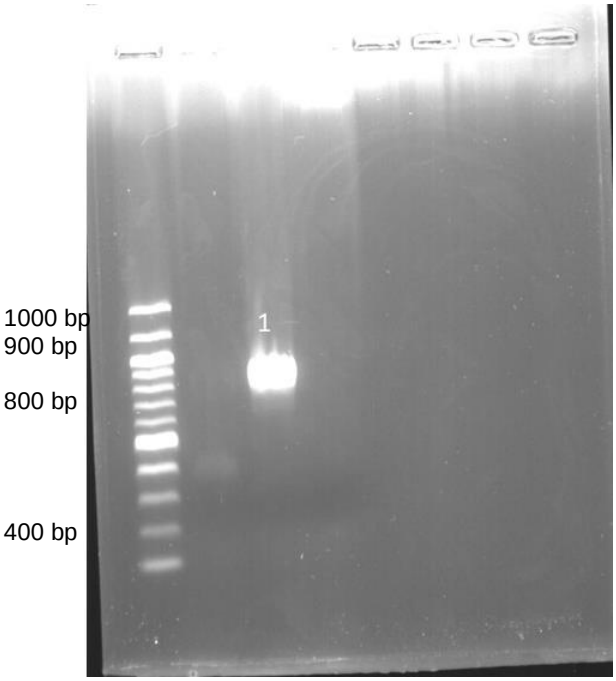


Figure 24-Electrophoresis in agarose gel with the products of PCR of the two-plasmid strain(source: Miguel Alves)

In Figure 23 the PCR products represented by the numbers are:

1. Sigma E with PcydA promoter.
2. Sigma E with PctaB promoter.
3. Sigma H with ParoF promoter.
4. Sigma H with Pcg1277 promoter.
5. Sigma H with PcydA promoter.
6. Sigma H with PctaB promoter.

In Figure 24 the PCR product represented by the number 1 is the Sigma H with ParoF promoter. Almost all the strains were confirmed with the electrophoresis represented in Figure 22 but in lane 3 the Sigma H with the promoter ParoF was poorly constructed so it was repeated and then the result was more satisfactory.

With all the electrophoresis presented before, it is possible to conclude that the transformation by electroporation was successfully made and the Fluorescence Assay for each one of the strains is now able to perform.

4.7 Fluorescence Assays

4.7.1 ParoF Promoter

As mentioned before, since the transformation by electroporation was successfully confirmed by Restriction with PstI and PCR, it was possible to proceed to the step where it was made the fluorescence assay to the several two-plasmid system strains that were constructed. The fluorescence assay was performed in 2xTY Complex Medium and the expression was induced by IPTG, that binds and inhibits the lac repressor without being degraded. Genes controlled by the *lac* or *tac* promotor/operator sequences are expressed to high levels in the presence of IPTG.

So, there will be four fluorescence assays, for each promoter, and it will consist by having two 500ml flasks of each two-plasmid system strain (Control Sigma, Sigma A, Sigma C, Sigma E, Sigma H). One flask will have the strain in the medium, and the other flask will have the same mix with the addition of IPTG, so for each experiment there will be 10 flasks. The growth of the cells will be performed in a large shaker (Annex 4).

For each promoter, the experiment will be repeated in order to have two results to see the average and standard deviation of such. In Figure 25 it is represented the average and standard deviation of the results of the fluorescence assays regarding the promoter ParoF.

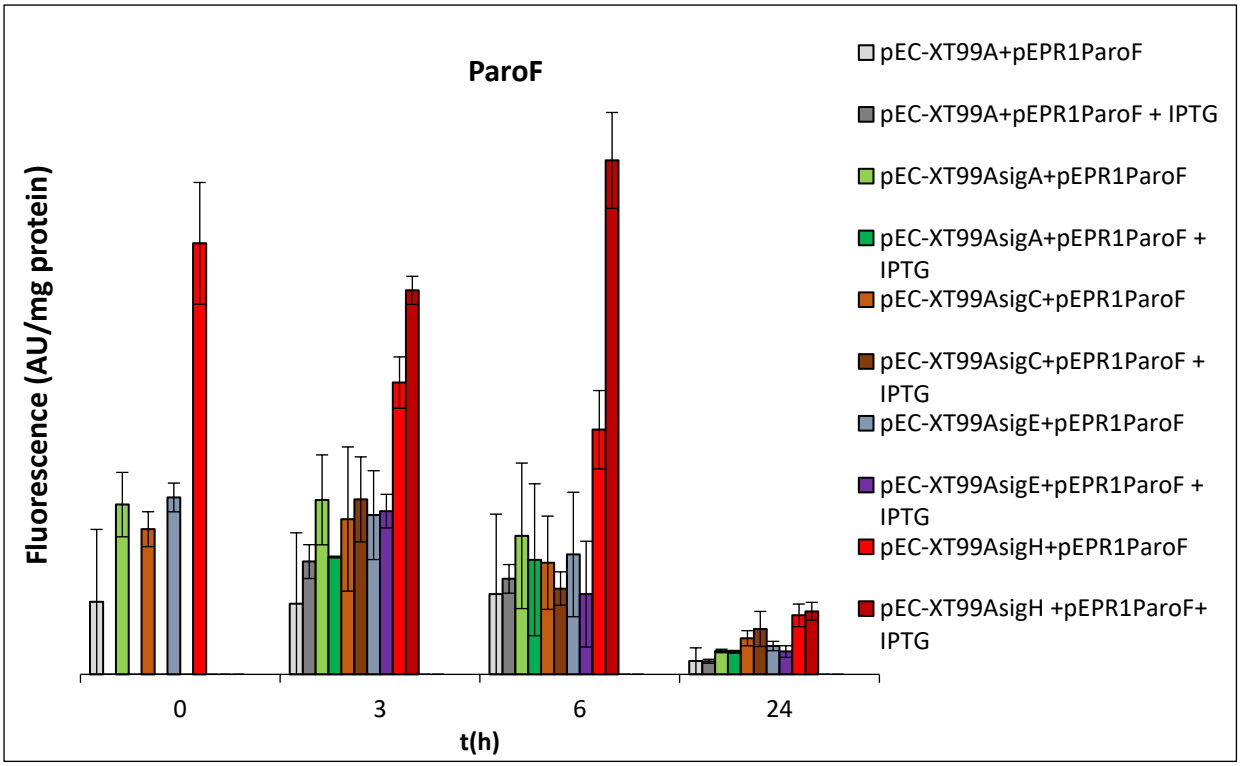


Figure 25 - Fluorescence values of the ParoF promoter

In previous experiments regarding these sigma factors and promoter, it was known that this promoter would recognize Sigma H and throughout time it would express it, and no other sigma factor.

By analysing the results in Figure 25, it is possible to see that in the first part of the cell growth, without the addition of IPTG, Sigma H responds entirely to the ParoF promoter, with much higher values of fluorescence compared to the other sigma factors studied. In the third and sixth hour of cell growth, the response is basically the same that was observed in the first part of the assay, with slightly higher values in the sixth hour of growth, which is a positive result.

However, in the last part of the cell growth there is a significant drop in the response of Sigma H to the ParoF promoter, although it still remains the higher response among the other sigma factors. Nevertheless, this should not happen, the response of the sigma factor to the promoter should remain in high values throughout the experiment, especially in the twenty-fourth hour. This drop in the response of the sigma factors could be related to the fact that the plate that contained the cells of the two-plasmid strain (Sigma H – ParoF promoter), produced very few colonies and when the colonies were transferred to a new plate by spreading, the cells didn't had vastly growth, resulting in a lower response period.

In the end, the response of Sigma H to the ParoF promoter was successfully confirmed, with Sigma H having response values much higher than the other sigma factors studied throughout the experiment period. As expected, the strains in which were added IPTG had a higher response compared to the strains that did not have the addition of IPTG.

Later in the extended work, this strain will be studied once more but this time with the addition of phenol to see what happens if the strain is put through stress, and because the addition of phenol will create a toxic environment for the cells.

4.7.2 Pcg1277 promoter

The promoter Pcg1277 has been previously studied as well, but there's not a clear settlement that this promoter makes a certain unique sigma factor respond. With the results of those studies it is known that this promoter makes whether Sigma H or Sigma E to respond, or maybe both.

In Figure 26 is represented the complete results of the fluorescence assay of the Pcg1277 promoter with the several sigma factors. Analysing the results that were obtained, the clear conclusion is that the sigma factor that has the higher response to the presence of the Pcg1277 promoter it is indeed Sigma E. As explained before, the results that are presented represent the junction of two fluorescence assays, showing the average and standard deviation of both measurements and it is clear to see that Sigma E is the predominant sigma factor that responds to the presence of the promoter in both assays. Sigma H is expressed as well but in much lower values.

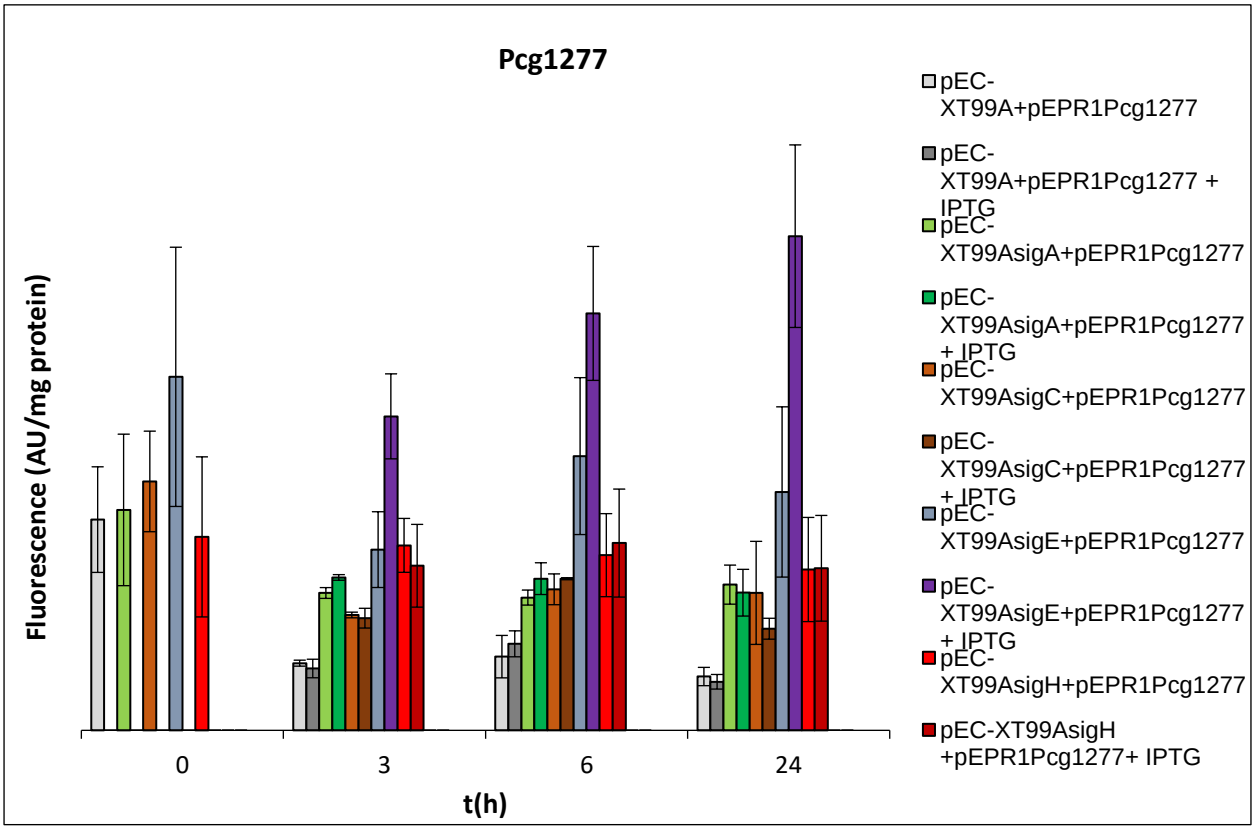


Figure 26 - Fluorescence values of the Pcg1277 promoter

In contrast to the results that were obtained regarding the previous promoter, throughout the period of the measurement, there was a constant increase in the response of Sigma E, including the twenty-fourth hour of the fluorescence assay.

Also, regarding Sigma E, the strain in which the IPTG was added, had a higher response than the strain that did not have the addition of IPTG. This was expected and confirmed by the results.

One intriguing factor regarding the response values in both of the measurements shown already is that the initial part of the experiment, the hour 0, has extremely high values of response, in some cases even higher that the third hour of cell growth. This was discussed with some laboratory experts and it was indeed a surprise but not a bad sign, because the response is visible and with the correct sigma factor, in this case, Sigma E.

4.7.3 PcydA promoter

The PcydA promoter has a different sigma factor that it is known to respond, regarding various experiment previously made. This promoter should have Sigma C has the primary and main response in this experiment.

In Figure 27 it is represented the complete results of the fluorescence measurement of the PcydA promoter with the various sigma factors. In the first time period of the experiment it is possible to see that Sigma C has the higher response values of all the sigma factors, but Sigma H has as well a significant response. However in this part of the assay there is still no addition of IPTG.

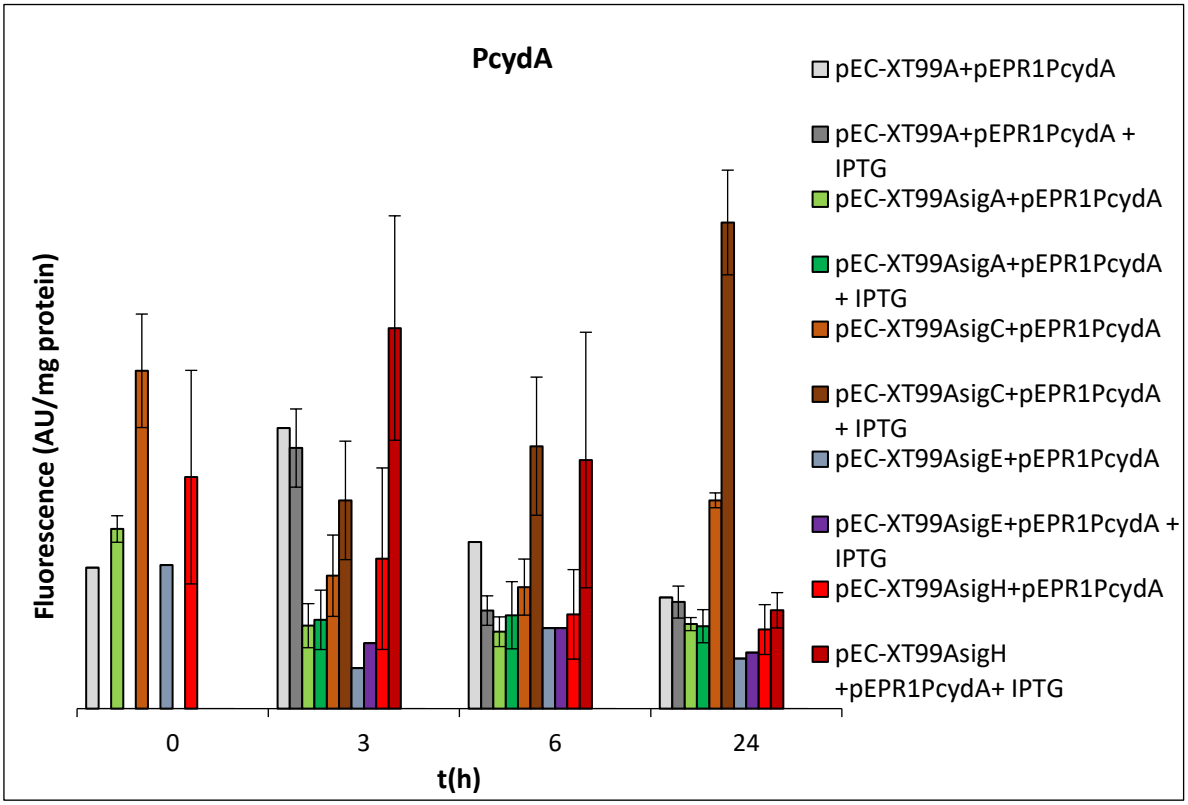


Figure 27 - Fluorescence values of the PcydA promoter

Surprisingly, in the third hour of cell growth the sigma factor that as the higher response is Sigma H with IPTG and also the control sigma in grey, whereas Sigma C

with IPTG has a slightly higher response than Sigma A and Sigma E, but significantly lower than the control sigma and sigma H.

By the sixth hour of cell growth, there is a significant change of scenery. The response of the control sigma decreased as well as the sigma H response, but as expected the response of Sigma C overtook all the other sigmas. However, it still somewhat equivalent but slightly higher than Sigma H.

In the last hour of recorded growth it is cleat that Sigma C represents the predominant response to the PcydA promoter, having a notorious advance compared to the remaining sigma factors. The strains in which the IPTG was added represented higher response values as expected.

The one factor that still remains unexplained is the fact that sigma H had a really high response to the promoter in the first hours of cell growth. This is a recurring phenomenon that happens regarding Sigma H, because it is known that the primary sigma factors of *C. glutamicum* are Sigma A and Sigma B, but several recent studies and experiments have shown that Sigma H could also be a primary factor and not a secondary one as it knows at his point in time.[3]

4.7.4 PctaB promoter

As well as the promoter presented before, the promoter PctaB also has Sigma C as the main sigma factor of response in previous consulted experiments. So, by examining Figure 28 that presents the final values of the fluorescence measurement regarding the PctaB promoter, it is possible to observe that in the first stage of the growth all the sigma factors had a similar response value to the presence of the promoter. In this step there is still no addition of IPTG.

When the induction begins in third hour of growth, the results show a small advantage in the response of Sigma C with the addition of IPTG, that clearly stands above the other sigma factors with the normal strain and induced as well.

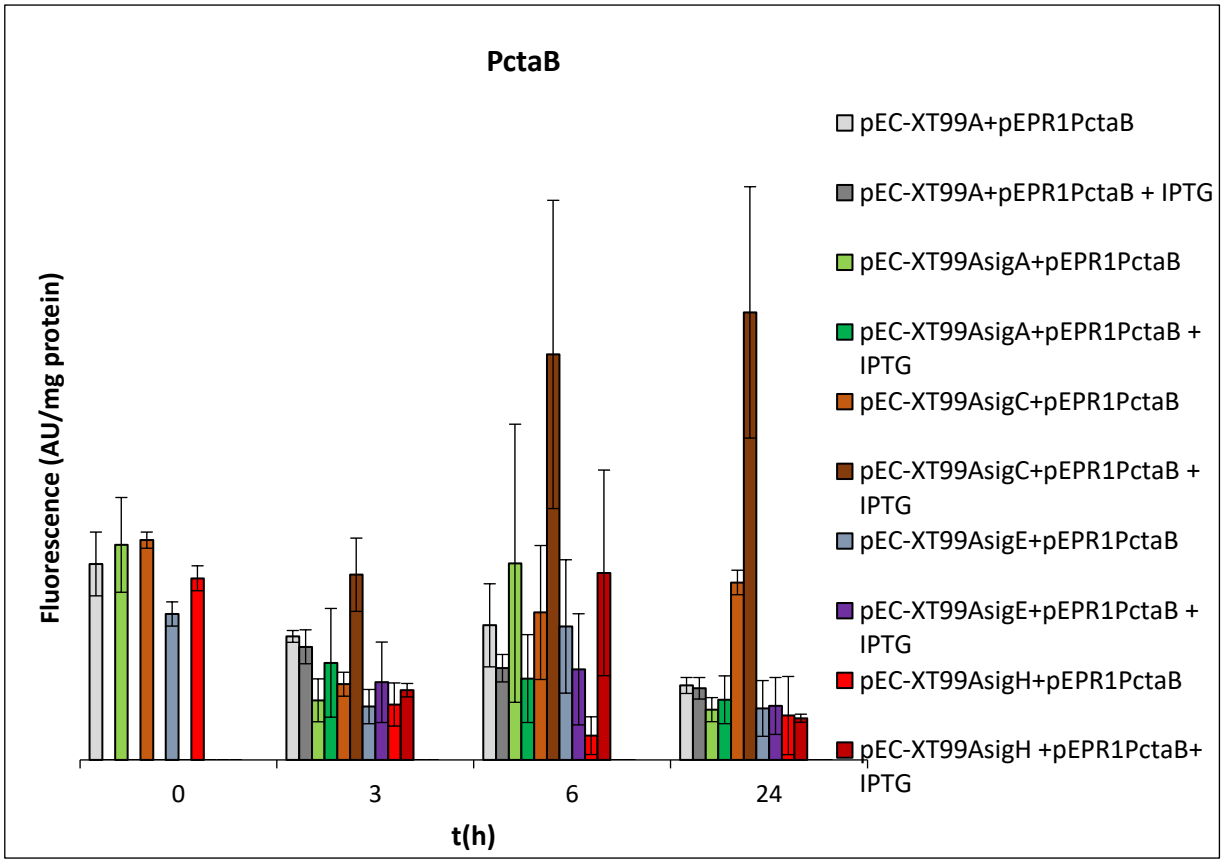


Figure 28- Fluorescence values of the PctaB promoter

Now in the sixth hour of growth, the strain of Sigma C that was induced by IPTG has an even bigger response than before and also a lot more compared to the other sigma factors. But it is possible to see too that there is a slight increase in some strains, which are Sigma A without induction and Sigma H inducted, however the Sigma H strain without induction has an extremely low response.

In the final phase of the measurement, the response values are clear, Sigma C has a big lead compared to the other sigma factors and the inducted strain has a really high response, even higher than in the last phase (sixth hour).

So, concerning the PctaB promoter, the fluorescence measurement proved correctly the consulted results previously recorded, showing that when the sigma factors are put through a exponential growth in the presence if this promoter, as well as the induction with IPTG, there is one sigma factor that has a definitely higher response that the others, being Sigma C.

4.8 Fluorescence assays with phenol

The results that were obtained by the fluorescence measurements made in point 4.7, confirm the previous provided information about the activity of the sigma factors with the promoter in a two-plasmid strain. So in this step, the measurements will be repeated but this time the cells will be cultivated in phenol, because with the presence of phenol the cells start co-existing in a toxic environment or as is it commonly known, stress.

But with the presence of phenol, the fluorescence measurement can't be carried out with the same medium. In this case, it will be used a different medium, CGXII (Annex 1), and to help the cell growth, it will be added glucose (10%) to the medium in the beginning of the measurement. The rest of the procedure is carried out the same way as previously noted.

Regarding this step of the study, there are no previous known results of this measurement with the addition of phenol.

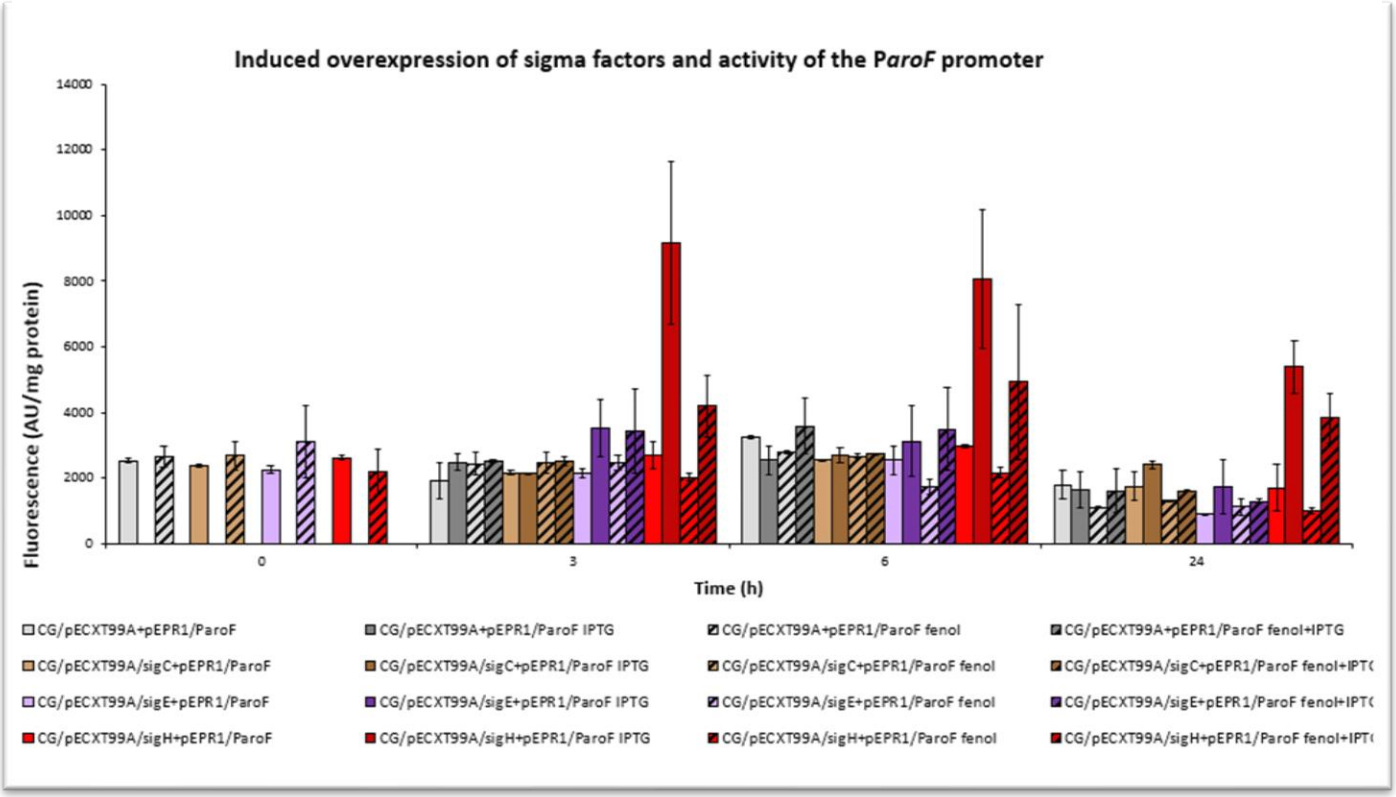
The way phenol is added to the medium along with the double-plasmid strains follows 3 important steps, being:

1. In the beginning of the growth at 30ºC, each strain has 2 100ml flasks, with 1 only containing the strain in the medium and the other with phenol, which is added the quantity of 3.28 ml (value previously calculated per 100 ml by the laboratory experts).
2. Then, there is waiting period until the OD of the flasks reach 1.00.
3. When the OD hits 1.00, it is added IPTG to the medium, giving the final count of four 100 ml flasks per sigma factor (control sigma, with IPTG, with IPTG and phenol, and just phenol)

4.8.1 ParoF Promoter

In Figure 29 it is represented the results of the fluorescence assay regarding the promoter ParoF, with the difference that this experiment was carried out using phenol as a stress factor during the cell growth at 30ºC, as previously mentioned. Also, another slight difference in this experiment was that is was only cultivated 4 double plasmid strains, a control sigma factor and Sigma C, E and H, in contrast to the previous fluorescence assay with the promoter ParoF which was also used Sigma A.

Figure 29 - Fluorescence values of the ParoF promoter, cultivation in Phenol



In the beginning of the experiment (0h) basically all strains have the same levels of response in terms of fluorescence. When the induction begins (3h) it is possible to observe that the sigma H strain with IPTG and IPTG along with phenol assert themselves with the higher response values while the other strains remain with similar responses. The same situation appears again in the next phase of analysis (6h). With 24 hours of growth, the sigma H strains with IPTG and IPTG with phenol, still are the primary responders to the promoter ParoF, but similarly to the fluorescence assay that was performed before with the same promoter, at the end of the experiment, the activity as significantly lower compared to the other times of the assay.

So, in conclusion, it is possible to affirm that the addition of phenol along with a different medium interfered with the expression of the sigma factors in the presence of the promoter ParoF, since the strains where it was added the IPTG remained as the ones that possesses the higher response values and the ones with phenol and both phenol and IPTG did not present high response.

4.8.2 Pcg1277 Promoter

The fluorescence assay previously carried out with this promoter, Pcg1277, provided the results that Sigma E is the primary sigma factor that shows a higher response. So, in Figure 30 it is possible to see the results of another fluorescence assay, but this time with the addition of phenol to the medium. In contrast to the previous assay in 4.8.1, in this one it was only studied 3 sigma factors, which are, a control sigma, sigma E and sigma H.

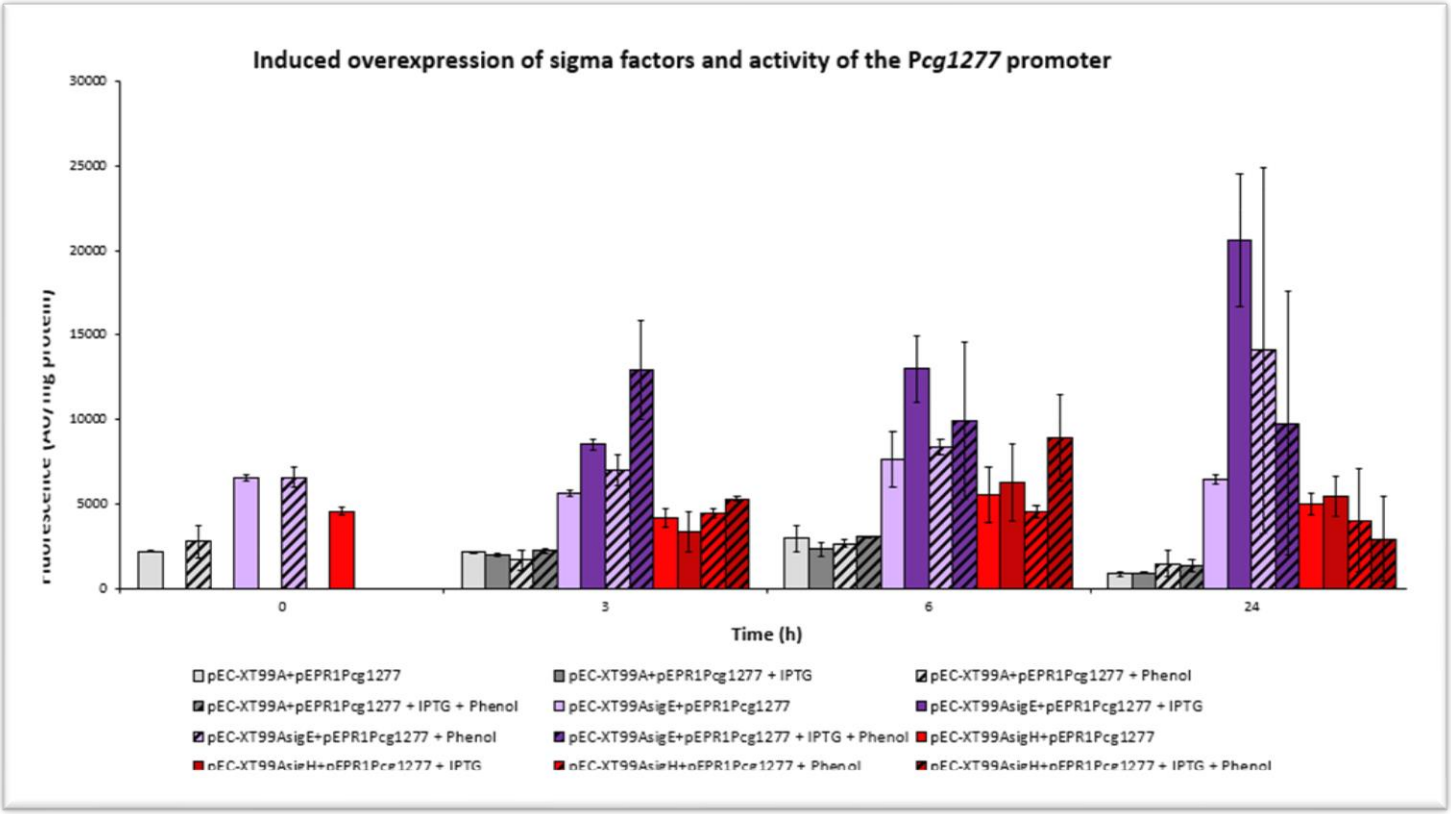


Figure 30 - Fluorescence values of the Pcg1277 promoter, cultivation in phenol

At the beginning of the experiment, the response was not similar for all strains, with sigma E and sigma H with phenol being the strains that presented a slightly higher response compared to the other strains. After 3 hours there were different results, while the control sigma and sigma H did not had significant response, sigma E with IPTG and sigma E with IPTG and phenol had the more noteworthy growth. In the next phase, at the 6-hour mark, there was a small change of scenery, in which the strain with sigma H with IPTG and phenol had a small progress, almost having the same response as the same strain with IPTG and phenol, but sigma E. Nevertheless, sigma E with IPTG still remained as the primary responder. Finally, at the end of the growth in medium, sigma E with IPTG asserted itself as the main responder to this promoter and the strain with phenol also had a notorious evolution, whereas sigma H did not maintain the same activity.

4.8.3 PcydA promoter

In Figure 31 it is represented the results of the fluorescence assay regrading the promoter, PcydA. As well as the previous promoter, in this assay it was only used 3 strains, which were, control sigma, Sigma C and Sigma H.

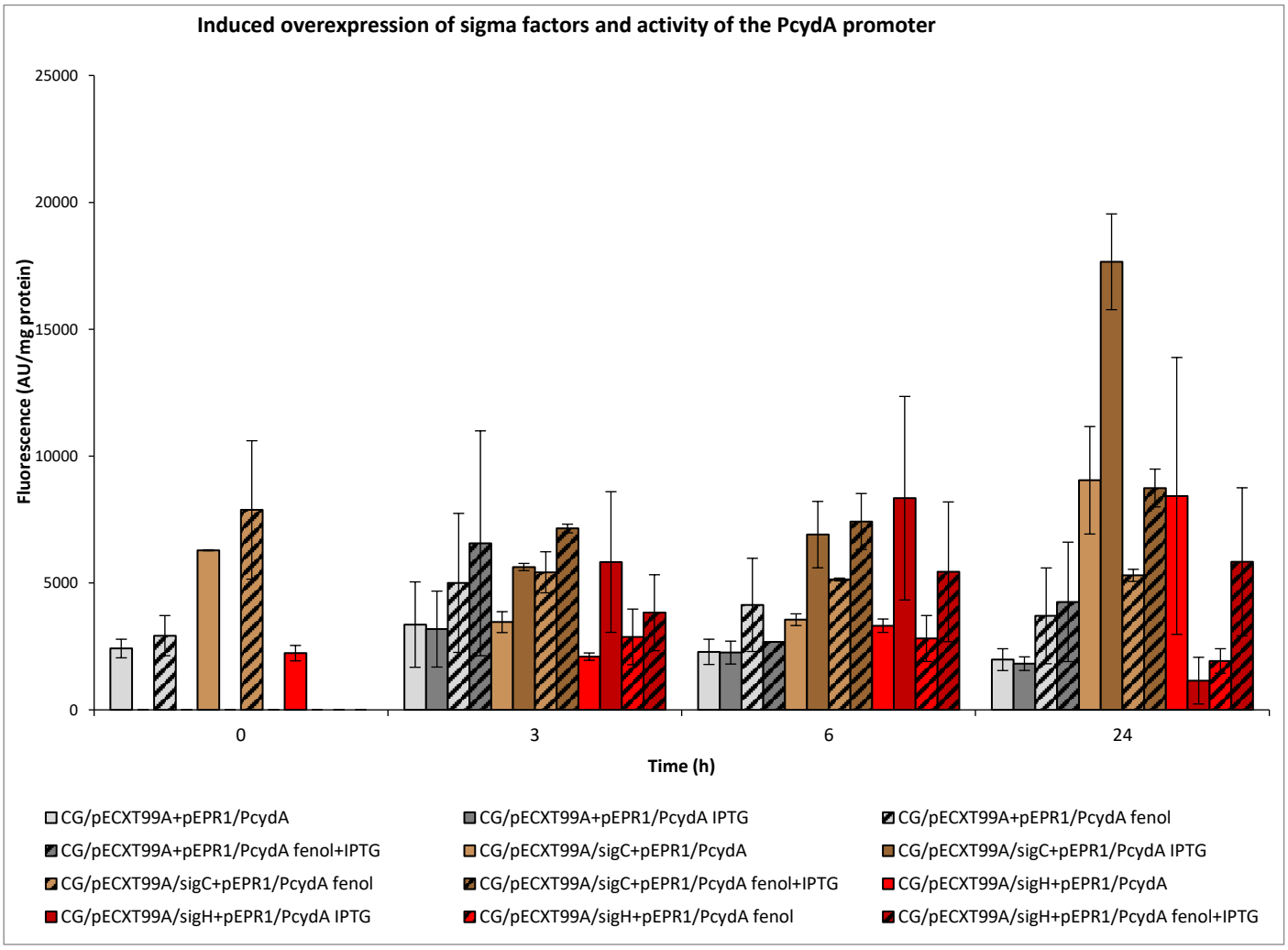


Figure 31 - Fluorescence values of the PcydA promoter, cultivation in phenol

The results show that in first part of the assay the strain with sigma C and phenol had a unusual high response to the promoter. But in the next two phases of evaluation, the values across the board (control sigma, Sigma C, and Sigma H) were vastly similar and relatively low, with no strain asserting itself as the primary responder.

In the last phase (24th hour) it's clear that the strain, Sigma C, is the one with the unique higher response. So, the phenol addition to some strains did not affect them in terms of having a higher response and the results were similar to the same experiment without the phenol.

4.8.4 PctaB promoter

The last promoter analysed is PctaB, in which the results are shown in Figure 32. As well as the previous two promoters, it was only used 3 strains during this final fluorescence assay.

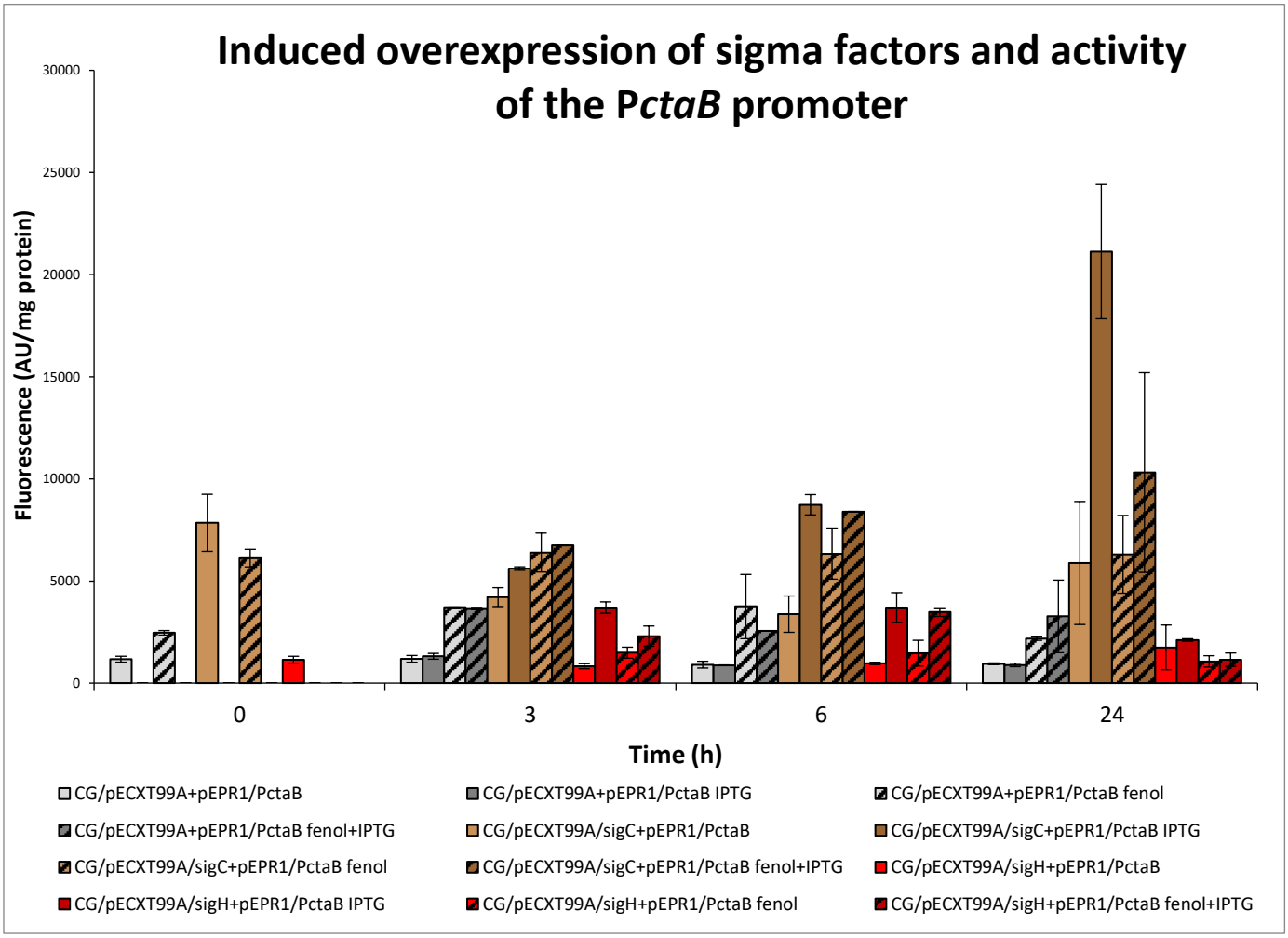


Figure 32 - Fluorescence values of the PctaB promoter, cultivation in phenol

In the first phase of the assay, there was a slight overexpression of the strains with Sigma C and Sigma C with phenol with a slight edge to the strain with phenol. During the next two phases (3rd and 6th hour) the values of the overexpression were similar, with the ones having the most activity being Sigma C with IPTG, an Sigma C with IPTG and phenol. In the last phase of cell growth, the strain Sigma C with IPTG had a huge spurt and in Figure 31 it's possible to see difference in values compared to the other strains, but the strain Sigma C with IPTG and phenol also had a slightly high expression compared to the other strains Sigma H and the control sigma.

So, the addition in phenol did not change the strain that had the high expression and response to the promoter but however, it didn’t inhibit the expression of other strains with Sigma C

5. Conclusion

The work that was presented in this thesis, was carried out to extend the knowledge regarding the four promoters (ParoF, Pcg1277, PcydA, PctaB) and the effect that they have in various strains with sigma factors of RNA polymerase, studies that are performed in the Czech Academy of Sciences.

In this thesis, it was studied particularly the effect that the promoters have in a set of different sigma factors. The study was divided in two parts, the first being having fluorescence assay with the strains only with the addition of phenol and second one different in the addition of IPTG and phenol. Regarding the first assay, there were some results that were done by a lab that is working together with CAS, so it was possible to confirm and compare the results obtained.

In the first part of the work, the results obtained are basically the same as the one ones previously analysed, so it was possible to conclude that the ParoF promoter induces an overexpression of Sigma H, Pcg1277 promoter induces the overexpression of Sigma E. The Pcg1277 promoter was a different case, because there was some doubt in which sigma was more expressed, Sigma E or Sigma H, but with the fluorescence assays done twice, it was possible to conclude it was Sigma E. The other promoters, PcydA and PctaB were believed to force an expression in Sigma C and that was confirmed. However, throughout this first experiment it was interesting to see that Sigma H had high responses in the promoters that didn't cause an overexpression of the same. This is being furtherly studied, because there are only two primary sigmas, Sigma A and B, but with this type of results, Sigma H can have an important impact in the transcription.

The second set of fluorescence assays, with the addition of phenol, did not change the outcome of the sigma factors that had the higher response to the promoters. However, this part was carried out to see the impact of the addition of phenol to the strains. It turns out that the phenol does not regress in any way the expression of the strains, and neither enhances it. This was not expected because phenol is toxic to cells, causing them to live in stress, but the results showed that it does not have a regression impact to the expression of the strains.

6. Bibliography

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[7] https://www.sciencelearn.org.nz/resources/2031-dna-cloning

Annex I

Culture media

(for solid medium add at least 15 g agar per 1000 ml)

Complex medium LB (1000 ml):

10 g Tryptone

5 g Yeast Extract

10 g NaCl

adjust to pH 7,2 - 7,5

Complex medium 2x TY (1000 ml):

16 g trypton

10 g yeast extract

5 g NaCl

Minimal medium CGXII (1000 ml):

5 g urea

20 g $(NH_4)_2SO_4$

1 g KH_2PO_4

1 g K_2HPO_4

0,25 g $MgSO_4 \times 7H_2O$

1 ml $CaCl_2$ (1 g/100 ml)

42 g MOPS

920 ml H_2O

adjust to pH 7,2

after sterilization:

1 ml of trace elements for *Corynebacterium glutamicum*

(100 ml: 1 g $FeSO_4$ + 1 g $MnSO_4$ + 0,1 g $ZnSO_4 \times 7H_2O$ + 0,02 g $CuSO_4$ + 0,002 g $NiSO_4$)

1 ml biotin (0,2 mg/ml)

80 ml 50% glucose (final concentration = 4%) or less

60 □l protocatechuic acid (30 mg/ml, for dissolution ad 1 ml 1N NaOH to 10 ml, sterilized by filtration)

Annex 2

GT 10% Glycerol
8mM Tris-HCl, pH 7.4

G 10% Glycerol

2x TY (1000 ml) 16 g Trypton
10 g yeast extract
5 g NaCl

Annex 3

PBS buffer: (1L) 8 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4 (3.6 g Na_2HPO_4 . 12 H_20)

0.24 g KH_2PO_4

adjust pH to 8.0 with H_3PO_4

sterilized by autoclave, storage in fridge

Final PBS: 100 ml PBS + 100 □l 0.1M PMSF (phenylmethylsulphonyl fluoride in 96% ethanol)

Bradford reagens: 5x concentrated solution in fridge,

dilute by water and filter

Annex 4

Large shaker at 30ºC

Annex 5

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