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Development of a genetic screen to identify hyperactive mutants of the protein Spo11

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Résumé

Dans les cellules méiotiques, un mécanisme appelé la recombinaison homologue est impliqué dans la diversité génétique, et est initié par la formation d'une cassure double brin dans l'ADN. Dû au caractère particulièrement dangereux de ce phénomène pour la cellule dont l'ADN doit être réparé pour conserver l'intégrité du génome, ce mécanisme est hautement régulé de manière spatiale et temporelle. Chez la levure, dix protéines sont impliquées dans la formation, la régulation et la réparation de ces cassures. La protéine Spo11, un homologue de la topoisomérase TopoVI, est la sous-unité catalytique qui va produire cette cassure.

Dans le but de développer un système pour mesurer la formation des cassures double brin dans l'ADN médiée par Spo11, ce projet a permis de faire deux grandes avancées. Premièrement, un vecteur d'expression hétérologue pour les protéines du « core complex » de *Saccharomyces cerevisiae* a été développé permettant ainsi leur expression dans *Escherichia coli* à partir d'un opéron. Une autre stratégie visant l'expression de ces protéines sous le contrôle de promoteurs individuels a également été développée, mais nécessite d'être terminée. Deuxièmement, plusieurs stratégies ont été utilisées pour développer un système rapporteur basé sur la réponse SOS. Dans un premier temps, des plasmides possédant un gène LacZ sous le contrôle d'un promoteur inductible par la réponse SOS ont été construits. Les phénotypes exprimés par ces plasmides et par un autre plasmide contenant un promoteur ne dépendant pas de la réponse SOS indiquent que ce système rapporteur n'est pas assez spécifique. Deux stratégies ont donc été commencées pour l'optimiser : soit par l'insertion de terminateurs dans la séquence d'ADN pour éviter une fuite de transcription, soit par l'intégration de ce système rapporteur dans le chromosome pour diminuer le risque de transcription par erreur. Dans un second temps, une souche rapportrice SOS développée dans une autre étude a également été utilisée. Les résultats obtenus par l'utilisation cette souche semblent indiquer une certaine incompatibilité entre la réponse SOS et certains antibiotiques. En utilisant ces vecteurs d'expression pour le « core complex » et ces systèmes rapporteurs, l'identification de mutants hyperactifs de Spo11 pourrait devenir possible.

Abstract

In the meiotic cells, a mechanism called the homologous recombination is implied in the genetic diversity, and is initiated by the formation of a DNA double strand break (DSB). Due to the particularly dangerous character of this phenomenon for the cell whose the DNA must be repaired to conserve the genome integrity, this mechanism is highly spatially and temporally regulated. In yeast, ten proteins are involved in the formation, the regulation and the repair of these DSBs. The protein Spo11, an homolog of the topoisomerase TopoVI, is the catalytic subunit which produces this break.

In order to develop a system to measure the Spo11-mediated DSBs formation, this project allows to make two headways. First, an heterologous expression vector for the core complex proteins of *Saccharomyces cerevisiae* has been developed allowing their expression in *Escherichia coli* from an operon. Another strategy to express these proteins under the control of individual promoters has been also developed, but needs to be finished. Second, several strategies have been used to develop a SOS reporter system based on the SOS response. In the beginning, plasmids containing a LacZ gene under the control of a SOS-inducible promoter have been constructed. The expressed phenotypes of these plasmids and of another plasmid that contains a promoter not SOS-inducible indicate that this reporter system is not specific enough. Therefore, two strategies have been started to optimize it: either by insertion of terminators in the DNA sequence to avoid transcription leak through phenomenon, or by insertion of the reporter system in the chromosome to avoid transcription by mistake. Afterwards, a SOS reporter strain developed in another study has been also used. The results obtained by using this strain seem indicate some incompatibility between the SOS response and some antibiotics. By using these expression vectors for the core complex and these reporter systems, the identification of Spo11 hyperactive mutants could become possible.

List of abbreviations

Amp	Ampicillin
BSA	Bovine serum albumin
Cm	Chloramphenicol
DNA	Deoxyribonucleic acid
DSB	Double strand break
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HR	Homologous recombination
HRP	horseradish peroxidase
Kan	Kanamycin
MBP	Myelin basic protein
MLE	Mariner-like element
MRX	Mre11-Rad50-Xrs2
NHEJ	Non-homologous end-joining
PAGE	Polyacrylamide gel electrophoresis
pAra	Arabinose-inducible promoter
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline – Tween
PCR	Polymerase Chain Reaction
PMSF	Phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene difluoride
RMM	Rec114-Mei4-Mer2
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SC	Synaptonemal complex
SDS	Sodium dodecyl sulfate
Strep	Streptomycin
TBE	Tris-Borate-EDTA
TE	Tris-EDTA

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1 Introduction

1.1 Meiosis and mitosis

Among all the mechanisms involved in cellular division, two occur in the eukaryotic cells. Mitosis allows cellular multiplication, and meiosis allows sexual reproduction. Both are preceded by an interphase (*figure 1*) that includes two growing phases (G1 and G2) separated by one DNA replication phase (S). After the S phase, each chromosome contains two identical sister chromatids. [Gottlieb *et al.* 2021]

Four steps compose mitosis (*figure 1*). During the prophase, the nuclear envelope is degraded and the chromatin condensates in chromosomes. After chromosomes alignment along the metaphasic plate during the metaphase, the chromatids are separated and pulled to opposite ends of the cell during the anaphase. Due to the physical link between the centrosomes localized on the two cell's poles and the kinetochores of the chromosomes, the cytoskeleton's microtubules allow these chromosomes alignment and chromatids segregation. During the telophase, the chromosomes gather at the poles and the cell divides by cytokinesis into two diploid identical daughter cells. Finally, the nuclear envelope reappears and the chromosomes decondensate in chromatin. [Gottlieb *et al.* 2021]

On the contrary, meiosis is defined as a specialised cellular division that generates gametes which are sexually reproducible cells in organisms. [Keeney *et al.* 2014, Page and Hawley 2003] Meiosis is composed by two cycles of chromosomal divisions (meiosis I and II) separated by an interkinesis phase, corresponding to a second interphase without DNA replication (*figure 1*). Meiosis I allows the segregation between the homologous (or parental) chromosomes, while the segregation of the sister chromatids occurs during meiosis II. [Kenney *et al.* 2014] Although meiosis II is composed by the same steps as the mitotic phase, meiosis I is similar but includes some differences. The tetrads (a pair of duplicated chromosomes) are aligned on the metaphasic plate instead of the chromosomes, the homologous chromosomes are separated instead of the sister chromatids, and diploid daughter cells are formed instead of haploid. [Gottlieb *et al.* 2021] The formation of haploid daughter cells during the last step of meiosis implies the reduction of the genome which can be restored by a conjugation phase corresponding to gamete fusion. [Keeney *et al.* 2014] This mechanism (described in 1.2 Homologous recombination) allows genomic diversity and genetic variation. Each daughter cell is genetically half-identical to its parent cell, yet distinctly different from both parent and other daughter cells. [Gottlieb *et al.* 2021]

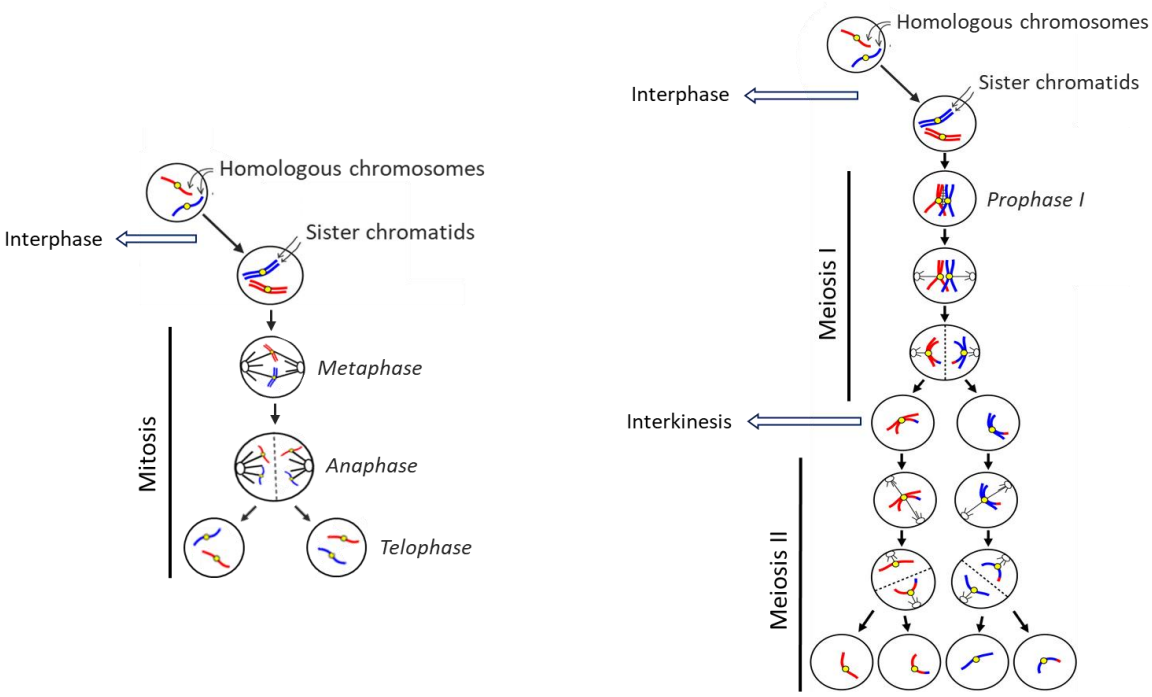


Figure 1: Comparison between mitosis and meiosis mechanisms. *Interphase: Single parental chromosomes (one in blue, other in red) replicate themselves to give sister chromatids bound by centromeres. Mitosis: Chromosome alignment in metaphase, chromatid segregation in prophase, and formation of identical daughter cells in telophase. Meiosis I: Homologous recombination in prophase I, exchange of homologous fragments between blue and red chromatids. Then, segregation of homologous chromosomes after exchange. Interkinesis: Cellular growth. Meiosis II: Splitting of sister chromatids leads to the formation of four gametes from which two possess a recombined chromatid. [Personal communication]*

1.2 Homologous recombination

Contrary to the direct repair that occurs in the mitotic cells through the sister chromatids or repeated sequence from the same chromosome, the homologous recombination that happens in the meiotic cells uses the non-sister chromatid from the homologous chromosome to complete the lacking DNA sequence. [Martini and Keeney 2002] This homologous recombination mechanism (*figure 2*) that occurs in prophase I is initiated by the formation of a DNA double-strand break (DSB) on the chromosomal axis. [Lam and Keeney 2014] After the homologous pairing's stabilization by a macromolecular protein structure: the synaptonemal complex (SC - *figure 3*), the recombination structures can be resolved as crossover or non-crossover products (*figure 2*). [Zhang *et al.* 2021] Although the sister chromatids born together during the DNA replication (S phase of meiosis), the pairing of homologous chromosomes depends on this homologous recombination mechanism. [Bhalla and Dernburg 2008, Lam and Keeney 2014]

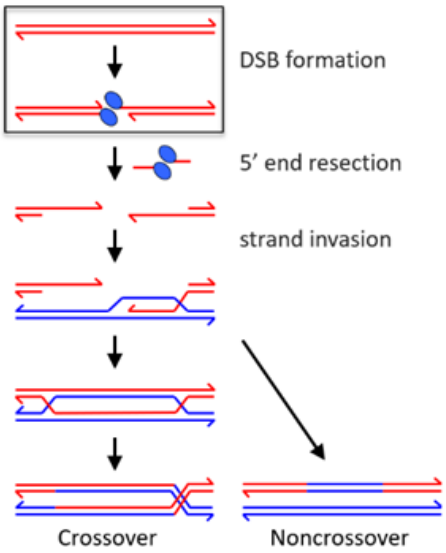


Figure 2: Pairing and recombination of parental chromosomes. *Spo11 introduces DSBs. After 5'-end resection, single strand DNA is used to search for homology into the parental chromosome. Resolution of the strand invasion product can lead to both crossover and non-crossover.*

The different steps of the meiotic recombination are associated with the structural organization of the chromosomes (*figure 3*). [Keeney 2001, Yadav and Claeys Bouuaert 2021] During the meiotic prophase, the chromosomes form several DNA loops which are anchored on a nucleoprotein axis. Due to this loop-axis structure, the proteins involved in the meiotic DSB formation, like Spo11 (see 1.4.1 The core complex structure and function), which are localized on the chromosomal axis can introduce DSBs in the DNA loop. When the DNA is cleaved, the strand searches homology on the opposite homologous chromosome. [Yadav and Claeys Bouuaert 2021] This will induce the polymerisation of the synaptonemal complex between the two lateral axes resulting in numerous transverse filaments of 100 nm, and the elongation along the entire axes length. Besides controlling the relocation of the recombination intermediates from axis to SC, the SC formation also assures the correct separation of the chromosomes, the DSB repair and the formation of crossovers. [Zhang *et al.* 2021] The homology searching occurs in the SC of euchromatic morphology corresponding to nodules and resulting in crossovers. [Carpenter 1975] When the recombination is done, the SC disassembles and the crossovers provide a physical connection between the homologs until their correct segregation during the anaphase. [Keeney *et al.* 1997]

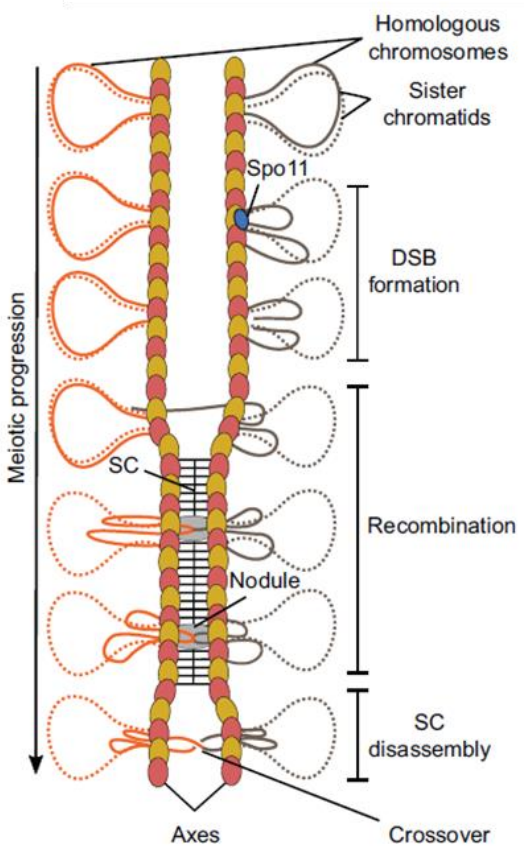


Figure 3: Relationships between meiotic recombination and high-order chromosome structure. *DSB formation happens in the context of the loop-axis structure. As recombination progresses, the SC polymerizes between the axes and is disassembled prior to chromosome segregation. [Yadav and Claeys Bouuaert 2021]*

1.3 DNA double-strand breaks

Several endogen and exogen factors induce DNA damage in the genome. To maintain genome integrity and function, all these mechanisms must be controlled and repaired. [Ceccaldi *et al.* 2016] Here, the formation, the regulation and the repair of the DNA double-strand break will be developed.

1.3.1 DSBs formation

In all eukaryotes that host the meiotic mechanism, the DSBs are formed during prophase I by Spo11, a highly conserved DNA topoisomerase II-like protein. [Bergerat *et al.* 1997, Keeney *et al.* 1997] After break formation, the Spo11 protein stays covalently bound in 5' of both strands of the broken DNA. [Liu *et al.* 1995] Two mechanisms have been considered for the Spo11 release from the DSB-ends (*figure 4*): [Keeney *et al.* 1997, Neale *et al.* 2005]

1. Direct hydrolysis of DNA-protein covalent link
2. Endonucleolytic cleavage of a single strand that liberates Spo11 bound to a short oligonucleotide

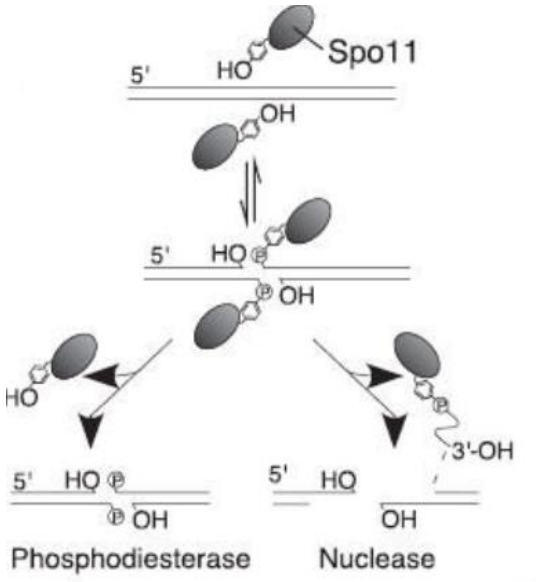


Figure 4: Endonucleolytic processing of covalent Spo11-DSB complexes. *Two possible mechanisms for Spo11 release: direct hydrolysis by phosphodiesterase or endonucleolytic cleavage by nuclease. [Neale et al. 2005]*

The DNA bases close to Spo11 cannot be degraded, therefore the protein removal occurs following the second mechanism, forming stable Spo11-oligonucleotide complexes. [Neale *et al.* 2005] After a 5'-ends resection by an endonuclease, Spo11 is liberated attached to a short fragment of DNA through a covalent 5'-phosphotyrosyl link. [Bergerat 1997, Garcia *et al.* 2011, Keeney 1997] After binding of the proteins Dmc1 and Rad51 (homologs of the *E. coli* RecA protein) on the two 3' single strands of approximately 600 nucleotides to form two nucleoprotein filaments, the single strand linked to Rad51 invades the homologous chromosome duplex to find homology and repair DNA (*figure 5*). [Lam and Keeney 2014, Keeney 1997]

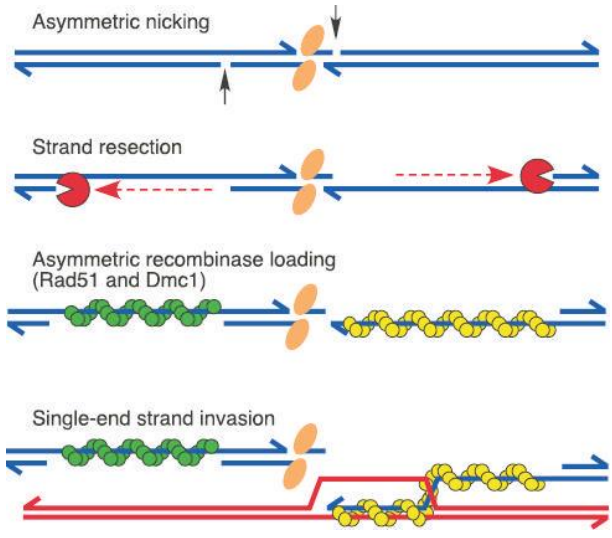


Figure 5: Asymmetric steps in meiotic recombination. *A Spo11 dimer (orange ellipses) creates a DSB which is processed by asymmetrically spaced nicks. Exonucleolytic resection initiates at these nicks, yielding single-stranded gaps. Rad51 and Dmc1 recombinases (Dmc1 in green and Rad51 in yellow) form nucleoprotein filaments on opposite sides of the DSB. Asymmetric strand invasion yields a stable strand exchange intermediate. Spo11-oligonucleotide complexes, stabilized by base pairing and protein-protein interactions, are proposed to remain associated with DSB ends until the strand exchange. [Neale et al. 2005]*

Independently from the DNA repair, the liberated Spo11-oligo complexes possess free 3'-OH ends and are quantitative by-products of DSB formation, one complex corresponding to one DSB. This allows to quantify the total level of DSB when direct molecular detection is not possible. [Lange *et al.* 2011, Keeney *et al.* 2014]

After the radiolabelling of the 3'-OH, the separation of the Spo11-oligo complexes on an electrophorese gel reveals two main protein-DNA complexes, corresponding to Spo11 attached to a long or a short oligonucleotide, one around 8-12 nucleotides and another around 25-35 nucleotides. [Johnson *et al.* 2021, Neale *et al.* 2005] If the protein Spo11 possesses a mutation that induces a lower affinity to the DNA, the DNA binding and the DSBs formation decrease, consistent with the Spo11-DNA complexes quantification (*figure 6*). Moreover, mutations in the protein Spo11 induce shorter oligonucleotides. [Claeys Bouuaert *et al.* 2021b] The nucleolytic cleavage of the DSBs depends on direct DNA binding by Spo11. [Claeys Bouuaert *et al.* 2021a]

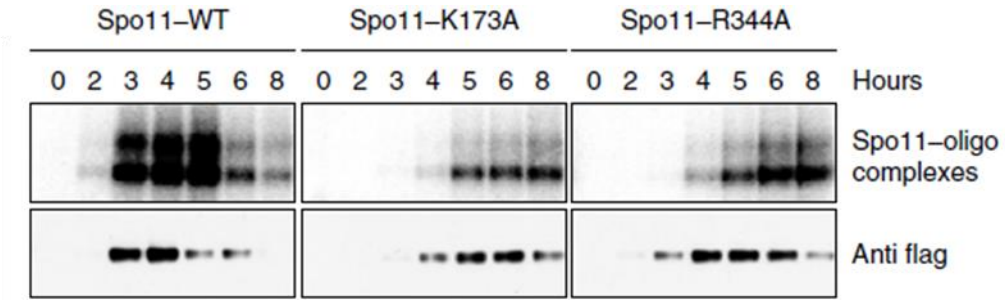


Figure 6: Mutations that affect Spo11–DNA binding compromise DSB formation. *Labelling of Spo11–oligo complexes on a polyacrylamide gel. Top panel: Detection of DNA marked radioactively. Bottom panel: Detection of Spo11 protein by Western blot. Comparison between Wild-type Spo11 and Spo11 with two types of mutations: the lysine 173 or the arginine 344 mutated in alanine. Different hours in meiosis have been used. [Claeys Bouuaert et al. 2021b]*

The DSB formation is a suicide reaction for Spo11 because the endonucleolytic release leaves tyrosine active residue from proteins covalently attached to the DNA. Although this could control the total number of DSBs, most Spo11 proteins don't introduce DNA breaks. [Lange *et al.* 2011, Neale *et al.* 2005] Some proteins of the meiotic DSB machinery remain on the chromatin after most DSBs are formed, therefore the mechanism that control the DSB number relies at least in part on the control of the Spo11 activity. [Keeney 2008]

1.3.2 DSBs regulation

Besides the essential role of both DSBs and homologous recombination which make physical connections between the homologous chromosomes to align and segregate them, these mechanisms also induce an increase of the genetic diversity due to the exchange of genetic material between two chromosomes. [Page and Hawley 2003] Therefore, the meiotic cells (unlike other cells) trigger the homologous recombination by formation of programmed DSBs, about 100 DSBs can happen during a meiosis event in yeast.[Yadav and Claeys Bouuaert 2021] However, the DNA double strand breaks can also lead to mutations (if there are mistakes into the DSB repair) or to the cellular death. Due to this potentially dangerous character, the DSBs formation is highly controlled in time, number and localization in order to assure essential functions and minimize deleterious effects. [Keeney *et al.* 2014]

The DSB formation is associated with the structure of the meiotic chromosomes (see 1.2 Homologous recombination), showing that the recombination initiation by the DSB formation is a robust and self-organized process which is physically and temporally regulated. [Hunter 2015, Keeney *et al.* 2014]

1.3.2.1 Spatial regulation of DSBs

The meiotic DSBs follow a non-random distribution on the chromosomes. The localization of the DSBs depends on several levels of the spatial organisation in the chromatin and the chromosomes. [Lam and Keeney 2015] Including open chromatin (not telomeres and centromeres) and DNA composition (including modifications), these factors act at different levels, but none is sufficient alone. Due to these factors, the DSBs localize preferentially on the chromosomal arms, in the chromatin loops enriched in GC which correspond to open chromatin (chromosomal domains from at least ten kilobase pairs) and close to regions cleaved by Spo11. [Petes 2001] These small regions of 150 to 250 base pairs are called hotspots. [Pan *et al.* 2011] These hotspots are located in nucleosome-depleted regions, typically within a promoter (in yeast). [Pan *et al.* 2011, Székvölgyi and Nicolas 2010] The DSB frequency on a hotspot depend on the accessibility to the chromatin structure. [Keeney *et al.* 2014] Regulatory mechanisms ensure that two DSBs are typically separated by the minimal distance of 30 to 60 kilobase pairs and the competition between strong hotspots avoids DSB activation on an adjacent hotspot. [Keeney *et al.* 2014]

1.3.2.2 Time regulation of DSBs

Several temporal factors have a high influence on the DSBs formation and repair, inducing a limited time during which the DSB proteins can access the chromosomes and therefore the DSBs can be formed. The formation of DSBs occurs only during the prophase I at a defined time after the premeiotic DNA replication, while the DSBs repair happens during the middle of the meiotic prophase I. [Mehrotra and McKim 2006]

According to these regulation mechanisms, the DSB's number is influenced by the cell and the species, and is therefore not genetically determined. [Keeney *et al.* 2014]

1.3.2.3 Link between DSBs and crossovers formation and regulation

To maintain the genome integrity and the continuity of the axis, the number of crossovers is limited according to the chromosome's size. Therefore, the repair of DSBs through the formation of crossovers don't systematically occur, indicating a high level of control and a higher number of DSBs than crossovers. Due to the chromosome's organization in hotspots or not, the formation of a crossover from a DSB depends on the chromosome's region where the DSB is formed. However, due to their requirement for the homologous segregation and the pairing of parental chromosomes in prophase I, each homologous pair contains at least one crossover. [Hunter 2015]

1.3.3 DSBs repair

To protect the integrity of the genome, the DNA repair is essential after the DSB formation and can occur through several mechanisms, like the non-homologous end-joining (NHEJ) and the homologous recombination (HR - see 1.2 Homologous recombination). The NHEJ mechanism repairs genome by ligation between two broken DNA strands inducing deletions in the genome, while the HR implies the homologous searching to recover the complete DNA strand. [Tsukamoto and Ikeda 1998]

During this project, one survival mechanism that allows DSBs repair has been particularly used: the SOS response (*figure 7*). The SOS response is a regulation mechanism which allows the bacterial adaptation and evolution when the environmental conditions require it. [Trémolières 2010] This conserved system is induced after stress that can cause damage to the DNA and is essential to repair DNA and recover the capacity of DNA replication. This system works according to an accurate mechanism. In homeostatic period, the repressor LexA (a regulator or inhibitor of transcription) is bound on the fixation site (SOS box) corresponding to the SOS promoter region, preventing the expression of the SOS genes. When the genome is damaged or the replication is interrupted after a DNA damage, the activator RecA (the sensor of the SOS response) fixes itself on the generated single strand DNA to form a nucleoprotein filament RecA-ssDNA. Besides inducing the cleavage of LexA by autoproteolysis to remove the repression of the SOS regulon, the RecA-ssDNA also prevents the supplementary degradation of the DNA. [Kelley 2006] According to this mechanism, the SOS genes expression occurs only when the DNA is damaged and depends on an accurate dynamic. [Friedman 2005] Moreover, different levels of stress can induce the SOS response. Each elements intervenes at the right time, and proportionally to the suffered damaged. The SOS regulon contains several proteins involved in the DNA repair, like nucleases, helicases, recombinases and polymerases. [Da Re and Ploy 2012]

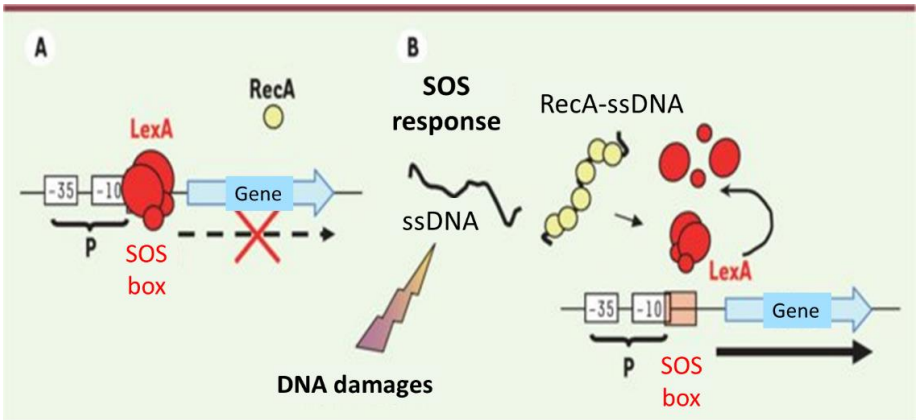


Figure 7: Mechanism of SOS response. *A. Without stress, a dimer of LexA proteins is fixed on promoter region of an SOS gene. Therefore, this gene is totally or almost inhibited. B. With DNA damages or interruption of replication, the RecA protein interacts with single strand DNA forming nucleoprotein filament and inducing autoproteolysis of LexA. It released the promoter and allows gene expression. [Da Re and Ploy 2012]*

The genes that intervene into the SOS response possess different sensitivities to the degradation of LexA. Due to different binding of LexA on the promoters, a low level of degradation can be sufficient when the binding is weak, but a higher level is needed when the binding is strong. [Da Re and Ploy 2012]

Several exogen and endogen agents can cause DNA damage, and therefore induce the SOS response, directly or not. The antibiotics make part of these agents, Reactive Oxygen Species (ROS) and UV radiations also. According to bacterial and antibiotic types, the antibiotics can induce directly the SOS response at low concentration. [Da Re and Ploy 2012] The indirect induction of the SOS response by the antibiotics is also possible by the production of ROS which induce oxidative stress in bacteria. [Kohansky *et al.* 2010] Independently, ROS can also make damage in the DNA and induce the SOS response. [Da Re and Ploy 2012]

Moreover, the SOS response can lead to antibiotic resistance by different ways: rise in the mutation frequency after the SOS response, expression of resistance genes that are normally repressed by LexA or transfer of genetic elements containing resistance genes. [Baharoglu and Mazel 2011]

1.4 Meiotic double-strand break machinery in *Saccharomyces cerevisiae*

In *Saccharomyces cerevisiae*, ten proteins that assemble into three subcomplexes intervene in the DSBs formation. [Keeney *et al.* 1997] The RMM complex is assembling a scaffold on the chromosome to recruit the other proteins. [Li *et al.* 2006] The core complex, which contain the catalytic subunit Spo11, produce the DSBs in the DNA strand. [Claeys Bouuaert *et al.* 2021a] Finally, the MRX complex is involved in both the DSB formation, and their subsequent repair by catalysis the DNA end resection. [Borde *et al.* 2004]

1.4.1 The core complex structure and function

Previous studies show functional and structural similarities between the core complex proteins, including Spo11, and the topoisomerase VI from the topoisomerase's family II. [Bergerat *et al.* 1997, Robert *et al.* 2016]

1.4.1.1 Topoisomerases and TopoVI

The topoisomerases are separated into two subgroups. The first family (TopoI) works independently of ATP, while the second (TopoII) couples ATP hydrolysis to the transport of one DNA segment through a transient break in another segment. [Bergerat *et al.* 1997] Besides relaxing supercoiled DNA by passing one (TopoI) or two (TopoII) strand of DNA, the TopoII also intervenes throughout the cellular cycle in cleavage, but also condensation and cohesion roles. [Lee and Berger 2019]

Belonging to the second family, the topoisomerase VI is composed by two subunits. The subunit A acts as a dimer with two active sites to cleave the DNA, the 5Y-CAP motif that contains a catalytic tyrosine, and the Toprim domain which possesses a metal-binding pocket. [Bergerat *et al.* 1997, Keeney 2001] Moreover, the subunit B corresponds to an ATPase GHKL-type and is involved in binding and hydrolysis of ATP. [Bergerat *et al.* 1997] One TopoVI is composed by two subunits A and two subunits B, forming an heterotetramer. The subunit B contains the binding domain for ATP, and also the transducer domain that interacts with the subunit A (*figure 8*). This transducer domain allows conformational changes and regulates interactions between the two homodimers. [Robert *et al.* 2016]

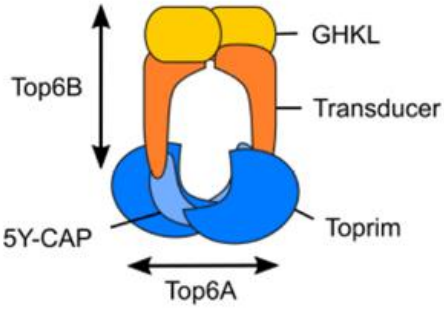


Figure 8: Cartoon of the TopoVI heterotetramer composed by two subunits. *Top6A, composed by the 5Y-CAP motif and the Toprim domain, interacts with the transducer of the Top6B. Top6B is composed by the transducer domain and the GHKL domain. [Yadav and Claeys Bouuaert 2021]*

To allow relaxation of the supercoiled DNA, the TopoVI uses ATP binding and hydrolysis to coordinate the formation of a transient DSB that will result in a heteroduplex. [Robert *et al.* 2016] Two double helices of DNA are involved in this topoisomerase's mechanism. The subunit A dimerizes, forming an U-shape complex that interacts with two DNA segments. After binding this complex, the G-segment is broken by ATP hydrolysis. The DNA strand cleavage involves the coordinated action of two tyrosine active sites that attack opposite strands of the phosphoribose DNA backbone, producing 5'-phosphotyrosyl intermediates (*figure 10*). Therefore, through the dimerization of the GHKL domain which allows a conformational change of the DNA topology, the T-segment will pass through this break before the break is resealed (*figure 9*). [Wendorff and Berger 2018, Yadav and Claeys Bouuaert 2021]

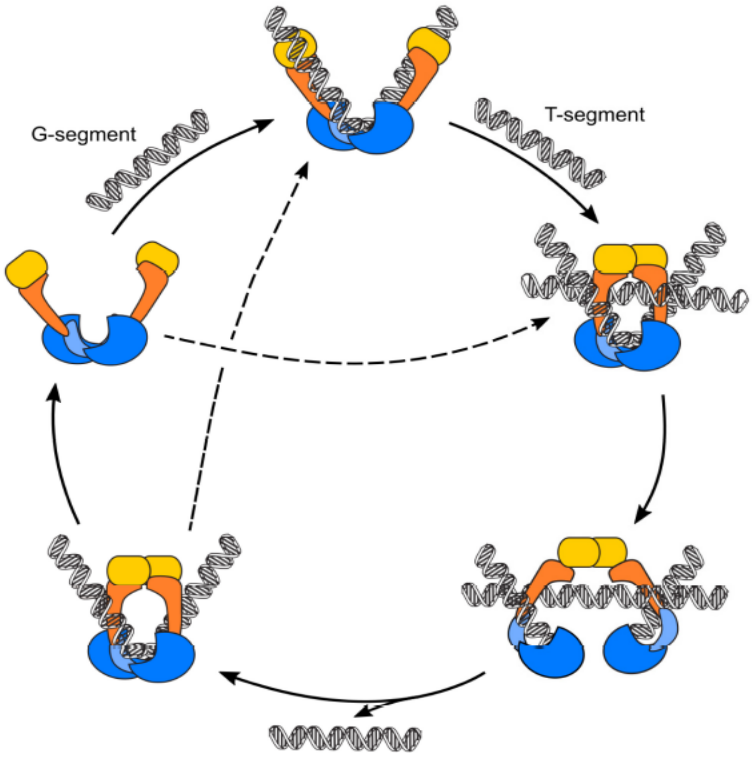


Figure 9: Catalytic cycle of TopoVI through a two-gate mechanism. *ATP-dependent dimerization of the GHKL domain upon sequential or simultaneous binding to gate (G) and transfer (T) DNA duplexes is communicated to the A subunit to activate DSB formation. TopoVI can undergo multiple catalytic cycles without dissociation from the G-segment. [Yadav and Claeys Bouuaert 2021]*

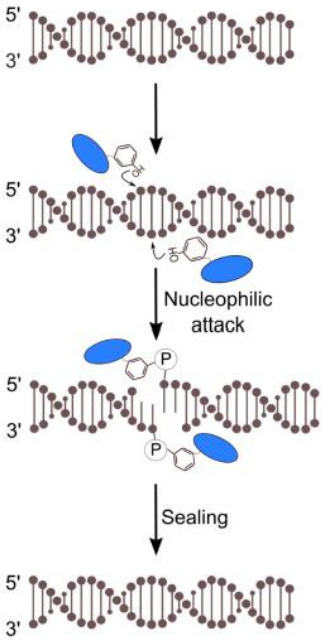


Figure 10: Chemistry of strand cleavage by a nucleophilic attack and DNA repair by sealing in TopoVI. *[Yadav and Claeys Bouuaert 2021]*

1.4.1.2 The core complex proteins and their homology to TopoVI

The core complex is composed of four proteins. The catalytic subunit Spo11 interacts with Rec104, Rec102 and Ski8 (*figure 11*). This complex is homologous to the topoisomerase TopoVI, Spo11 corresponding to the Top6A domain, Rec102 to the transducer, and Rec104 to the GHKL domain (*figure 12*). Despite structural and functional similarities, Spo11 core complex and TopoVI possess distinct biological roles. [Claeys Bouuaert *et al.* 2021a]

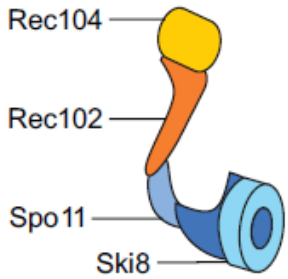


Figure 11: Cartoons illustrating the arrangement of the different subunits in the core complex. *Spo11 protein interacts with Rec104, Rec102 and Ski8. [Yadav and Claeys Bouuaert 2021]*

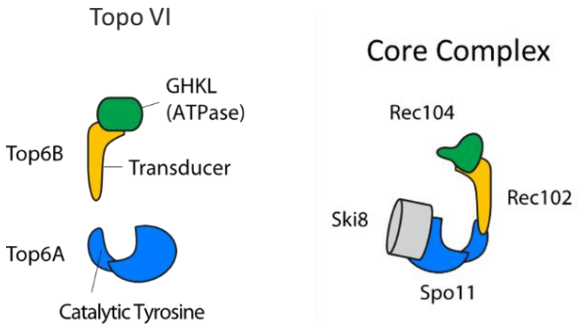


Figure 12: The meiotic DNA DSB core complex. *Structure of TopoVI compared to core complex model based on homology with TopoVI. [Claeys Bouuaert et al. 2021a]*

Homolog of the subunit TopoVIA, Spo11 is an essential gene for the recombination and is specific to meiosis. [Jiao *et al.* 2003] Due to Spo11's role, a mutations in the Spo11 gene prevents meiotic DSBs formation, and therefore meiotic recombination. Moreover, a mutagenesis on Spo11 has shown that the residue tyrosine 135, highly conserved in TopoVI and homologs, is essential for Spo11's activity. [Bergerat *et al.* 1997]

The homologs of subunit B of the archaea's topoisomerase VI, Rec104 et Rec102 are recruited on the meiotic chromosomes by Spo11 and Ski8. [Kee *et al.* 2004] Rec104 is not required for interaction between Spo11 and Rec102, and same for Spo11 and interaction between Rec104 and Rec102. Therefore, Spo11 and Rec104 don't interact directly. [Jiao *et al.* 2003]

Rec102 and Rec104 interact with both Spo11-Ski8 and Rec114-Mei4 complexes, indicating that they could be the connection between the RMM complex and the Spo11-Ski8 complex. [Arora *et al.* 2004] They form a functional unit localized on the axis, which is required for the Spo11 localization into the nucleus, the chromatin association and the binding to the hotspots. [Kee *et al.* 2004]

The non-meiosis specific protein Ski8 usually works in the cytoplasm. It is involved in the RNA metabolism of the vegetative cells, but is displaced in the nucleus during meiosis where it stabilizes the core localization and the chromatin association between Spo11 and Rec102-

Rec104. Besides a meiotic role highly conserved, Ski8 is notably required for RNA degradation and to inhibit RNA transcription. Partners of Ski8 vary according to its function: it interacts with Ski2 and 3 during the non-meiotic roles, and with Spo11 during the meiotic roles (inducing an indirect interaction between Ski8 and the other core complex proteins). Ski3 and Spo11 possess a common motif allowing their direct interaction with Ski8. These observations indicate that the roles of Ski8 are distinguished between the meiotic cells and the vegetative cells. Switching of Ski8 from the non-meiotic roles to the meiotic roles is made possible by the Ski8's relocalization into the nucleus and its association with the chromosomes during meiosis, and is facilitated by several partners like Spo11. [Arora *et al.* 2004]

During meiosis, Ski8 stabilizes the association between Spo11 and the meiotic chromatin, and serves with Spo11 as a scaffold protein mediating assembly for all proteins needed for the DSBs formation. The Rec104 and Rec102 proteins usually localize in the nucleus and are associated with the chromosome during the meiotic prophase. In the absence of Ski8, this association rate decreases a lot, supporting the hypothesis that Ski8 promotes and stabilizes the interaction between Spo11 and Rec102-Rec104 during the DSBs formation. [Arora *et al.* 2004] Therefore, the principal meiotic role of Ski8 is to promote the assembly of Spo11, Rec104 and Rec102. When other proteins are bound together, Ski8 may dissociate from hotspots and not be detected anymore when DSBs are done. This observation could indicate that DSB efficiency decreases when dissociation of Ski8 not occur. [Arora *et al.* 2004, Koehn *et al.* 2009]

There is an accurate structural model of the core complex where N-terminal Spo11 and C-terminal Rec102 are bound together, like Spo11 and Ski8. The position of Rec104 is determined by its similarity to the GHKL domain of TopoVI and its binding to Rec102. [Claeys Bouuaert *et al.* 2021a]

Although TopoVI has a stoichiometry A_2B_2 (*figure 8*), the core complex is monomeric with a stoichiometry 1:1:1:1, consistent with the hypothesis that the Spo11 dimerization participates in the control of DSB formation. [Claeys Bouuaert *et al.* 2021a]

1.4.2 The RMM and MRX complexes

The RMM complex is composed by three proteins: Rec114, Mei4 and Mer2. Rec114 and Mei4 form an heterotrimer with a 2:1 stoichiometry and Mer2 forms an homotetramer. [Claeys Bouuaert *et al.* 2021b, Yadav and Claeys Bouuaert 2021] Besides serving as a scaffold on the chromatin to recruit Spo11 and the other proteins of the core complex, the highly conserved RMM proteins are also involved in activating the protein Spo11. [Li *et al.* 2006]

Three other proteins (Mre11, Rad50 and Xrs2) compose the MRX complex that is involved in both the formation and the resection of the DSBs. The MRX complex is also used in somatic cells where it serves to maintain telomeres, induce cellular cycle response to DNA damages and initiate DNA repair by NHEJ or HR (see 1.2 Homologous recombination). [Li *et al.* 2006] Possessing an 3'-5' double strand exonuclease and a single strand endonuclease activities, the Mre11 protein binds the DNA strand and allows the resection of the strand involved in the DNA repair. [Borde 2007] The Mre11 recruitment occurs only when all the other DSB proteins

are associated on the chromosome, but before the cleavage by Spo11. [Borde *et al.* 2004] Mre11 possesses a N-terminal nuclease domain containing five conserved phosphoesterase motifs, and dimerizes through the phosphodiesterase domain to form an U-shape structure which binds the DNA and Rad50. [Arthur *et al.* 2004, Williams *et al.* 2008] The ABC-type ATPase Rad50 possesses two defined domains giving a specific conformation to bind and hydrolyse DNA. [Hopfner *et al.* 2001] Consistent with the requirement of both to form and repair the DSB, the mutants for Mre11 or Rad50 are not able to remove Spo11 from the DSB-ends. [Keeney 2001, Neale *et al.* 2005] The protein Rad50 controls the MRX function by a specific mechanism: with ATP, Rad50 has a dimeric conformation inducing the inaccessibility of Mre11's nuclease domain. However, when ATP is hydrolysed, Rad50 dimers are dissociated, allowing the active site of Mre11 to access to the DNA. [Hopfner *et al.* 2001] The chaperone molecule Xrs2 connects Mre11 with other repairer proteins, and is needed for molecular localization of Mre11. Therefore, if one mutation prevents the interaction between Xrs2 and Mre11, their meiotic and vegetative functions are abolished. [Tsukamoto *et al.* 2005] The presence of MRX during the DSB formation will facilitate the rapid coordination with the repair and allow the effective treatment of all breaks. [Borde *et al.* 2004]

1.4.3 Interactions between the three complexes

Depending of Rec102, Rec104 and Rec114, the Spo11 self-association and therefore the core complex dimerization occurs when the Rec114 protein is recruited on the chromosomal axis. [Claeys Bouuaert *et al.* 2021a] In the RMM complex, the phosphorylation of Mer2 allows to recruit Rec114, Mei4 and Xrs2. [Henderson *et al.* 2006] In the MRX complex, besides its interaction with Rec104 and Rec102, Mre11 induce 3' to 5' resection to liberate Spo11 attached to a short oligonucleotide corresponding to the single strand adjacent to the DSBs. [Keeney 2001, Neale *et al.* 2005]

2 Objectives

The formation of DSBs by Spo11 is potentially harmful to the cell, suggesting that this activity is tightly regulated. However, the meiotic cells induce a high level of breaks in their DNA, hundreds during a meiosis event in yeast. Despite their manifest importance, the mechanisms that control the break formation are not fully understood. Therefore, the purpose of this project was to investigate if the Spo11 protein possesses an intrinsic regulatory mechanism preventing the introduction of excessive or unwanted DNA breaks.

The auto-inhibition is a recurrent phenomenon in nature for enzymes that catalyse potentially dangerous activities. It has already been described in numerous other systems including DNA transposition, enzymes that mediate post-translational modifications, transcription factors, chromatin remodelers, signalling receptors and others.

One previous study has shown potential evidence to support the hypothesis that Spo11 activity is controlled by auto-inhibition. [Neale *et al.* 2005] After break formation due to the DNA cleavage by MRX complex, Spo11 is liberated bound to a short or a long oligonucleotide, providing Spo11-oligo complexes as quantitative by-products of Spo11 activity (*figure 13*). In the study's experiment, Flag-tagged Spo11 are immunoprecipitated during meiosis and the DNA is labelled radioactively. After the segregation on a SDS-Page gel, the proteins have been blotted on a membrane and revealed either by autoradiography (to visualize the labelled DNA) or by Western blotting (to visualize Spo11). By autoradiography, two main complexes present in approximately 50-50% ratio can be observed (*figure 13, top panel*), that correspond to Spo11 bound to a long or a short oligonucleotide. If all the Spo11 proteins were bound to an oligonucleotide, the same two bands should be detected by Western blotting, but only one band is detected (*figure 13, bottom panel*). The presence of an excess of Spo11 proteins that are not attached to an oligonucleotide is a likely interpretation of these results. This indicates that the vast majority of Spo11 molecules never engage in catalysis and didn't make a break. Therefore, this is consistent with the idea of an auto-regulatory mechanism that must be unlocked.

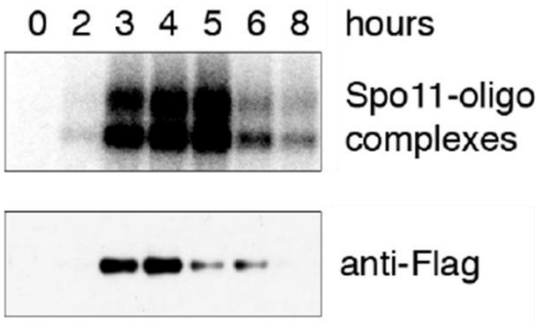


Figure 13: Labelling of Spo11–oligo complexes on a polyacrylamide gel. *Top panel: Detection of DNA marked radioactively. Bottom panel: Detection of Spo11 protein by Western blot. Different hours in meiosis have been used. [Claeys Bouuaert et al. 2021a]*

Different studies have shown that in case of enzymes controlled by auto-inhibition, hyperactive mutants are often readily isolated. For example, in the case of the DNA transposition, different genetic screens have successfully identified hyperactive mutants for a variety of transposase families. [Lohe and Hartl 1996, Mátés *et al.* 2009, Yusa *et al.* 2011] Identifying such mutants would provide a new insight into the catalytic mechanism of Spo11.

However, despite the requirement of a semi-quantitative reporter system to identify hyperactive mutants, no such reported system exists to measure Spo11-mediated DSB formation. Therefore, the strategy of this project will be to develop an assay to measure the Spo11-dependant break formation in *E. coli*, and this project was divided into two parts. First, the generation of a bacterial plasmid to express the core complex proteins of *Saccharomyces cerevisiae* into *Escherichia coli*. Second, the development of a reporter system to detect the Spo11-mediated DSB formation in *E. coli*. These two constructs could allow future identification of hyperactive mutants of Spo11.

3 Material and methods

3.1 Bacterial strains and plasmids

Several bacterial strains and plasmids have been used during this project. The *E. coli* strains are referenced in table 1, and plasmids in table 2. For transformations, the three first strains are transformed by heat shock, the other strains are transformed by electroporation.

***E. coli* strains**			
	Strain's genotype	**RM**	**Reference and comments**
Top10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$*		
BL21	F$^-$ *ompT gal dcm lon hsdS$_B$*($r_B^-m_B^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K\text{-}12}$(λ^S)		
DH5α	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* ϕ80d*lacZ* ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^-m_K^+$), λ$^-$		
Marionette-Wild	MG1655+marionette cluster transduced in glvC	Low Cm (5µg/ml)	Contain 12 optimized regulators [Meyer *et al.* 2019]
MW LacZ-	Marionette-Wild with lacZ deleted (lacZ::KanR)	Low Cm (5µg/ml) + KanR	
MG1655	K-12 F$^-$ λ$^-$ *ilvG$^-$ rfb-50 rph-1*		"Wild type" sequenced K-12 strain [Blattner *et al.* 1997]
MG1655 ΔlacZ (KanR)	K-12 F$^-$ λ$^-$ *ilvG$^-$ rfb-50 rph-1 ΔlacZ(KanR)*	Kan	LacZ ORF replaced by KanR cassette (Insertion of PCR product by lambda red in MG1655)
JH139	F- λ- Pro$^+$ lacΔU169 tif-1 sfiA11 thr leu his arg ilv^{ts} gal str dinDI::Mu dI1734(KmR lac)	Kan	Promoter-SOS::lacZ fusion strains [Heitman and Model 1991]

Table 1: *E. coli* strains used for this project. *Genotypes, resistance marker (RM) and origin paper are referenced.*

Plasmids			
	Strain's genotype	**RM**	**Reference and comments**
pBAD24	pBAD promoter from araBAD operon + araC gene's regulator	Amp	[Guzman *et al.* 1995]
pCCB888	pBAD24 + core complex proteins	Amp	Constructed by Corentin
pCCB912	pCCB888 with Cm instead Amp	Cm	Constructed by Corentin
pCCB911	pCCB912 with EndoI instead core complex	Cm	Constructed by Corentin
pCCB875	pCCB888 without core complex		Constructed by Corentin
pCCB878	pCCB912 without core complex		Constructed by Corentin
pOC123	Derived from pCCB888 (RBS replacement)		Constructed by Corentin
pAJM716 (pOC136)	araBAD promoter + EYFP gene	Kan	[Meyer *et al.* 2019]
pOC158	pAJM716 + Rec104 instead of EYFP	Kan	
pOC159	pAJM716 + Rec102 instead of EYFP	Kan	
pOC160	pAJM716 + Ski8 instead of EYFP	Kan	In construction
pOC161	pAJM716 + Spo11 instead of EYFP	Kan	
pOC164	pAJM716 + Rec104, Rec102, Spo11 and Ski8	Kan	
pCCB877	LacZ gene + non-SOS promoter	Kan	Constructed by Corentin
pOC120	pCCB877 + SulA promoter	Kan	
pOC121	pCCB877 + RecA promoter	Kan	
pOC122	pCCB877 + UmuDC promoter	Kan	
pUC19	Lac promoter + LacZα gene	Amp	[Norrander *et al.* 1983]
pRC1632	Used to construct a LacZ- strain	Amp	Thermo-sensitive plasmid (Ronald Chalmers)
pBlueScript	LacZ gene + lac promoter	Amp	(reference n°212207, Stratagene)
p15A	Control for transformation with a p15a origin of replication	Kan	B. Hallet team
pCCB873	Lambda Red Genes under the control of the temperature-inducible Lambda promoter (42°C)	Amp	

Table 2: Plasmids used for this project. *Genotypes, resistance marker (RM) and origin paper are referenced.*

3.2 Usual media and antibiotics used

Several liquid media and plates have been used during this project. They are referenced in table 3.

	LB medium	SOC medium	LB plates
Bacto-tryptone	10 g/L	20 g/L	10 g/L
Bacto-yeast extract	5 g/L	5 g/L	5 g/L
NaCl	10 g/L	10 mM	10 g/L
KCl	/	2.5 mM	/
$MgCl_2$	/	10 mM	/
$MgSO_4$	/	10 mM	/
Glucose (dextrose)	/	20 mM	/
Agar	/	/	20 g/L
5N NaOH	To adjust pH at 7.0	/	To adjust pH at 7.0
H_2O_{mq}	Until 1L	/	Until 1L

Table 3: Composition of media used during this project. *LB medium is used for cellular culture and SOC medium for regeneration during transformation. LB plates corresponds to LB medium supplemented with agar.*

Before using these media, they must be sterilized in an autoclave.

Concerning the LB plates medium, 1L can serve for 40 plates, corresponding to approximately 25 ml per plate.

Different compounds have also been used. They are referenced in table 4.

	Stock concentration	Usual concentration for experiments	Interest of this compound
Amp	100 mg/ml in H_2O	100 µg/ml	Used for bacterial expression
Cm	34 mg/ml in ethanol	35 µg/ml	Used for bacterial expression
Kan	50 mg/ml in H_2O	50 µg/ml	Used for bacterial expression
Strep	10 mg/ml in H_2O	50 µg/ml	Used for bacterial expression
X-gal	40 mg/ml	100 µg/ml	Sugar cleaved by β-galactosidase coded by LacZ that induces a blue coloration
Mitomycin	1 mg/ml	1 µg/ml	Induce SOS response
Arabinose	20 g/L	0.2 g/L	Activate arabinose inducible promoters
Glucose	20 g/L	0.5 g/L	Catabolic repressor that avoid X-gal cleavage by endogen LacZ

Table 4: Antibiotics used for this project. *Mention of stock concentration, working concentrations, and interests.*

When needed for agar plates, antibiotics where added around 60°C, a temperature that does not promote degradation of the compound while maintaining the agar liquid.

3.3 Machines and specific material

Several machines and other specific materials have been used in the different following protocols. They are referenced in table 5.

	Use	Producer	Reference
Mini-centrifuge	Centrifugate small samples	VWR	MiniStar
Microcentrifuge		Eppendorf	Eppendorf/5425, /5417C and /5430
Centrifuge	Centrifugate huge samples	Beckman	Beckman coulter allegra X-22R (rpm max = 6000)
		Eppendorf	Eppendorf/5804R (rpm max = 4200)
Autoclaves (two different sizes)	Sterilize media or materials	Analis	SYSTEC/VB-95 (159258)
		Gemini Lab	PBI (02564)
pH meter	Measure and adjust pH	HANNA Instruments	HANNA/PH209
Electroporator	Allow insertion of plasmid into electrocompetent cells	Eppendorf	Eppendorf (035683)
Ultrasonic sonicator	Lyse cells	Sonics & Materials	VIBRA CELL/75022
Protein electrophoresis machine	Run proteins on a polyacrylamide gel	Bio-Rad	Power Pac HC High-Current Power Supply (1645052)
Protein electrophoresis vat	Contain the polyacrylamide gel and the running buffer	Bio-Rad	Mini-PROTEAN Tetra Vertical electrophoresis (1658005)
Protein electrophoresis gel	Constitute the matrix for running proteins	Bio-Rad	4-15% Mini-PROTEAN TGX Precast Protein Gels (4561081)
Protein ladder	Scaling runed proteins	Bio-Rad	Precision Plus Protein Dual Color Standards (1610374)
Western blot machine	Transfer proteins from polyacrylamide gel to a membrane	Bio-Rad	Trans-Blot Turbo Transfer System
Western blot membrane	Allows chemiluminescence detection after transfer	Bio-Rad	Trans-Blot Turbo Transfer Pack (1704156)
Proteins revelation machine	Reveal presence of proteins on gels (colorimetric) or membranes (chemiluminescence)	GE Healthcare Life Sciences	Amersham Imager 600
DNA electrophoresis machine	Run DNA on an agarose gel	Bio-Rad	Power Pac Basic Power Supply (1645050)

DNA electrophoresis vat	Contain the agarose gel and the running buffer (TBE)	Bio-Rad	Sub-Cell GT Horizontal Electrophoresis System (1704401)
			Mini-Sub Cell GT Horizontal Electrophoresis System (1704406)
DNA ladder (Ladder:Tris-EDTA:charge buffer in 1:7:2)	Scaling runed DNA fragments	Bioke (New England Biolabs)	1 kb Plus DNA Ladder (N3200)
UV imager	Reveal intercalant agent which is bound to DNA	Bio-Rad	Molecular Imager Gel Doc XR+ Imaging System
PCR machine	Amplify DNA fragments	Thermo Fisher Scientific	AB APPLIED BIOSYSTEMS/VERITI

Table 5: Machines and other specific materials used for this project. *Mention of use, producer and reference.*

3.4 Transformation of a plasmid in thermocompetent cells

This method allows insertion of 25 to 50 ng of DNA plasmid containing a selection marker into an *Escherichia coli* bacterial strain (40 µl). After 30 minutes on ice, mixed bacteria and DNA are placed in a water bath at 42°C for 45 seconds, and then on ice for 2 minutes. Next, the mix is supplemented with 1 ml of SOC (table 3), transferred in a 14 ml tube and regenerated for 1 hour at 37°C and 120 rpm for oxygenation. Finally, the culture is spread out on plates with the appropriate antibiotic (table 4) at different dilutions (10^{-1}, 10^{-3} and 10^{-5}), and incubated at 37°C.

3.5 Transformation of a plasmid in electrocompetent cells

The plasmid (25 to 50 ng) is mixed with the electrocompetent cells (40 µl, see 3.6 Electrocompetent cells) on ice, and the mix is transferred in an electroporation cuvette. After the electroporation, 980 µl of SOC (table 3) is added and the mix is transferred in a 14 ml tube. After 1 hour at 37°C and 120 rpm (to oxygenate cells) for regeneration, the culture is spread out on plates with the appropriate antibiotic (table 4) at three different dilutions (without dilution, 10^{-1} and 10^{-2}). The temperature's adaptation to 30°C instead of 37°C is needed for thermo-sensitive plasmids, like pRC1632 and pCCB873 (table 2).

3.6 Electrocompetent cells

After inoculation of 1 colony into 7 ml of LB (table 3) and the appropriate antibiotic (table 4) at 37°C overnight, 100 µl of this culture are transferred into an Erlenmeyer of 500 ml that contains 100 ml of LB (table 3) and the appropriate antibiotic (table 4), and shaken at 37°C. When the optic density reaches 0.6, the culture is transferred into 50 ml falcons and centrifugated at rpm maximum (around 4200 or 6000 rpm according to the machine) for 5 minutes. To protect the bacteria while avoid de novo cell wall synthesis, the samples and the glycerol solutions must stay on ice during the next steps. After the supernatant removal, the cell pellet is washed with 40 ml of ice cold 10% glycerol and resuspended with a pipette before a centrifugation of 4 minutes. This step is repeated 5 times. Finally, the pellet is

resuspended into 500 µl of ice cold 80% glycerol and divided into aliquots of 50 to 100 µl. The tubes are frozen in liquid nitrogen directly, and placed at -80°C.

3.7 Protein induction in *E. coli*

In a 14 ml tube, 2 ml of liquid LB medium (table 3) containing the appropriate antibiotic (table 4) is inoculated with a colony from cells freshly transformed with the expression vector (see 3.4 Transformation in thermocompetent cells and 3.5 Transformation in electrocompetent cells). After overnight growth at 37°C (or 30°C for thermo-sensitive plasmids), the starter is diluted 50 times into liquid LB (table 3) and antibiotic (table 4), and placed at 37°C under the agitation of 150 rpm until the optic density reaches 0.7. After cooling conical flasks to 30°C which is the incubation temperature, the culture is induced with 0.2% arabinose (table 4) for 4 hours or overnight. To analyse the sample, a fraction of the culture is taken and centrifuged at 3000 rpm for 2 minutes. After the supernatant removal, the SDS sample buffer 4x (table 6) is added to the sample. The tube are then incubated during 3 minutes at 95°C, cooled on ice, grated on a rack to shear the DNA and briefly centrifugated. The sample can be directly loaded on a polyacrylamide gel or conserved at -20°C.

Compound	Working concentration
0.5M Tris-HCl pH6.8	50mM
20% SDS	2%
100% Glycerol (without if already in the sample)	40%
Bromophenol blue	0.1%
β-mercapto ethanol	50 µg/ml

Table 6: SDS sample buffer, also called laemmli buffer, composition.

3.8 Analysis of protein expression

The sample with laemmli buffer is deposed into a well of a polyacrylamide gel 4-15% (table 5), and run at 200 Volts for 30 minutes. The precast gel is previously deposed in a running chamber and coated by Tris-glycine buffer (table 7). During this project, three methods have been used to analyse these SDS-Page gels (see next).

Compound	Working concentration
Tris-base	25mM
Glycine	250mM
10% SDS	0.1%
H_20	Until 4L

Table 7: Tris-glycine buffer composition.

3.8.1 Western blot

After running, the proteins are transferred from the gel onto a 0.2 µm PolyVinyliDene Fluoride (PVDF) membrane (table 5) at 25 Volts during 30 minutes. After a wash in PBS-T (PBS 1x (table 8), Tween 0.1%), the membrane is incubated with a blocking buffer (PBS-T, milk 5%) for 1 hour at ambient temperature. After three other washes, the membrane is incubated into the blocking buffer supplemented with primary antibody, Anti-MBP Monoclonal Antibody (reference n°E8032, produced by New England Biolabs) or Penta-his Antibody BSA-free (reference n°34660, produced by QIAgen), at dilution 10 000x for 1 or 3 hours (according to the used antibody) at ambient temperature. After three other washes of 10 minutes, the membrane is incubated into the blocking buffer supplemented with the secondary antibody (Goat Anti-Mouse IgG Antibody, (H+L) HRP conjugate (reference n°AP308P, produced by Sigma-Aldrich)) at dilution 10 000x which is fused with the horseradish peroxidase (HRP). Finally, after three other washes of 10 minutes, Lumi-Light Western Blotting Substrate (the Luminol/Enhancer and the Stable Peroxide solutions (reference n°12015200001, produced by Roche)) are added and will interact with HRP proteins to product photons detectable by chemiluminescence.

Compound	Working concentration
NaCl	80 g/L
KCl	2 g/L
Na_2HPO_4	14.4 g/L
KH_2PO_4	2.4 g/L
HCl	To adjust pH at 7.4
H_2O_{mq}	Until 1L

Table 8: PBS 10x composition. *This medium needs to be autoclaved before use.*

3.8.2 Coomassie blue

After running, the gel is incubated into Coomassie blue (table 9) for 30 to 45 minutes. The gel is then destained into a specific solution (table 9), and then incubated in water to remove background noise.

Compound	Working concentration	
	Coomassie blue	Destain solution
Coomassie 250	1 g/L	/
Glacial acetic acid	100 ml/L	100 ml/L
Methanol	400 ml/L	400 ml/L
H_2O_{mq}	Until 1L	Until 1L

Table 9: Coomassie blue and destain solutions composition.

3.8.3 Silver staining

After running, the gel is washed twice in water before fixing proteins in 30% ethanol and 10% acetic acid during 15 minutes (also twice). After washing twice in 10% ethanol solution and twice in water, the gel is incubated for 1 minute in a solution that contains 50 µl Sensitizer and 25 ml of water, and directly washed two times one minute in water. After incubation during 30 minutes or more in a solution containing 25 ml of Stain and 500 µl of Enhancer, the gel is washed in water and coloured in a solution composed by 25 ml of Developer and 500 µl of Enhancer (30 seconds to 2 or 3 minutes). The coloration is stopped with a solution of acetic acid 5% (in two times). Sensitizer, Stain, Enhancer and Developer solutions come from the Pierce Silver Stain Kit (reference n°24612) produced by Thermo Fisher Scientific.

3.9 Purification of proteins on Nickel (or amylose) column

After the transformation (see 3.4 Transformation in thermocompetent cells), 1 colony is placed into 4 ml of LB (table 3) and the appropriate antibiotic (table 4) at 37°C overnight. After inoculation of this culture (500 µl) into 50 ml of LB (table 3) with the appropriate antibiotic (table 4) at 37°C, cells grow until the optic density reaches 0.7. After the induction with arabinose (table 4) at 30°C overnight to produce the proteins, the culture is centrifuged 7 minutes at 4200 rpm and the cell pellets can be conserved at -80°C or used directly. For all the following steps of this protocol, the experiment take place on ice and all solutions are ice cold (4°C). The pellets are resuspended into 5 ml of Ni-NTA wash buffer (table 10) with protease inhibitors (table 11) and PMSF (table 11). After the sonication according to different parameters: amplitude 50, timer 2 and pulse 5, the lysed cells are centrifuged during 30 minutes at rpm maximum (around 4200 or 6000 rpm according to the machine) and 4°C. During the centrifugation, the HisPur Ni-NTA Resin (reference n°88222, produced by Thermo Fisher Scientific) is prepared by taking 300 µl of slurry and 10 ml of Ni-NTA wash buffer (table 10), mixing by turnaround and then the supernatant removal after a centrifugation (1 minute at 1000 rpm). After the addition of the cells supernatant to the resin with more proteases inhibitors (table 11, dilution 2x) and PMSF (table 11, dilution 2x) , the solution is incubated on a tube roller at 4°C for 30 minutes. After another centrifugation (1 minute at 1000 rpm), the Flow Through is discarded and the resin is washed again by adding 10 ml Ni-NTA wash buffer (table 10) and incubating during 10 minutes on the tube roller. After another centrifugation, the supernatant is again removed. After the transfer on the column, the resin is washed with 10 ml Ni-NTA wash buffer, and then eluted in two times with 100 µl and 400 µl of Ni-NTA elution buffer (Wash buffer with 500 mM imidazole, table 10). To analyse the different samples harvested during this experiment, the proteins segregation on a polyacrylamide gel (see 3.8 Analysis of protein expression) can be used. Then, the protein expression can be detected by Western blot (see 3.8.1 Western blot), Coomassie blue (see 3.8.2 Coomassie blue) or Silver staining (see 3.8.3 Silver staining) analysis.

To purify the proteins on an amylose column, the same protocol can be followed with the appropriate buffers (table 10) and the Amylose Resin (reference n°E8021, produced by New England Biolabs).

	Ni-NTA column		Amylose column	
Compound	Wash buffer	Elution buffer	Wash buffer	Elution buffer
HEPES 1M pH7.5	25 mM	25 mM	25 mM	25 mM
NaCl 5M	50 mM	50 mM	50 mM	50 mM
Glycerol 50%	10%	10%	10%	10%
Imidazole 3M	21 mM	500 mM	/	/
Dithiothreitol (DTT) 1M	0.1 mM	0.1 mM	1 mM	1 mM
Ethylenediaminetetraacetic acid (EDTA) 0.5M	/	/	2 mM	2 mM
Maltose 1M	/	/	/	10 mM
H_2O_{mq}	Until 1L	Until 100 ml	Until 1L	Until 100 ml

Table 10: Composition of wash and elution buffers for Ni-NTA and amylose purification's column. *HEPES and NaCl allow physiological pH maintain and regulation in cell's culture; Glycerol helps protein solubility and stability; Imidazole allows fixation and elution of his-tagged proteins through interaction with metallic ions; DTT is a reducing agent; EDTA inhibits nucleases by capturing ions like Mg^{2+}; Maltose interacts with MBP-tag taking it down from the column.*

Proteases inhibitor 1 (Red)			Proteases inhibitor 2 (Green)			PMSF		
Compound and references	Concentration		Compound and references	Concentration		Compound	Concentration	
	Stock	Working		Stock	Working		Stock	Working
Leupeptin hemisulfate (Roth – CN33.4)	8.4 mM	8.4 µM	Pepstatin A (Roth – 2936.3)	5.8 mM	5.8 µM	Phenylmethyl-sulfonyl fluoride (serine protease inhibitor)	100 mM	333 µM
Aprotinin (Roth – A162.3)	0.61 mM	0.61 µM	Chymostatin (Sigma – C7268-100MG)	6.6 mM	6.6 µM			
Antipain dichlorhydrate (Roth – 2933.2)	6.6 mM	6.6 µM	In DMSO					
In water								

Table 11: Proteases inhibitors composition. *Name, reference and concentration (stock and working) of compounds.*

3.10 Plasmid DNA purification

One colony is cultured into liquid LB (4 ml, table 3) and the appropriate antibiotic (table 4) overnight at 37°C. After the sampling and the centrifugation (at 10 000 rpm during 1 minute) of 3 ml of this culture, the cell pellet is resuspended in 250 µl of buffer P1 that contains RNAses. After adding 250 µl of the buffer P2 and mixing the tubes 4 to 6 times by turnaround to lyse the cells, 350 µl of the buffer N3 are added and the tubes are mixed again 4 to 6 times gently to neutralize the buffer P2 and stop the cell lysis. The mix is centrifuged at 13 000 rpm during 10 minutes, and 800 µl of the supernatant is applied on the column. After the centrifugation to 8000 rpm during 1 minute, 500 µl of the buffer PB are added. After another centrifugation, 750 µl of the buffer PE are added. After two successive centrifugations and a tube's changing, 50 µl of the buffer EB are added and the tubes are centrifuged after 1 minute

of incubation. For this protocol, the QIAprep Spin Miniprep Kit (reference n°27106) produced by QIAgen has been used.

3.11 Agarose gel electrophoresis

To analyse the DNA, the plasmids can be run on an agarose gel. According to the gel's size, 30 to 120 ml of TBE buffer (table 12) containing 0.8% of agarose are heated to dissolve agarose, and poured in a box with combs to form wells. After polymerisation, the gel is placed in an electrophorese tank and coated by TBE buffer (table 12).

Compound	Concentration
Tris-base	108 g/L
Boric Acid	55 g/L
Ethylenediaminetetraacetic acid (EDTA)	58 g/L
H_2O_{mq}	Until 2L

Table 12: TBE composition.

After charging the samples in the wells, the gel is run at 100 to 130 Volts for 30 to 60 minutes according to the length of the gel. After running, the gel is incubated 30 minutes in a bath of water containing Midori Green Direct (reference n°NG MG06, produced by Filter Service) according to proportions 5µl of Midori for 100ml of water, and then in water without Midori during 10 minutes. Midori green is a intercalant agent which marks DNA like ethidium bromide. Finally, the gel is revealed by photography under UV light.

3.12 DNA analysis by restriction endonuclease

The DNA of interest is incubated with the CutSmart buffer 10x (reference n°B6004, produced by New England Biolabs), a restriction enzyme and H_20 to a final volume of 15 or 20 µl. After at least 15 minutes in a water bath at 37°C, the loading buffer 6x (reference n°B7024, produced by New England Biolabs) is added and the sample can run on an agarose gel (see 3.11 Agarose gel electrophoresis). The undigested plasmid is run on the same gel and serves as a negative control.

3.13 Polymerase Chain Reaction

This method allows the amplification of a specific DNA fragment. It implies mixing of the DNA template with several components (table 13). The PCR machine will then follow defined parameters to amplify the fragment in this mix (table 13). Finally, the PCR product can be analysed by running on an agarose gel (see 3.11 Agarose gel electrophoresis), and then extracted (see 3.15 Purification of PCR products by extraction on agarose gel).

Mix composition		Machine's parameters		
Compound	Concentration	Step's name	Specific conditions	
DNA template	25-50 ng/µl	Lid's heating	Temperature raise to 105.5°C to avoid overflow during reaction and activate polymerase	
Primer (x2)	0.5 µM	Temperature stabilizing	98°C during 30 seconds (95°C during 2 minutes for GoTaq)	
Polymerase (Phusion, GoTaq or Q5)	0.3, 0.25 and 0.5µl respectively	Denaturation	98°C during 10 seconds (95°C during 30 seconds for GoTaq)	35 cycles
Corresponding polymerase buffer (+ high GC buffer for Q5)	Stock diluted 5x	Oligonucleotides hybridation	T° correspond to estimated T° for oligos (depends on size and composition) - 3°C, during 20 seconds	
dNTP	200 µM	Elongation	72°C during 2 minutes (3 minutes for GoTaq)	
H_2O_{mq}	Until 50 µl	Last step	72°C during 2 minutes (5 minutes for GoTaq) to finish elongation of strands, and conservation at 16°C until the end	

Table 13: PCR mix composition and PCR machinery parameters. *Phusion and Q5 High-Fidelity DNA polymerase are produced by New England Biolabs (2000 units/ml, reference n°M0530 and n°M0491), while GoTaq DNA Polymerase is produced by Promega (5 units/µl, reference n°M3005). Corresponding buffers are produced by the same companies: Phusion HF Buffer Pack (5x, reference n°B0518) and Q5 Reaction Buffer Pack (5x, reference n°B9027) by NEB, and 5x Green/Colourless GoTaq Reaction Buffer (reference n°M791 and n°M792).*

3.14 Overlap PCR

This method allows the annealing of two DNA fragments by PCR (see 3.13 Polymerase Chain Reaction). The mix composition is similar to the PCR mix (table 13) with two PCR products in proportions (according to the molecular weights and the concentrations) to form a final volume of 30 µl instead of one DNA template. For this reaction, the Q5 polymerase is more adapted than the Phusion. The parameters of the machine correspond to "Machine's parameters" in the table 13. Finally, the overlap PCR product can be analysed by running on an agarose gel (see 3.11 Agarose gel electrophoresis), and then extracted (see 3.15 Purification of PCR products by extraction on agarose gel).

3.15 Purification of PCR products by extraction on agarose gel

This method allows to purify the amplified DNA fragments (see 3.13 Polymerase Chain Reaction). After running on agarose gel (see 3.11 Agarose gel electrophoresis), the DNA bands of interest are selected and sliced under UV light. The agarose slice is weighted and incubated at 50°C with a 3x excess of QG buffer (w:v). QG buffer contains an enzyme that will degrade the agarose slice in less than 30 minutes. Due to its capacity to induce the DNA precipitation, which allows the DNA fixation on the column's silica membrane, the isopropanol is then added 1 time compared to agarose slice weight, and the mix is transferred on the column. The used column, like all solutions, provides from the QIAquick Gel Extraction Kit (reference n°28706) produced by QIAgen, and functions through a salt's gradient. The supernatant is removed by centrifugation at 12 000 rpm during 40 seconds. The column is washed successively with

500 µl of QG buffer and 750 µl of PE buffer before vacuum centrifugation. Finally, after tube's changing, 30 µl of EB buffer are added for the elution. The EB buffer will act during 1 minute before the last centrifugation.

3.16 Purification of PCR products by digestion with DpnI, PCR clean-up and ethanol precipitation

The restriction enzyme DpnI 50x (reference n°R0176, produced by New England Biolabs) is added to the PCR mix (44 µl, see 3.13 Polymerase Chain Reaction) with the CutSmart buffer 10x (reference n°B6004, produced by New England Biolabs) to a final volume of 50 µl, and the mix is placed in a water bath at 37°C overnight. During this step, DpnI will cut the methylated DNA, corresponding to all DNA fragments except those amplified by PCR.

For the PCR clean-up, 5 volumes of the PB buffer are added to the PCR-Dpn1 mix before transferring on the column. After a centrifugation during 1 minute at 13 000 rpm, 750 µl of the PE buffer are added. After a second centrifugation and a tube's change, 50 µl of the EB buffer are added and act during 1 minute before the last centrifugation. PB, PE and EB buffers, like columns, come from the QIAquick PCR Purification Kit (reference n°28106) produced by QIAgen.

For the ethanol precipitation, 3 volumes of 10% ethanol and 1/5 volume of NaOAc are added to the eluted DNA. After a centrifugation at 14 000 rpm and 4°C during 15 minutes, 200 µl of 70% ethanol are added. After another centrifugation at 14 000 rpm and 4°C during 10 minutes, the DNA is dried under a laminar flow hood during 15 minutes and then resuspended in 10 µl of TE buffer (table 14) before storage at -20°C.

Compound	Concentration
1M Tris-HCl pH8	10 mM
0.5M Ethylenediaminetetraacetic acid (EDTA)	1 mM
H_2O_{mq}	Until 1L

Table 14: TE composition. *This medium needs to be autoclaved before use.*

3.17 Gibson's cloning Assembly of DNA fragments by Gibson's cloning method

This method allows the insertion of a fragment into a plasmid vector. According to their molecular weights and their concentrations, the insert and the matrix formed a total volume of 2 µl that contains a twofold molar excess of insert. The mix is supplemented by 2 µl of the Gibson Assembly Master Mix 2x (reference n°E2611) which is produced by New England Biolabs. After the incubation in a water bath at 50°C for 15 to 20 minutes, the mix is transformed into DH5α cells (70 µl, table 1). Then, the tubes undergo thermal shock (see 3.4 Transformation in thermocompetent cells) until the cells are spread out on LB-agar plates (table 3) at different dilutions (without dilution, 10^{-1} and 10^{-2}). The Gibson mix contains three different enzymatic activities that are performed in a single buffer. An exonuclease to create single-stranded 3' overhangs that facilitate the annealing of the fragments that share

complementarity at one end (overlap region), a polymerase to fill in gaps within each annealed fragment, and a DNA ligase to seal nicks in the assembled DNA.

4 Results

4.1 Test of core complex expression in *E. coli*

The first purpose of this project was to express the four proteins of *Saccharomyces cerevisiae*'s core complex (Spo11, Rec102, Rec104, Ski8) in *Escherichia coli*. To reach this goal, the first approach was to express these proteins as an operon in plasmids with different resistance markers, and then detect them by Western blotting or purification on Nickel and Amylose columns. The second approach was to construct another plasmid which expresses each subunits under the control of individual promoters, instead of as an operon. During this project, four intermediate plasmids which express each subunits individually have been constructed for this second strategy.

4.1.1 Is it possible to express the core complex in *E. coli* ?

The first step of the strategy consists in testing the expression of the four proteins of core complex in *E. coli* with two different resistance markers: chloramphenicol (see pCCB912 in table 2) or ampicillin (see pCCB888 in table 2) (*figure 14*). This test is a heterologous expression of yeast proteins that come from *Saccharomyces cerevisiae*, an eukaryotic organism, in *E. coli* bacterial strain.

Like shown on the figure 14, in these two plasmids, the operon is under the control of an arabinose inducible promoter. Each subunits is preceded by a Ribosome Binding Site (RBS). The four RBS were different, which allowed the simultaneous cloning of four PCR products (each containing the open reading frame of one subunit preceded by a RBS) into a pBAD-derived vector, by Gibson assembly (see 3.17 Gibson's cloning Assembly of DNA method by Gibson's cloning method). Moreover, each Open Reading Frame is flanked by two unique restriction sites. Finally, the first protein (Rec104) is fused with a N-terminal MBP-tag, while the last (Ski8) with a C-terminal his-tag. These tags will be used to detect protein expression with antibodies (see 3.8.1 Western blot).

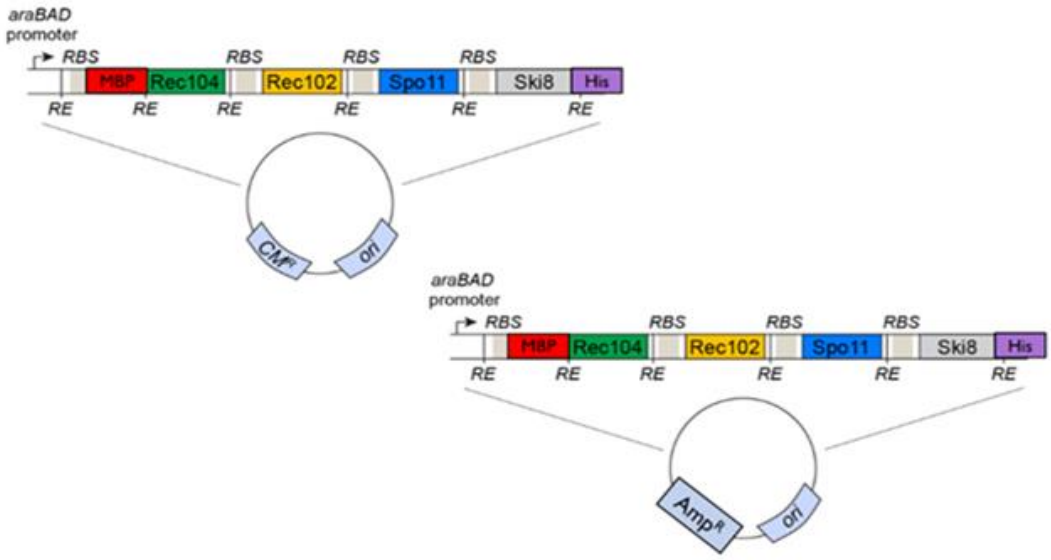


Figure 14: Expression vectors for CORE complex. *Top left: Expression vector containing proteins of core complex (Rec104, Rec102, Spo11 and Ski8), several Ribosome Binding Sites allowing their expressions, a MBP-tag fused with Rec104 and a his-tag fused with Ski8, a chloramphenicol resistance marker (CmR), an inducible arabinose promoter and an origin of replication. Bottom right: Same vector with an ampicillin resistance marker instead of the chloramphenicol resistance marker.*

As a control, a plasmid containing the *E. coli* endonuclease EndoI fused to a MBP-tag (*figure 15*, see pCCB911 in table 2) has been designed. This control will be used for two purposes:

• EndoI will serve as a positive control to verify the protein expression in bacteria.

• EndoI's DNA cleavage activity should activate the SOS response (see 1.3.3 DSBs repair). Therefore, this vector will be used to validate the reporter system (see 4.2 Construction of a reporter system) based on the detection of the SOS response.

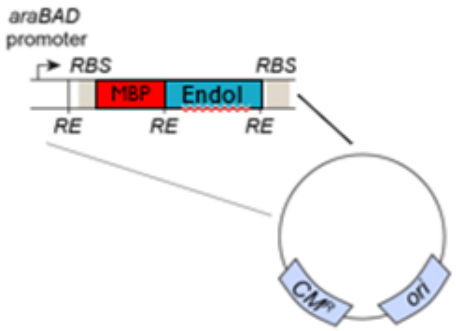


Figure 15: Expression vector of Endonuclease I. *Similar vector than the first on figure 14 with an Endonuclease I gene fused to the MBP-tag instead of the core complex proteins operon.*

To check the protein expression with these three plasmids, two different analyses have been performed: a Western blot analysis (see 3.8.1 Western blot) and a purification test (see 3.9 Purification of proteins on Nickel (or amylose) column).

To analyse the protein expression by Western blotting, Top10 or BL21 *E. coli* strains (table 1) containing one of the expression vectors previously described have been cultured (see 3.7 Protein induction in *E. coli*). After 4 hours or overnight, the protein expression has been induced, or not, by adding arabinose (table 4) in the medium to activate the arabinose inducible promoter. After harvesting, the cellular extracts have been separated on a SDS-Page gel (see 3.8 Analysis of protein expression) and then blotted on a PDVF membrane (see 3.8.1 Western blot). Finally, the proteins have been detected with an antibody against the MBP-tag or the his-tag (see 3.8.1 Western blot). Two types of *E. coli* strains and periods have been used to maximize the cellular growth and the protein expression.

The results provided by the Western blot analysis against the MBP-tag (*figure 16*) show that, in Top10 with arabinose induction, both the MBP-EndoI fusion and the MBP-Rec104 fusion are detected at their expected size, around 65 kDa. Besides, in Top10 without arabinose induction, lighter bands are detected at the same size. However, in BL21, proteins are detected lower.

On the Western blot against the his-tag (*figure 16*), the purified Ski8-his, which serves as a positive control, is detected around 45 kDa. Moreover, consistent with the absence of fusion with an his-tag, the EndoI which serves a negative control is not detected. Despite these two observations, in both Top10 and BL21, bands at the same size as purified Ski8-his are not detected for the core complex proteins. These results indicate that the Western blot analysis is valid, but the Ski8's expression is lower than the detection limit of the experiment.

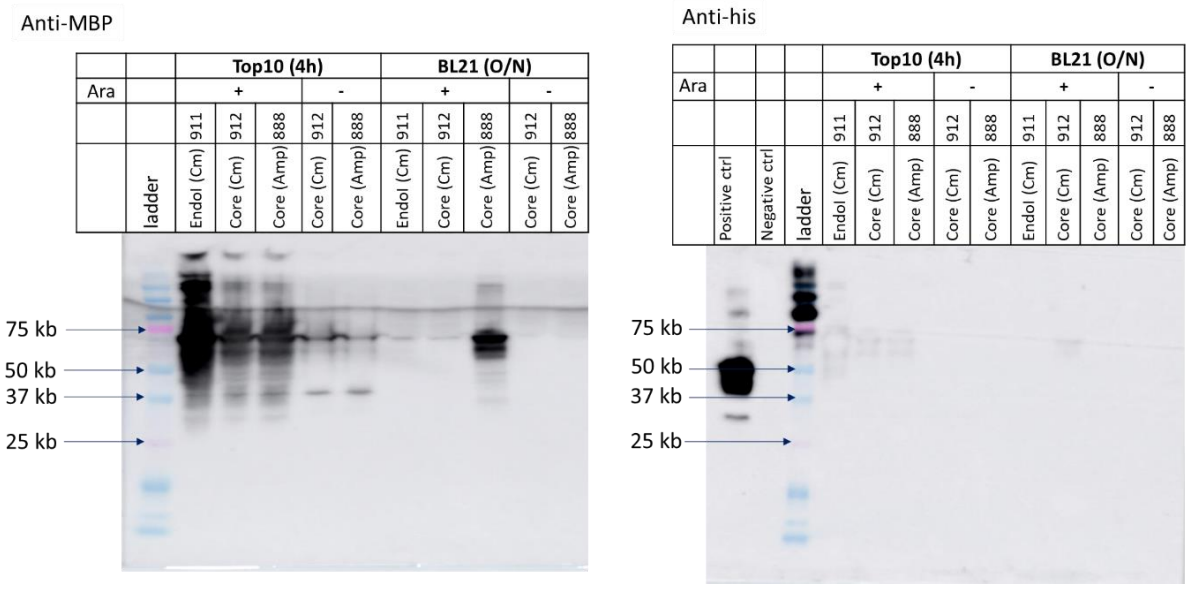


Figure 16: Western blot against MBP-tag or his-tag. *Detection of core complex proteins and EndoI by two different antibodies, and with or without arabinose induction. Top10 and BL21 E. coli strains have been used.*

Therefore, from this Western blot analyses, it appears that the first subunit of the core complex expression vectors is expressed, but no evidence exists for the last one, indicating a potential expression under the detection limit or any expression. To increase the sensitivity level, a purification attempt has been performed.

For this purification test, Top 10 and BL21 *E. coli* strains (table 1) containing expression vectors have been cultured (see 3.7 Protein induction in *E. coli*) and protein expression has been induced with arabinose (table 4). After isolation by clearing cellular extracts from core complex and EndoI, the proteins have been purified on a Nickel column to retain the his-tagged proteins and interactants, or on amylose column, to do same with the MBP-tag (see 3.9 Purification of proteins on Nickel (or amylose) column). After the separation by SDS-Page gel (see 3.8 Analysis of protein expression), the purified proteins have been analysed by a Silver staining (see 3.8.3 Silver staining) analysis.

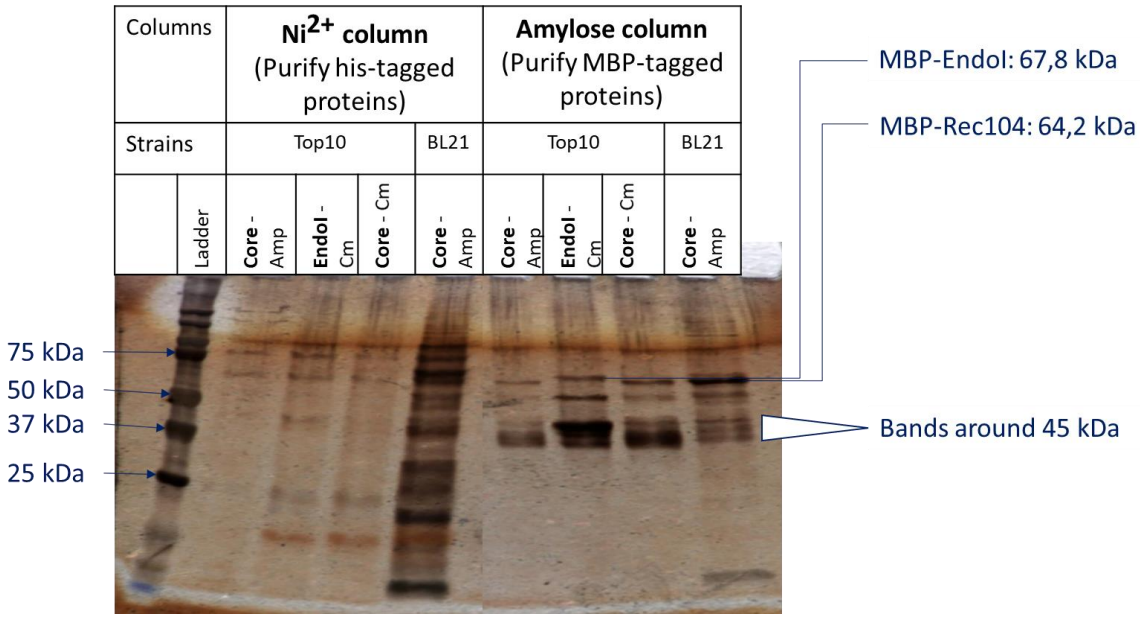


Figure 17: Silver staining on eluates obtained by purification and separated on a SDS-Page gel. *Detection of separated core complex proteins and EndoI after purification on Nickel column or amylose column. Top10 and BL21 E. coli strains have been used.*

After the purification on an Nickel column (*figure 17*), the results indicate that the detected bands don't correspond to the molecular weights of the core complex proteins.

After the purification on an amylose column (*figure 17*), heavy bands are detected around 65 kDa in both EndoI and Rec104 tracks. This can correspond to the MBP-EndoI and the MBP-Rec104 fusions whose theoretical molecular weights correspond to 67,8 kDa and 64,2 kDa respectively. Moreover, several bands are detected around 45 kDa, around the theoretical sizes of Spo11 and Ski8-his whose molecular weights correspond to 45,2 kDa and 45,8 kDa respectively. However, another Western blot analysis against the his-tag (*figure 18*) revealed the absence of Ski8-his proteins in the eluates.

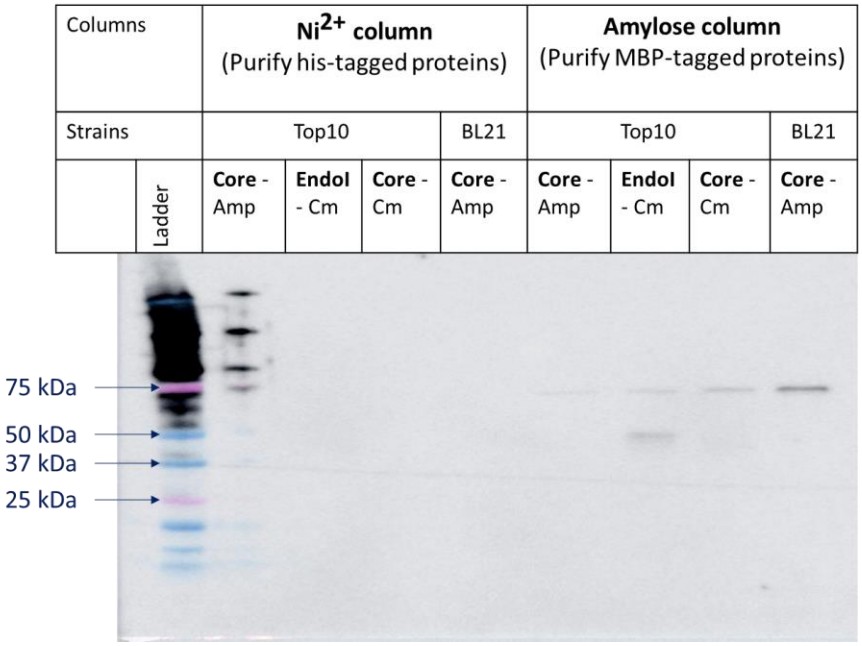


Figure 18: Western blot against his-tag on eluates obtained by purification and separated by SDS-Page gel. *Detection of separated his-tagged proteins of core complex and EndoI after purification on Nickel column or amylose column. Top10 and BL21 E. coli strains have been used.*

On this Western blot analysis against the his-tag (*figure 18*), bands around 65 kDa seem detectable after the purification on an amylose column. Besides, a band around 45 kDa seems detected in the EndoI rack. The sizes corresponding to the MBP-tagged proteins and the band at expected size for Ski8-his being present in EndoI which not possesses a his-tag, both these two observations seem to correspond to non-specific signal, indicating that his-tagged protein are not detectable. Moreover, any his-tagged proteins can be detected after purification on Nickel column. The bands that appear in the first Ni column's rack seems to correspond to protein ladder.

Therefore, similar conclusions can be drawn from the Western blot analysis and the purification experiment, indicating that Rec104, the first subunit of the core complex operon, is significantly expressed while the last one (Ski8) is not.

According to these observations and to our construct's design which uses four different Ribosome Binding Sites (RBS), one hypothesis can be stated. The expression of the first subunit Rec104 indicate that the corresponding RBS is functional. On the contrary, the absence of Ski8 expression could indicate that the last RBS is non-functional. To maximize the chances of expression, the three last RBS have been replaced by the first one.

4.1.2 Is the lack of expression due to suboptimal RBS sequence ?

Since the expression of Rec104 is effective, the next purpose was to express the other proteins of the core complex. In this goal, the ampicillin resistance plasmid from previous experiment

was used as a base to construct a new expression vector with the RBS from Rec104 in front of each subunits of the core complex (see pOC123 in table 2).

After Top10 or BL21 *E. coli* strains (table 1) containing pOC123 (table 2) were cultured during 4 hours or overnight, protein production has been induced by arabinose (table 4). Due to the unknown activity of the core complex, two *E. coli* strains and two growth's times have been used to maximize the production and the protein expression. After harvesting, the cellular extracts have been separated by SDS-Page gel (see 3.8 Analysis of protein expression) and proteins have been detected by Western blotting (see 3.8.1 Western blot) or Silver staining (see 3.8.3 Silver staining) analysis.

The Western blotting on cellular extracts analysis (*figure 19*) shows that the protein expression is better with arabinose induction. On the Western blot against the MBP-tagged proteins, bands around 65 kDa are detected for all conditions with an arabinose induction, consistent with the expression of Rec104. Besides, due to bands around to 45 kDa in BL21-4 hours and Top10-overnight with an arabinose induction, the Western blot against the his-tagged proteins shows that Ski8 is expressed.

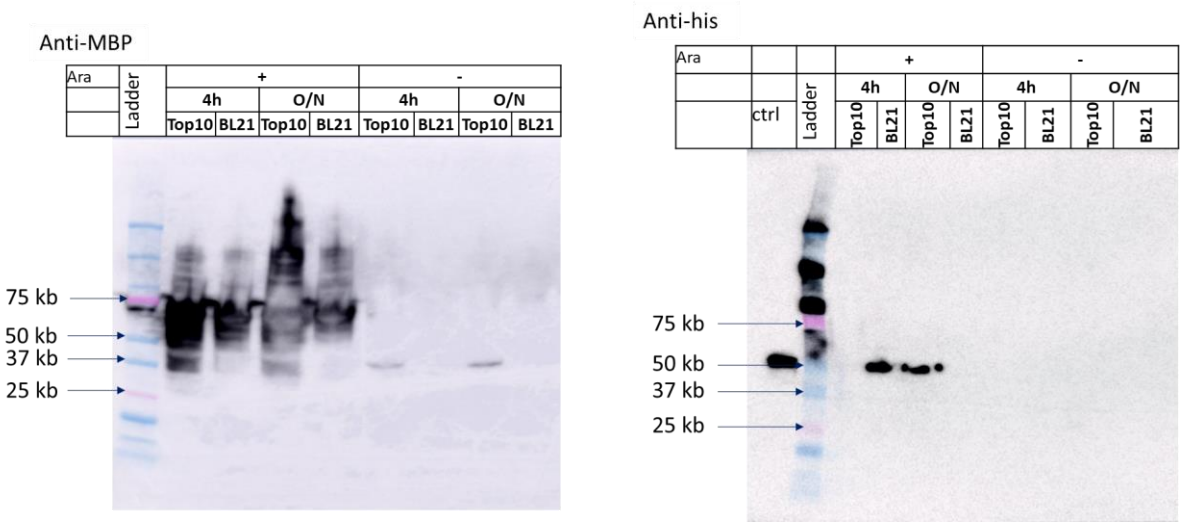


Figure 19: Western blot against MBP-tag or his-tag. *Detection of core complex proteins by two different antibodies after culture under different conditions: Top10 or BL21 E. coli strains, growth during 4 hours or overnight, and with or without arabinose induction.*

To confirm the results obtained by the previous experiment, evaluate the solubility of the proteins and demonstrate the assembly of the four subunits into a complex, a purification attempt (see 3.9 Purification of proteins on Nickel (or amylose) column) has been performed on proteins expressed from pOC123 (table 2). The cellular extracts have been prepared, and loaded on two columns successively. Either the Ni-NTA column then the amylose column, or the opposite (*figure 20*).

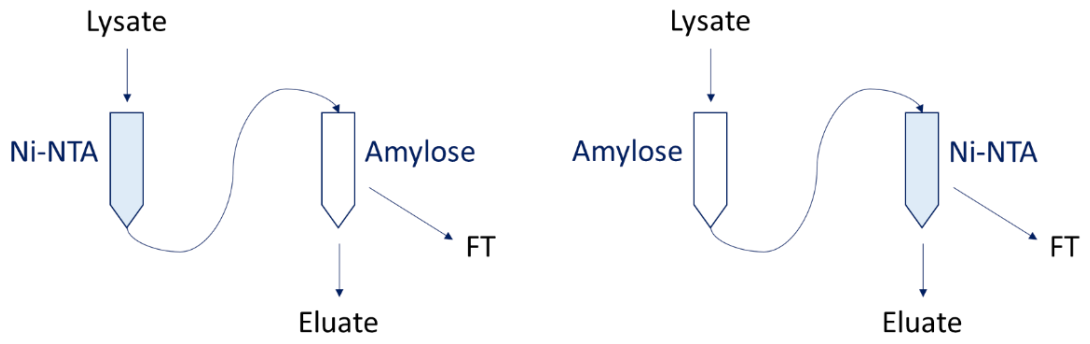


Figure 20: Schematic representation of purification through two successive columns. *The cellular extract (lysate) is purified on a Nickel column, then amylose column (left, opposite on the right). The Flow Through of the second column and the final eluate have been harvested.*

In the two cases, the lysate (corresponding to the cellular extract after sonication), the Flow Through of the second column (corresponding to the eluate of the first column passed through the second), and the final eluate (corresponding to all proteins which have been fixed on the two resins) have been analysed by Western blotting (see 3.8.1 Western blot) and Silver staining (see 3.8.3 Silver staining). As shown on figure 21, the purification on the Nickel column retains all the his-tagged DNA fragments, while the purification on amylose column retains all the MBP-tagged DNA fragments. The use of a second column allows to purify only the double-tagged DNA fragments, corresponding to the complete complex.

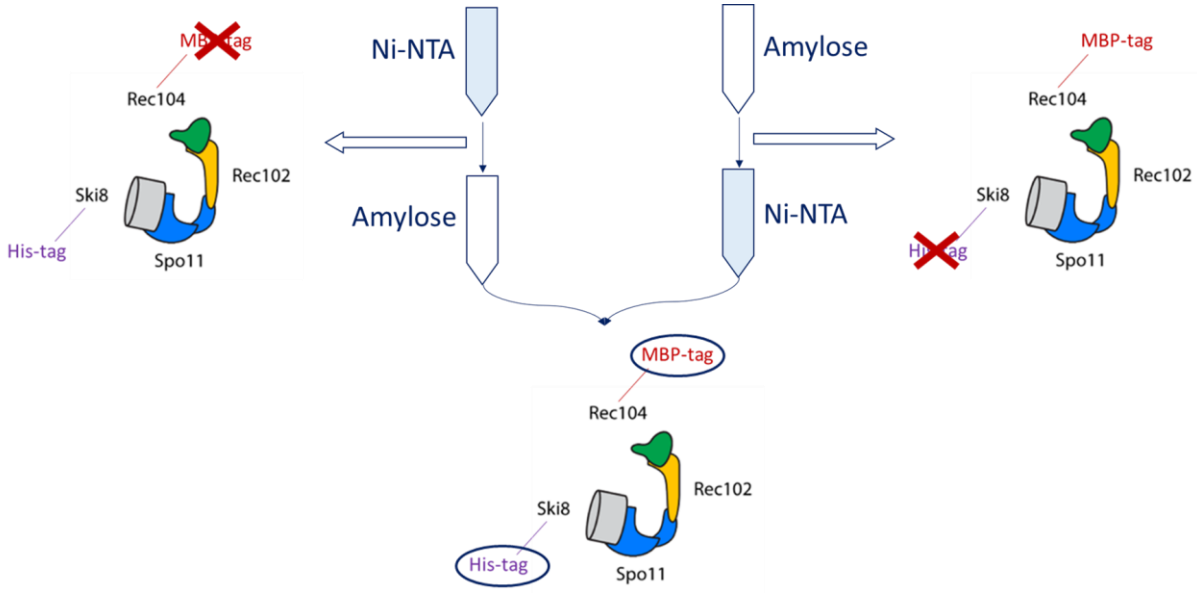


Figure 21: Schematic representation of core complex purification through two successive columns. *The purification of the cellular extract on the Nickel column retains all his-tagged proteins, while the purification on the amylose column retains all MBP-tagged proteins. The use of the second column allows purification of double-tagged fragments.*

When the Ni-NTA column is used before the amylose column (*figure 22*), a small band is detected in the lysate on the anti-his Western blot, and another small band is detected in the

eluate around 45 kDa corresponding to Ski8-his size. Moreover, the heavy band detected in the Flow Through at the same size could be due to the soluble character of Ski8 which can be produced without the other proteins of the core complex. Besides, with the anti-MBP antibody, a heavy band around 65 kDa is detected in the lysate. Moreover, two bands are detected in the eluate. The band around 65 kDa could correspond to the MBP-Rec104 fusion, while the band around 60 kDa could correspond to a truncated form of the same protein.

When the amylose column is used before the Ni-NTA column (*figure 22*), the same band is detected in the lysate on the anti-MBP Western blot. However, a heavy band is detected in the Flow Through and there is no signal in the eluate. This could indicate that the proteins are not able to interact with the second resin, and therefore are lost. Besides, with the anti-his antibody, a band is detected in the lysate, but only light bands can be detected in the Flow Through and the eluate.

These results could indicate that the purification of the core complex is better when the Ni-NTA column is used before the amylose column than the opposite. These observations are consistent with non-published experiments of Hajar Ait Bella who purifies the core complex from insect cells.

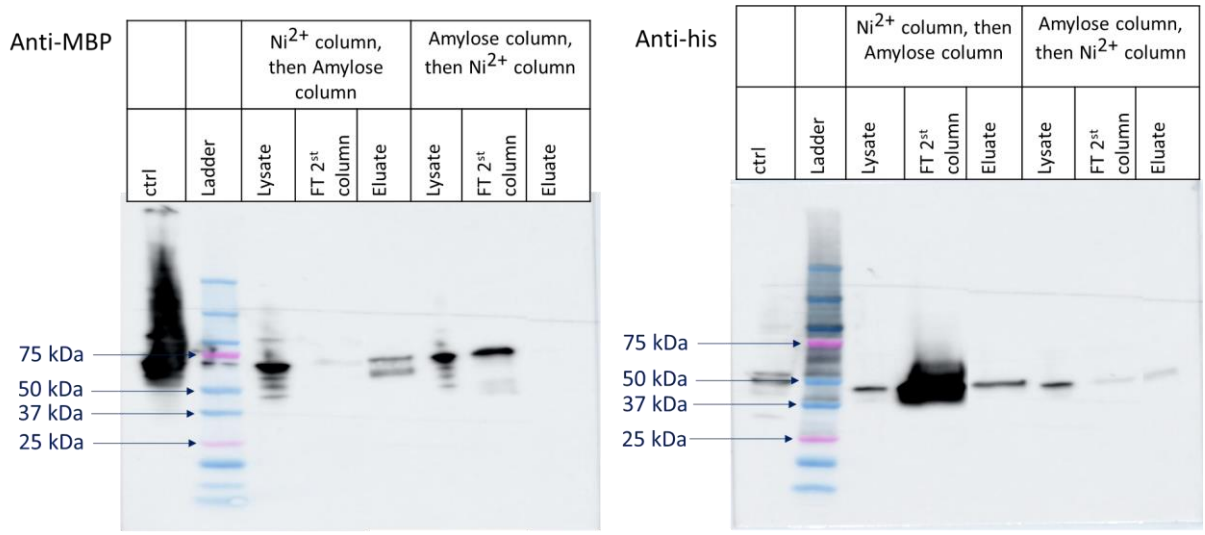


Figure 22: Western blot against MBP-tag or his-tag. *SDS-Page separation and detection by antibodies on lysate, Flow Through of the second column and eluate obtained by purification on two columns: Nickel, then amylose or the opposite.*

Since when the Ni-NTA column is used before the amylose column, the eluate can be detected with the anti-MBP and the anti-his antibodies, proteins are probably in a protein complex. This is consistent with the figure 21 which shows that the eluate contains only the double tagged complexes.

To detect all proteins with or without a tag, the samples (lysate, Flow Through and eluate) have been analysed by Silver staining (see 3.8.3 Silver staining) (*figure 23*). As shown on the Western blot, the purification seems better when the Ni-NTA column is used before the

amylose column. A lot of bands corresponding to the proteins that haven't interacted with the second column have been detected in the Flow Through. This could be consistent with the excess of his-tagged proteins shown by the anti-his Western blot. Besides, several bands can be detected in the eluate. One around 30 kDa that could correspond to Rec102, another around 65 kDa probably corresponding to Rec104, and two bands around 45 kDa indicating that Spo11 and Ski8 may both be expressed. However, the expression of Spo11 should be confirmed using an anti-Spo11 antibody.

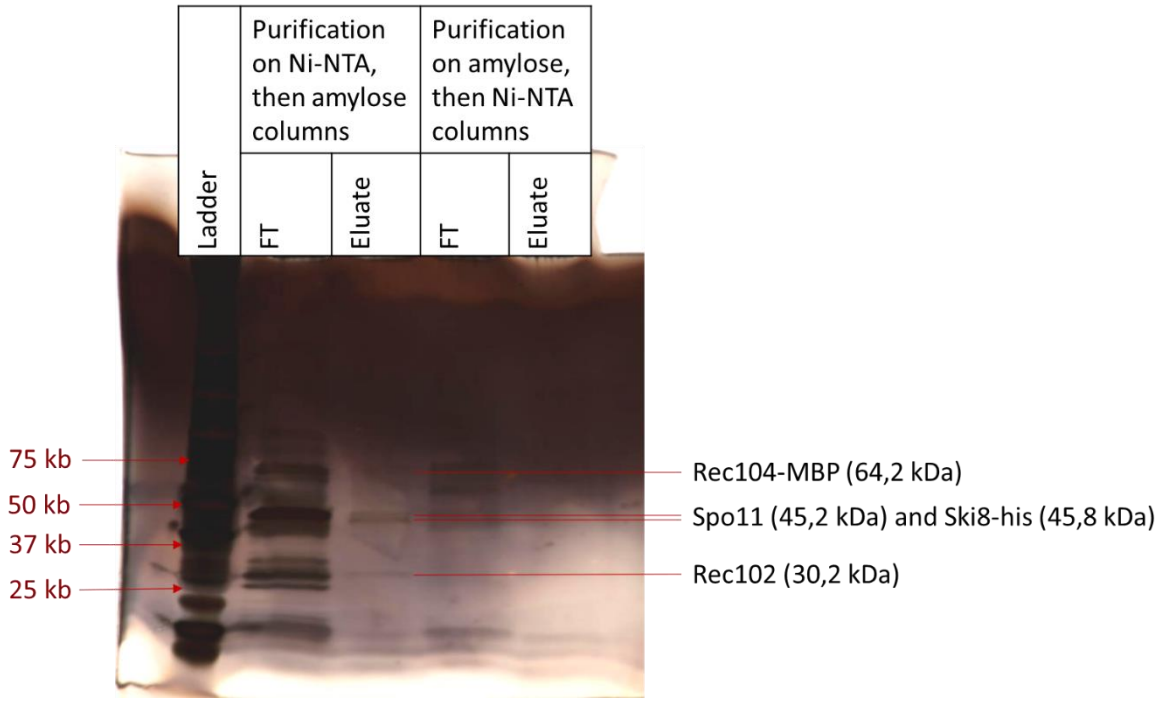


Figure 23: Silver staining on polyacrylamide gel. *SDS-Page separation and detection of second column's Flow Through and eluate obtained by purification on two columns: Nickel, then amylose or the opposite.*

In summary, while the original operon failed to allow expression of the four subunits of the core complex, this issue was solved by replacing the three last RBS.

4.1.3 Is the core complex better expressed in *E. coli* as an operon or with each subunits under the control of individual promoters ?

As a complementary strategy, the plasmid pOC164 (table 2) shown on figure 24 has been designed in order to optimize the core complex expression. The kanamycin resistance matrix comes from pAJM716 (Table 2). [Meyer *et al.* 2019]

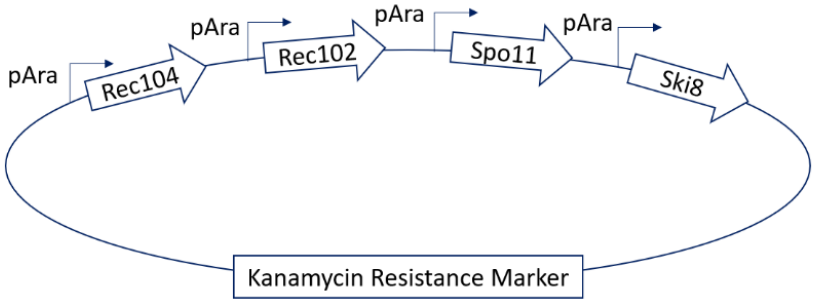


Figure 24: Vector to express the four proteins of core complex under the control of individual promoters (pOC164, table 2). *This plasmid contains a kanamycin resistance marker in addition to the four proteins and their arabinose inducible promoters.*

To obtain this plasmid which allows expression of the four proteins of the core complex under the control of individual promoters, a cloning strategy by means of intermediate individual plasmids (*figure 25*) has been designed. These plasmids (table 2) contain a kanamycin resistance marker, an origin of replication, an arabinose inducible promoter and the gene of one subunit.

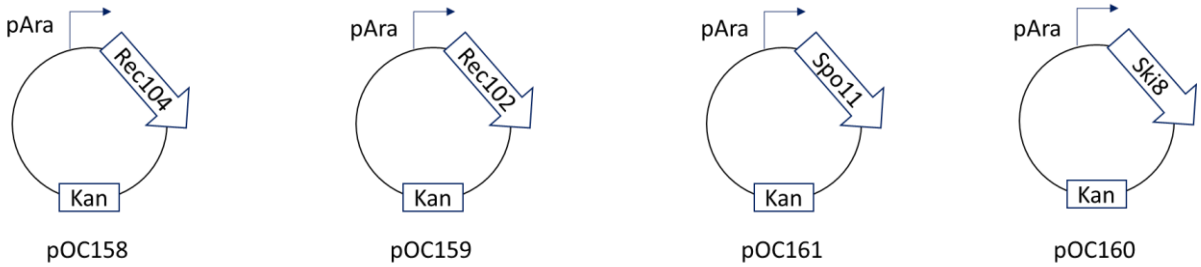


Figure 25: Intermediate plasmids in the cloning strategy to express core complex under the control of individual promoters. *These plasmids contain a kanamycin resistance marker in addition to the gene of one core complex protein and an arabinose inducible promoter.*

After expression in Top10 (figure 26, see 3.17 Gibson's cloning Assembly of DNA method by Gibson's cloning method), these four plasmids have been sequenced.

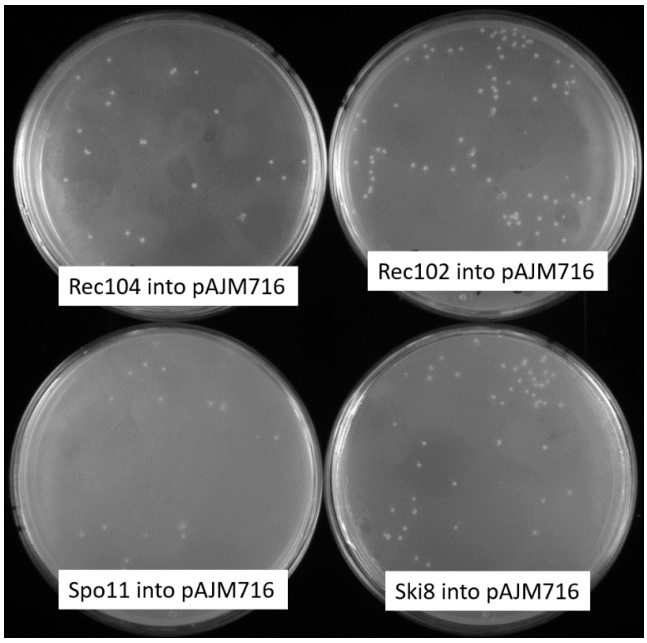


Figure 26: Transformation of four individual plasmids containing one core complex subunit. *Expression made in Top10 after insertion into pAJM716 (table 2) matrix by Gibson assembly. [Meyer et al. 2019]*

Sequencing allows to identify correct clones for pOC158 (Rec104), pOC159 (Rec102) and pOC161 (Spo11). However, the cloning for the plasmid pOC160 that contains Ski8 needs to be restarted. The next step will be to assemble the four subunits into a single vector.

4.2 Construction of a reporter system

The second part of my project was to generate a DSB reporter system, where a LacZ reporter gene is placed under the control of a SOS-inducible promoter, which becomes active when there DNA damage. In this goal, a matrix containing a kanamycin resistance marker and a LacZ gene that come from the pCCB877 plasmid (table 2) has been linearized. Moreover, three SOS-inducible promoters, derived from SulA, RecA and UmuDC genes, have been amplified with primers containing homology to the vector. These promoters possess different sensitivities to the SOS-response, and would therefore produce different levels of LacZ upon DNA damages. Linearized matrix and amplified SOS-inducible promoters have been assembled by Gibson assembly (see 3.17 Gibson's cloning Assembly of DNA method by Gibson's cloning method), forming pOC120 (SulA), pOC121 (RecA) and pOC122 (UmuDC) (table 2). In future experiments, these three SOS-reporter plasmids could allow to isolate Spo11 hyperactive mutants by the formation of blue colonies.

4.2.1 Is my SOS response reporter functional ?

After the transformation in BL21 (table 1, see 3.4 Transformation in thermocompetent cells), the cells containing these plasmids pOC120, 121 and 122 (table 2) have been spread out on plates containing X-gal, kanamycin and glucose, and with or without mitomycin (table 4), to test the SOS response (*figure 27*). The mitomycin is a mutagen agent that is known to activate the SOS response. The original plasmid (pCCB877, table 2) containing another promoter not SOS-inducible has been used as control. The results show that these plasmids can give blue colonies when the SOS response is induced with mitomycin. However, the control vector that lacks the SOS promoter also gave blue colonies in the presence of mitomycin, corresponding to background noise. Despite a color differential between the cells with and without mitomycin, a slight bluing also appears on the plates without mitomycin.

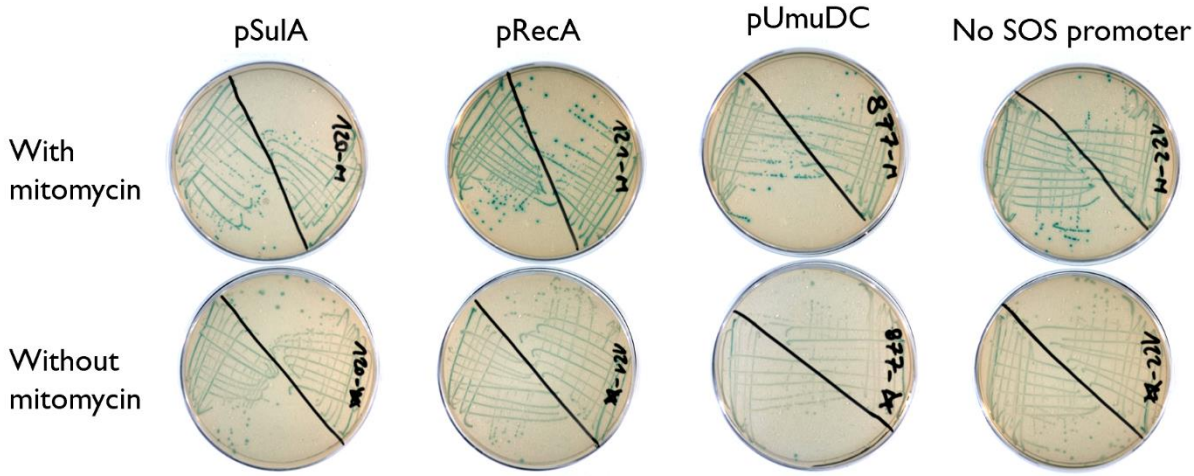


Figure 27: Test of constructions with SOS-inducible promoters. *Constructions on plates containing X-gal, kanamycin and glucose, with or without mitomycin. The same vector with another promoter not SOS-inducible serves as control.*

Two factors can lead to this background noise : a transcription leak through phenomenon, or a too high gene copy number.

4.2.2 Design of a strategy to avoid transcription leak through phenomenon

To avoid this transcription leak through phenomenon, the strategy was to insert terminators before the SOS-inducible promoter and after the LacZ gene in pOC120, 121 and 122 (table 2). The terminators are loops in the DNA which will block the mRNA transcription by the polymerase, inducing a higher specificity for the SOS response. In this goal, the PCRs (see 3.13 Polymerase Chain Reaction) with oligonucleotides that contain terminators in their floating tails have been realized (*figure 28, first strategy*). Due to the presence of secondary structures in these DNA loops, the DNA fragments cannot be amplified with these primers. Three polymerases (Phusion, GoTaq and Q5, table 13) have been used without providing amplified DNA fragments.

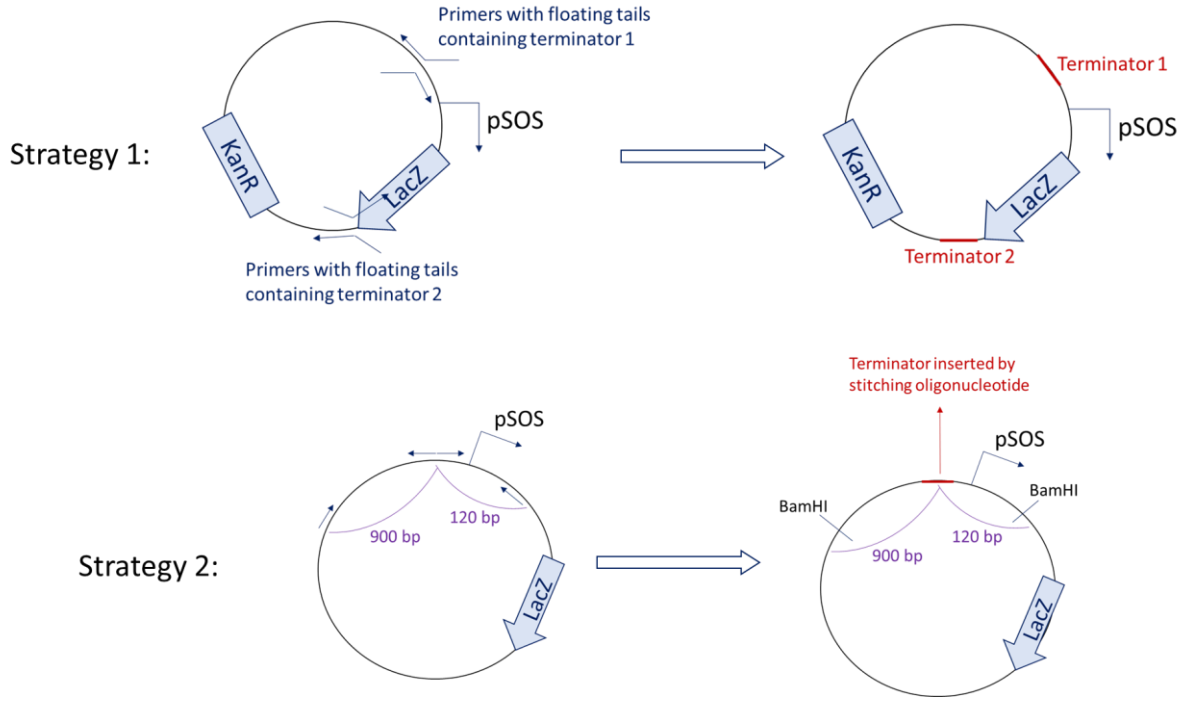


Figure 28: Two strategies for terminators insertion into the plasmids containing SOS-inducible promoters. *Insertion of two terminators through floating tails of primers, or insertion of one terminator through a stitching oligonucleotide.*

Therefore, another strategy (*figure 28, second strategy*) has been designed to amplify shorter DNA fragments without the terminators. In order to realize this strategy, other PCRs with shorter oligonucleotides (*figure 29*) have been performed (see 3.13 Polymerase Chain Reaction), and the DNA has been extracted (see 3.15 Purification of PCR products by extraction on agarose gel). The first DNA fragment corresponds to the promoter region with a start codon and a specific restriction site (BamHI), while the second fragment contains the same specific restriction site (BamHI) allowing the future insertion in the matrix, after the annealing of the two fragments.

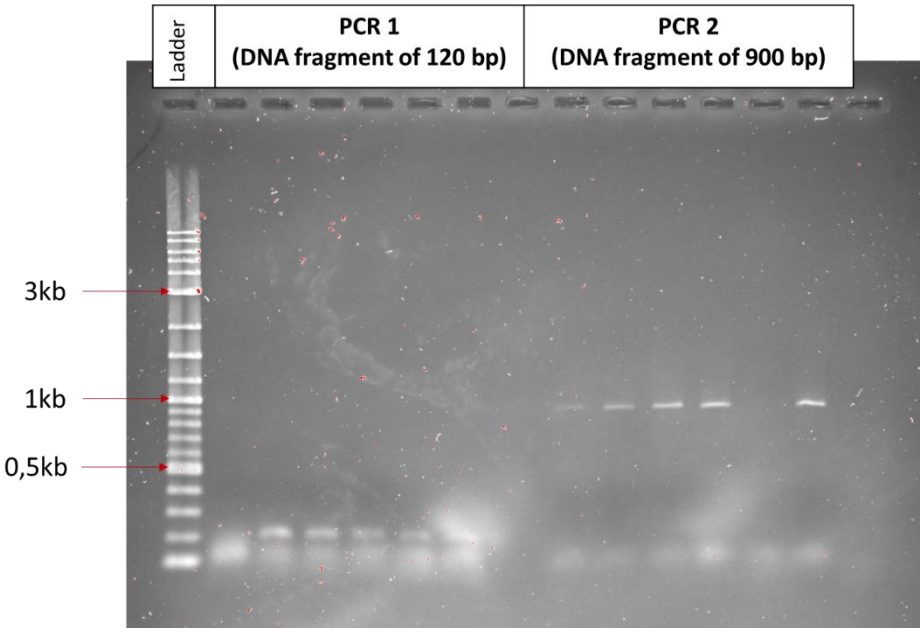


Figure 29: Short PCRs that will allow future insertion of terminators during overlap PCR.

Afterward, the two PCR fragments obtained will be assembled by overlap PCR (see 3.14 Overlap PCR) with stitching oligonucleotide containing the terminator, and then inserted again in the pCCB877 (table 2) matrix by a Gibson assembly (see 3.17 Gibson's cloning Assembly of DNA method by Gibson's cloning method).

4.2.3 Design of a strategy to reduce gene copy number

After the construction of a functional reporter system, it will be inserted into the chromosome to reduce the gene copy number by reducing the probability that it will be transcribed by mistake. In this goal, the Marionette-Wild bacterial strain (table 1, [Meyer *et al.* 2019]) has been used, and endogen LacZ has been deleted. After the production of electrocompetent cells (see 3.6 Electrocompetent cells), the thermo-sensitive plasmid pCCB873 (table 2) which contains an ampicillin resistance marker has been inserted in this strain and induced at 42°C. This new strain, called Marionette-Wild λ-red, serves to produce new electrocompetent cells (see 3.6 Electrocompetent cells). Besides, the pCCB877 plasmid (table 2) which possesses a kanamycin resistance marker has been amplified by PCR (see 3.13 Polymerase Chain Reaction) and purified by digestion with DpnI restriction enzyme, PCR clean-up kit and ethanol precipitation (see 3.16 Purification of PCR products by digestion with DpnI, PCR clean-up and ethanol precipitation). After the electroporation of the PCR product in electrocompetent cells Marionette-Wild λ-red (see 3.5 Transformation of a plasmid into electrocompetent cells), the clones have been selected by plating cells on plates containing either kanamycin or ampicillin antibiotics (table 4) to confirm the kanamycin resistance and the loss of the pCCB873 plasmid (*figure 30*). The correct clones have been conserved and used to produce electrocompetent cells (see 3.6 Electrocompetent cells), called Marionette-Wild LacZ-. After genotyping to confirm strain's identity, the future experiments will allow to construct a functional reporter strain.

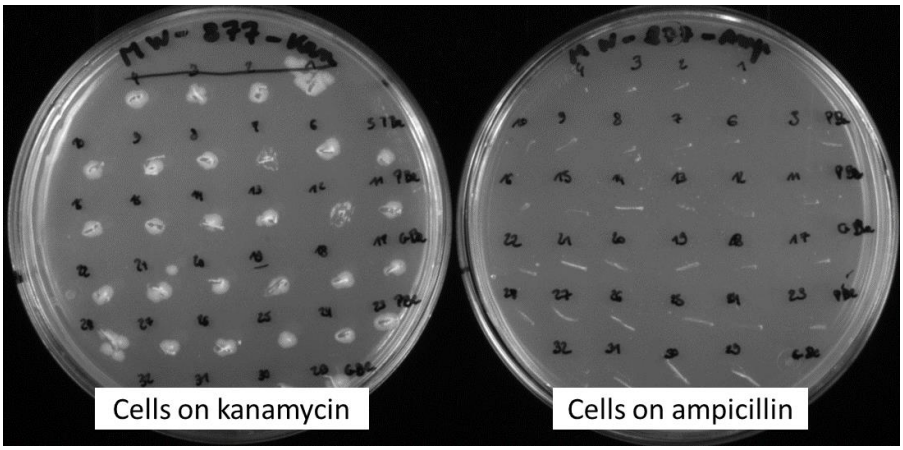


Figure 30: Marionette-Wild λ-red cells transformed with pCCB877 PCR product plated on kanamycin or ampicillin. *This allows to identify the correct Marionette-Wild LacZ- clones in which the LacZ- gene has been replaced by a kanamycin resistance marker.*

4.2.4 A SOS reporter strain developed and used in previous studies

Another strain could be used to transform a functional SOS-reporter system. The JH139 strain (table 1) is a sensitive indicator which uses SOS-LacZ fusion to respond to DNA damage by producing β-galactosidase. Uses and applications of this strain include the characterization of a restriction system, screening mutants for endonuclease's restriction and studying other enzymes involved in DNA metabolism. [Heitman and Model 1991] This strain contains the *dinD1::lacZ* reporter construct inducing a low basal level of β-galactosidase that largely increases when a DNA damage is introduced, like the SOS response. The introduction of the SOS/LacZ+ transformants in JH139 strain (table 1) without *recA* gene allows to establish that the SOS response is RecA-dependant. [Perkins *et al.* 1999] Due to results obtained by this previous study, this strain could allow future identification of Spo11 protein hyperactive mutants. Moreover, a similar experiment has been performed in Perkins *et al.* (1999) in which the *E. coli* SOS induction assay (see 1.3.3 DSBs repair) has been used to screen the yeast cDNA to evaluate the involvement in the genome stability.

To evaluate the potential use of this strain, electrocompetent cells have been made (see 3.6 Electrocompetent cells) and used to measure the transformation rate. After the transformation of the ampicillin resistance marker pUC19 (table 2) in JH139 and Top10 electrocompetent cells (see 3.5 Transformation of a plasmid into electrocompetent cells), JH139 shows very low level of transformation, almost 3.10^6 lower than Top10 (*figure 31*).

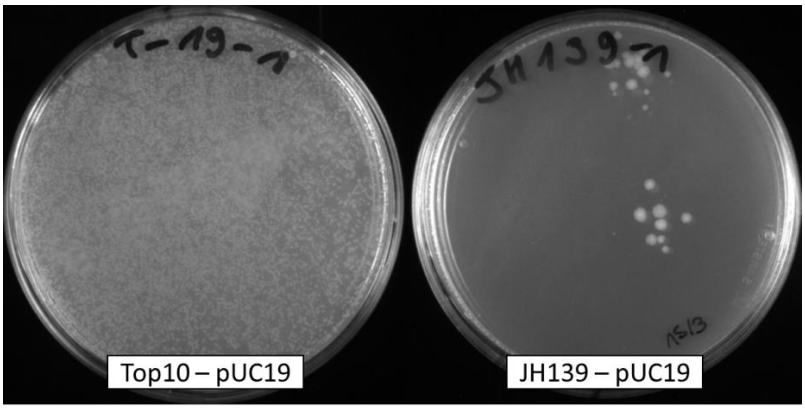


Figure 31: Comparison between transformation ratio without dilution of Top 10 and JH139 with the ampicillin resistance plasmid pUC19.

Other transformation tests (*figure 32*) with pBlueScript and pBAD24 (table 2) show similar results. However, with p15A (table 2) which possesses the same resistance marker as the strain, the transformation ratio is clearly higher.

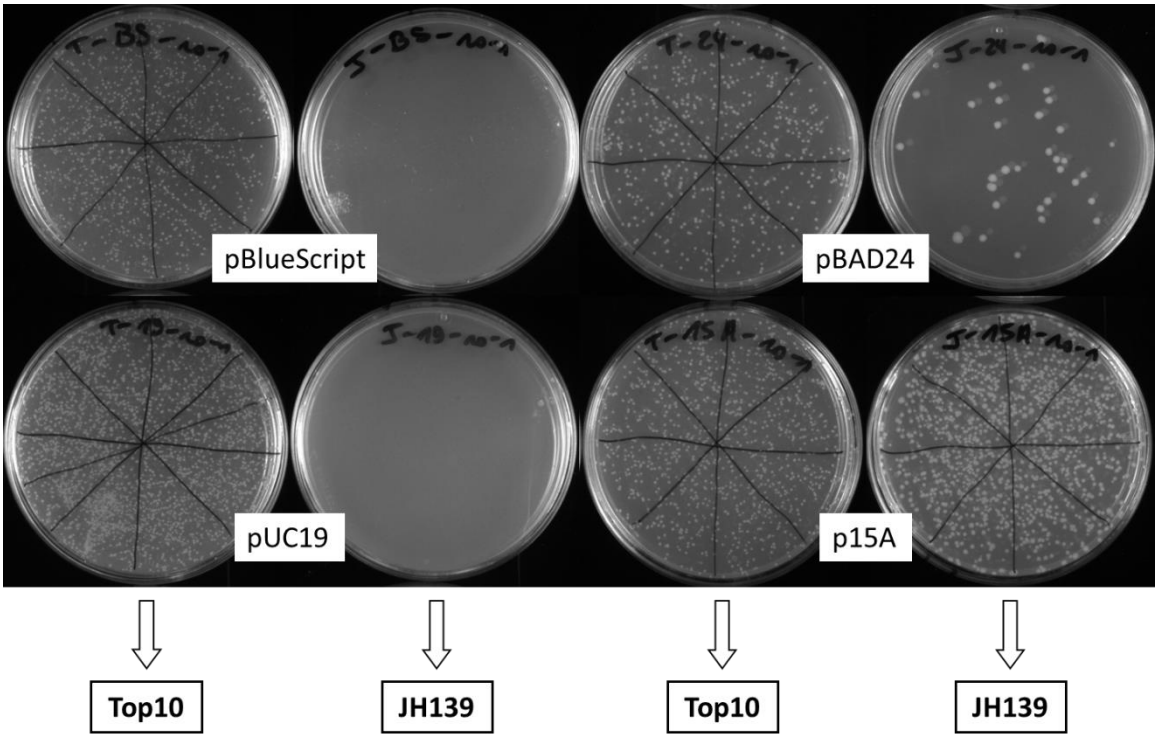


Figure 32: Comparison between transformation ratio without dilution of Top 10 and JH139 with the ampicillin resistance plasmids pBlueScript, pBAD24 and pUC19 and with the kanamycin resistance plasmid p15A.

Despite this low transformation rate, the JH139 electrocompetent cells have been transformed with pCCB888, pCCB911 and pCCB912 plasmids (table 2) (see 3.5 Transformation of a plasmid into electrocompetent cells). After the successful transformation for pCCB911 and pCCB912 (not for pCCB888), the colonies have been spread out on plates containing X-gal

and chloramphenicol (table 4), with different concentrations in arabinose to induce production of proteins: 0.02%, 0.002% or 0% (*figure 33*).

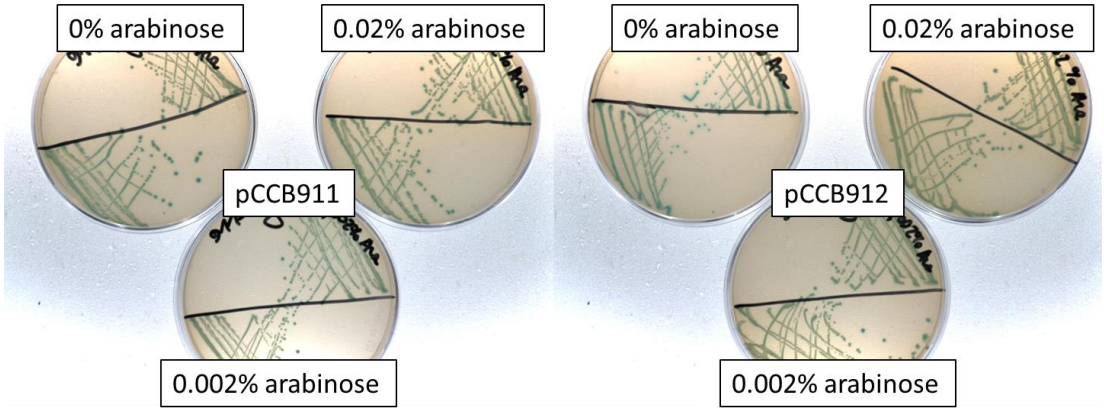


Figure 33: pCCB911 and pCCB912 on plates containing X-gal, chloramphenicol and different concentrations of arabinose.

The results shown by these plates indicate that all the conditions induce a blue coloration in the colonies, whether for pCCB911 which possesses the endonuclease EndoI or for pCCB912 which express the four proteins of core complex (table 2). The arabinose induction inducing the protein production, a gradient of coloration should be visible between the plates at different concentrations, whether with the protein EndoI or the proteins of the core complex. However, the condition without arabinose induction (0%) give also blue colonies.

To evaluate the effectiveness of this strain for this project, the effects of different conditions have been tested on the JH139 strain empty or containing pUC19 plasmid (table 1 and 2) (*figure 34*). All the plates contain X-gal, but only a part contains mitomycin (table 4), and another part ampicillin (table 4, dilution 2x).

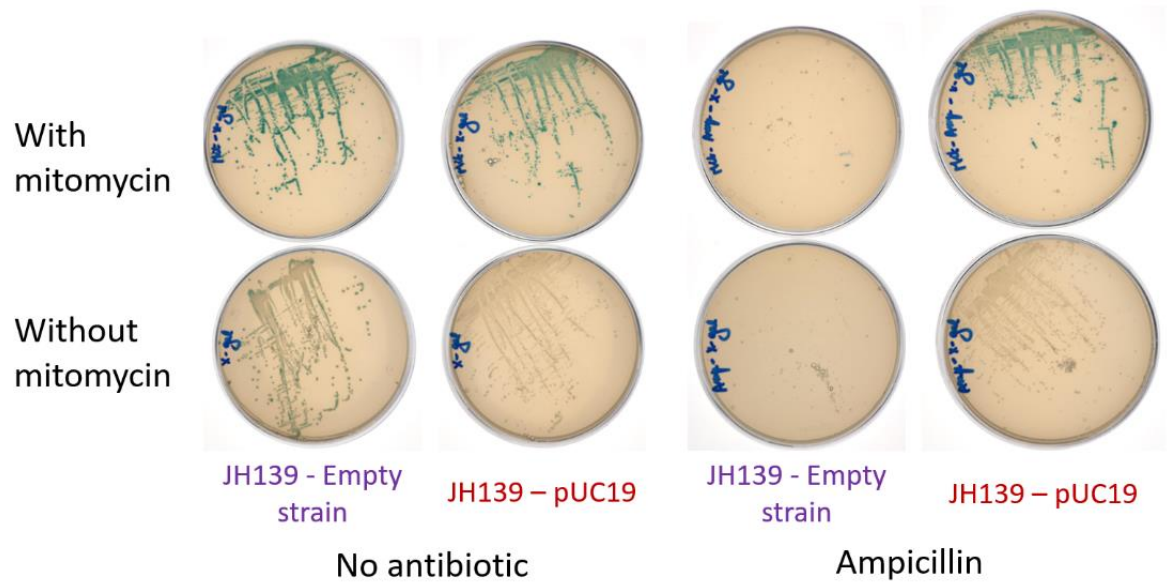
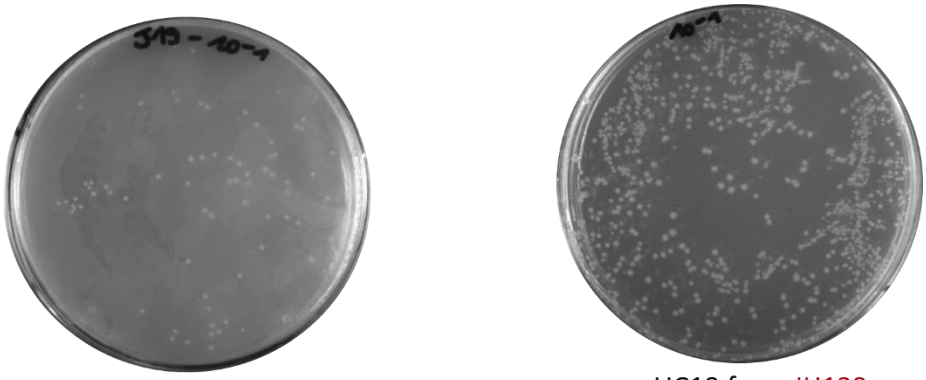


Figure 34: Plating of the SOS-reporter strain with different conditions. *With and without mitomycin, the empty strain or the strain with an ampicillin resistance marker and with or without ampicillin.*

As expected, for the strain containing the ampicillin resistance marker, the colonies become blue with mitomycin and remain white without. However, the background noise is higher without mitomycin for the empty strain. Besides, the empty strain don't grow with ampicillin, consistent with the absence of this resistance marker in the genome.

After all these observations, the JH139 strain has been transformed (see 3.5 Transformation of a plasmid into electrocompetent cells) with the two plasmids pCCB888 (core complex expression with different Ribosome Binding Site, table 2) and pOC123 (core complex expression with same RBS, table 2). However, no colony has been produced with these two transformations. To test if the transformation rate is higher when the plasmid used comes from the same strain, the transformation rate of pUC19 (table 2) coming from Top10 (table 1) and of pUC19 coming from JH139 (table 1) have been compared. The results (*figure 35*) show that the transformation rate is 200 times higher when pUC19 come from JH139. This ratio has been calculated based on the number of colonies at the same dilution and the DNA quantities in ng per ml.



pUC19 from Top10
transformed into JH139

pUC19 from JH139
transformed into JH139

Figure 35: Comparison of transformation rates at dilution 10^{-1} in JH139 between pUC19 coming from Top10 and pUC19 coming from JH139.

Despite a low transformation rate, the results obtained with this strain JH139 (table 1) show a potential link between the SOS response and the antibiotic type that should be investigate. Moreover, future experiments could allow to optimize the transformation rate.

5 Discussion and perspectives

5.1 Test of core complex expression in *E. coli*

After the attempts to express the core complex in *E. coli* by using pCCB888 and pCCB912 (table 2) which possess four different RBS, the results obtained by Western blot (*figure 16*) showed that the first subunit of the operon is expressed, while the last not. After the purification tests on the same plasmids, these results have been confirmed by Silver staining and Western blot (*figures 17* and *18*) analysis. The detection of the first subunit shows that the Ribosome Binding Site before Rec104 is functional. This RBS is a reference sequence that allows to demonstrate the protein expression. On the contrary, the absence of detection signal for the last subunit (Ski8) suggests that the divergences with the reference sequence have affected the RBS function. These results don't provide accurate information for the two intermediate subunits, and therefore the intermediate RBS. Following these results, a new expression vector (pOC123, table 2) has been constructed with the RBS with proven functionality before each of the four subunits.

Moreover, of interest for subsequent experiments, the protein expression is higher in BL21 than in Top10 (table 1). Due to the lack of knowledge about the core complex effects on the DNA, these two strains which possess different uses and phenotypes have been used to maximize the chances of expression. Top10 serves for cloning and transformation with a high fidelity, while BL21 possesses a more specific promoter which avoids the leak through phenomenon. [Durfee *et al.* 2008, Studier *et al.* 1990] Moreover, BL21 is mutated for different major proteases and contains the RecA gene, making it more stable and less sensitive to DNA damage.

After the attempts to express the core complex in *E. coli* by using pOC123 (table 2) which possesses the same functional RBS before each of the four subunits, the results obtained by Western blotting (*figure 19*) suggest the expression of both Rec104 and Ski8 subunits. To increase the sensitivity, a purification test has been performed on a bacterial culture containing this plasmid. The analysis of these samples by Western blotting and Silver staining (*figures 22* and *23*) indicate a high probability that the first and the last subunits are both expressed. Moreover, the gel revealed by Silver staining could indicate the presence of the Spo11 proteins. Due to possible interactions between Rec104-Rec102 and Ski8 (see 1.4.1.2 The core complex proteins and their homology to TopoVI [Arora *et al.* 2004]), the presence of Spo11 should be verified by Western blotting against Spo11 using an anti-Spo11 antibody.

Four intermediate plasmids have been constructed in order to express the four subunits independently. The validity of the three plasmids pOC158, pOC159 and pOC161 (table 2) which express Rec104, Rec102 and Spo11 respectively has been confirmed by their transformation into Top10 *E. coli* strain (table 1, *figure 26*) and by sequencing. After the construction of pOC160 (Ski8) (table 2), the next step will be to construct the plasmid pOC164 (table 2) by assembling these four plasmids.

The construction of both these two different plasmids (pOC123 and pOC164, table 2) could allow to develop two different tools which can be both tested to implement the screening

assay (see 5.3 Toward the screening of core complex hyperactive mutants). Until now, it cannot be predicted what will be the best suited solution.

5.2 Construction of a reporter system

Due to the background noise obtained (*figure 27*) with a plasmid which possesses a promoter not SOS-inducible (pCCB877, table 2), terminators have been inserted before the SOS-inducible promoter and after the LacZ gene to optimize the SOS reporter system. It could prevent the transcription leak through phenomenon (see 4.2.2 Design of a strategy to avoid transcription leak through phenomenon) occurring when a promoter is induced when it should not, this phenomenon decreases the specificity of the SOS response.

Different experimental conditions have been performed to test the potential use of the SOS-reporter strain JH139 (table 1). While the experiment with the chloramphenicol resistance plasmids (pCCB912 and pCCB911, table 2) shows a high level of background noise (*figure 32*) with blue colonies at a 0% rate of arabinose, the ampicillin resistance plasmid pUC19 (table 2) doesn't induce this background noise (*figure 33*). These two antibiotics can induce the SOS response by inducing a DNA damage in the cell (see 1.3.3 DSBs repair), the ampicillin acting on the cell wall synthesis and the chloramphenicol limiting the protein synthesis. However, white colonies can be observed with the ampicillin resistance marker (*figure 33*), suggesting that the SOS response is not induced. Due to the difference of phenotypes between the chloramphenicol and the ampicillin plasmids, future experiments could allow to test the effect of the different antibiotics on the SOS response to prevent a potential incompatibility between the JH139 strain which possesses a resistance to kanamycin and the plasmid's resistance marker.

Moreover, three ampicillin resistance plasmids (pBlueScript, pBAD24 and pUC19, table 2) and one kanamycin resistance plasmid (p15A, table 2) have been transformed (see 3.6 Electrocompetent cells) into the JH139 strain (table 1) to measure the transformation rate. The results have shown a large difference between the transformation rate in Top10 and in JH139 (*figure 31*). However, no issue was mentioned in the article presenting the construction of the JH139 strain. [Heitman and Model 1991] To optimize the transformation rate, the plasmid pUC19 (table 2) has been extracted (see 3.10 Plasmid DNA purification) from a Top10 strain (table 1) and transformed (see 3.6 Electrocompetent cells) into the JH139 strain (table 1). The results (*figure 34*) shown an increase of 200 times in the transformation rate which will have to be investigate.

5.3 Toward the screening of core complex hyperactive mutants

When the core complex expression vector and the reporter system will be efficient, the next steps will be to construct a library of mutants, and then screen this library with the reporter system to identify Spo11 hyperactive mutants, corresponding to hyper-cleavage alleles of the Spo11 core complex. Random mutations into the four subunit will be introduced through error prone PCR or mutagenesis PCR by using a low fidelity Taq polymerase. These random mutations could highlight a structure-function link or a DNA-protein interaction. Besides, this

library will be screened by spreading out the cells on plates containing X-gal, like the reporter systems have been tested until now. The final objective will be to characterize the Spo11 hyperactive mutants *in vitro* and *in vivo*. One possible issue for both the screen and this final step could be the toxicity of Spo11. Indeed, is it possible to develop hyperactive mutants if the expressed protein is toxic? The preliminary results seem indicate that the experiment works and that it is possible to produce the core complex in sufficient quantities to detect it, indicating that it could be toxic but not sufficiently to kill all the cells within the time where it is induced. A possible experiment to verify it could be to measure the optic density after the arabinose induction to observe if it raises, decreases or stays stable. To conclude, unleashing the catalytic activity of Spo11 will yield important new mechanistic insights into the control of the meiotic DSB formation mediated by Spo11.

References

Arora C, Kee K, Maleki S, Keeney S. Antiviral protein Ski8 is a direct partner of Spo11 in meiotic DNA break formation, independent of its cytoplasmic role in RNA metabolism. Mol Cell. 2004 Feb 27;13(4):549-59. doi: 10.1016/s1097-2765(04)00063-2. PMID: 14992724.

Arthur LM, Gustausson K, Hopfner KP, Carson CT, Stracker TH, Karcher A, Felton D, Weitzman MD, Tainer J, Carney JP. Structural and functional analysis of Mre11-3. Nucleic Acids Res. 2004 Mar 26;32(6):1886-93. doi: 10.1093/nar/gkh343. PMID: 15047855; PMCID: PMC390353.

Baharoglu Z, Mazel D. Vibrio cholerae triggers SOS and mutagenesis in response to a wide range of antibiotics: a route towards multiresistance. Antimicrob Agents Chemother. 2011 May;55(5):2438-41. doi: 10.1128/AAC.01549-10. Epub 2011 Feb 7. PMID: 21300836; PMCID: PMC3088271.

Bergerat, A., de Massy, B., Gadelle, D. *et al.* An atypical topoisomerase II from archaea with implications for meiotic recombination. *Nature* **386,** 414–417 (1997). https://doi.org/10.1038/386414a0

Bhalla N, Dernburg AF. Prelude to a division. Annu Rev Cell Dev Biol. 2008;24:397-424. doi: 10.1146/annurev.cellbio.23.090506.123245. PMID: 18597662; PMCID: PMC4435778.

Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y. The complete genome sequence of Escherichia coli K-12. Science. 1997 Sep 5;277(5331):1453-62. doi: 10.1126/science.277.5331.1453. PMID: 9278503.

Borde V, Lin W, Novikov E, Petrini JH, Lichten M, Nicolas A. Association of Mre11p with double-strand break sites during yeast meiosis. Mol Cell. 2004 Feb 13;13(3):389-401. doi: 10.1016/s1097-2765(04)00034-6. PMID: 14967146.

Borde V. The multiple roles of the Mre11 complex for meiotic recombination. Chromosome Res. 2007;15(5):551-63. doi: 10.1007/s10577-007-1147-9. PMID: 17674145.

Carpenter AT. Electron microscopy of meiosis in Drosophila melanogaster females: II. The recombination nodule--a recombination-associated structure at pachytene? Proc Natl Acad Sci U S A. 1975 Aug;72(8):3186-9. doi: 10.1073/pnas.72.8.3186. PMID: 810799; PMCID: PMC432946.

Ceccaldi R, Rondinelli B, D'Andrea AD. Repair Pathway Choices and Consequences at the Double-Strand Break. Trends Cell Biol. 2016 Jan;26(1):52-64. doi: 10.1016/j.tcb.2015.07.009. Epub 2015 Oct 1. PMID: 26437586; PMCID: PMC4862604.

Claeys Bouuaert, C., Tischfield, S.E., Pu, S. *et al.* Structural and functional characterization of the Spo11 core complex. *Nat Struct Mol Biol* **28,** 92–102 (2021). https://doi.org/10.1038/s41594-020-00534-w

Claeys Bouuaert, C., Pu, S., Wang, J. *et al.* DNA-driven condensation assembles the meiotic DNA break machinery. *Nature* **592,** 144–149 (2021). https://doi.org/10.1038/s41586-021-03374-w

S. Da Re & M.-C. Ploy (2012) *Antibiotiques et réponse SOS bactérienne.* Med Sci (Paris) 28:179–184.

Durfee T, Nelson R, Baldwin S, Plunkett G 3rd, Burland V, Mau B, Petrosino JF, Qin X, Muzny DM, Ayele M, Gibbs RA, Csörgo B, Pósfai G, Weinstock GM, Blattner FR. The complete genome sequence of Escherichia coli DH10B: insights into the biology of a laboratory workhorse. J Bacteriol. 2008 Apr;190(7):2597-606. doi: 10.1128/JB.01695-07. Epub 2008 Feb 1. PMID: 18245285; PMCID: PMC2293198.

Friedman N, Vardi S, Ronen M, Alon U, Stavans J. Precise temporal modulation in the response of the SOS DNA repair network in individual bacteria. PLoS Biol. 2005 Jul;3(7):e238. doi: 10.1371/journal.pbio.0030238. Epub 2005 Jun 21. PMID: 15954802; PMCID: PMC1151601.

Garcia V, Phelps SE, Gray S, Neale MJ. Bidirectional resection of DNA double-strand breaks by Mre11 and Exo1. Nature. 2011 Oct 16;479(7372):241-4. doi: 10.1038/nature10515. PMID: 22002605; PMCID: PMC3214165.

Gottlieb SF, Gulani A, Tegay DH. Genetics, Meiosis. 2021 Aug 11. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan–. PMID: 29494069.

Guzman LM, Belin D, Carson MJ, Beckwith J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol. 1995 Jul;177(14):4121-30. doi: 10.1128/jb.177.14.4121-4130.1995. PMID: 7608087; PMCID: PMC177145.

Heitman J, Model P. SOS induction as an in vivo assay of enzyme-DNA interactions. Gene. 1991 Jul 15;103(1):1-9. doi: 10.1016/0378-1119(91)90383-m. PMID: 1908806.

Henderson KA, Kee K, Maleki S, Santini PA, Keeney S. Cyclin-dependent kinase directly regulates initiation of meiotic recombination. Cell. 2006 Jun 30;125(7):1321-32. doi: 10.1016/j.cell.2006.04.039. PMID: 16814718; PMCID: PMC1950680.

Hopfner KP, Karcher A, Craig L, Woo TT, Carney JP, Tainer JA. Structural biochemistry and interaction architecture of the DNA double-strand break repair Mre11 nuclease and Rad50-ATPase. Cell. 2001 May 18;105(4):473-85. doi: 10.1016/s0092-8674(01)00335-x. PMID: 11371344.

Hunter N. Meiotic Recombination: The Essence of Heredity. Cold Spring Harb Perspect Biol. 2015 Oct 28;7(12):a016618. doi: 10.1101/cshperspect.a016618. PMID: 26511629; PMCID: PMC4665078.

Jiao K, Salem L, Malone R. Support for a meiotic recombination initiation complex: interactions among Rec102p, Rec104p, and Spo11p. *Mol Cell Biol*. 2003;23(16):5928-5938. doi:10.1128/MCB.23.16.5928-5938.2003

Johnson, D., Crawford, M., Cooper, T. *et al.* Concerted cutting by Spo11 illuminates meiotic DNA break mechanics. *Nature* **594,** 572–576 (2021). https://doi.org/10.1038/s41586-021-03389-3

Kee K, Protacio RU, Arora C, Keeney S. Spatial organization and dynamics of the association of Rec102 and Rec104 with meiotic chromosomes. *EMBO J*. 2004;23(8):1815-1824. doi:10.1038/sj.emboj.7600184

Keeney S, Giroux CN, Kleckner N. Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. Cell. 1997 Feb 7;88(3):375-84. doi: 10.1016/s0092-8674(00)81876-0. PMID: 9039264.

Keeney S. Mechanism and control of meiotic recombination initiation. Curr Top Dev Biol. 2001;52:1-53. doi:

10.1016/s0070-2153(01)52008-6. PMID: 11529427.

Keeney S. Spo11 and the Formation of DNA Double-Strand Breaks in Meiosis. Genome Dyn Stab. 2008 Jan 1;2:81-123. doi: 10.1007/7050_2007_026. PMID: 21927624; PMCID: PMC3172816.

Keeney S, Lange J, Mohibullah N. Self-organization of meiotic recombination initiation: general principles and molecular pathways. Annu Rev Genet. 2014;48:187-214. doi: 10.1146/annurev-genet-120213-092304. PMID: 25421598; PMCID: PMC4291115.

Kelley WL. Lex marks the spot: the virulent side of SOS and a closer look at the LexA regulon. Mol Microbiol. 2006 Dec;62(5):1228-38. doi: 10.1111/j.1365-2958.2006.05444.x. Epub 2006 Oct 17. PMID: 17042786.

Koehn DR, Haring SJ, Williams JM, Malone RE. Tethering recombination initiation proteins in Saccharomyces cerevisiae promotes double strand break formation. Genetics. 2009 Jun;182(2):447-58. doi: 10.1534/genetics.109.102640. Epub 2009 Mar 30. PMID: 19332879; PMCID: PMC2691754.

Kohanski MA, DePristo MA, Collins JJ. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Mol Cell. 2010 Feb 12;37(3):311-20. doi: 10.1016/j.molcel.2010.01.003. PMID: 20159551; PMCID: PMC2840266.

Lam I, Keeney S. Mechanism and regulation of meiotic recombination initiation. Cold Spring Harb Perspect Biol. 2014 Oct 16;7(1):a016634. doi: 10.1101/cshperspect.a016634. PMID: 25324213; PMCID: PMC4292169.

Lange J, Pan J, Cole F, Thelen MP, Jasin M, Keeney S. ATM controls meiotic double-strand-break formation. Nature. 2011 Oct 16;479(7372):237-40. doi: 10.1038/nature10508. PMID: 22002603; PMCID: PMC3213282.

Lee JH, Berger JM. Cell Cycle-Dependent Control and Roles of DNA Topoisomerase II. Genes (Basel). 2019 Oct 30;10(11):859. doi: 10.3390/genes10110859. PMID: 31671531; PMCID: PMC6896119.

Li J, Hooker GW, Roeder GS. Saccharomyces cerevisiae Mer2, Mei4 and Rec114 form a complex required for meiotic double-strand break formation. Genetics. 2006 Aug;173(4):1969-81. doi: 10.1534/genetics.106.058768. Epub 2006 Jun 18. PMID: 16783010; PMCID: PMC1569690.

Liu J, Wu TC, Lichten M. The location and structure of double-strand DNA breaks induced during yeast meiosis: evidence for a covalently linked DNA-protein intermediate. EMBO J. 1995 Sep 15;14(18):4599-608. PMID: 7556103; PMCID: PMC394552.

Lohe AR, Hartl DL. Autoregulation of mariner transposase activity by overproduction and dominant-negative complementation. Mol Biol Evol. 1996 Apr;13(4):549-55. doi: 10.1093/oxfordjournals.molbev.a025615. PMID: 8882498.

Martini E, Keeney S. Sex and the single (double-strand) break. Mol Cell. 2002 Apr;9(4):700-2. doi: 10.1016/s1097-2765(02)00512-9. PMID: 11983162.

Mátés L, Chuah MK, Belay E, Jerchow B, Manoj N, Acosta-Sanchez A, Grzela DP, Schmitt A, Becker K, Matrai J, Ma L, Samara-Kuko E, Gysemans C, Pryputniewicz D, Miskey C, Fletcher B, VandenDriessche T, Ivics Z, Izsvák Z. Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. Nat Genet. 2009 Jun;41(6):753-61. doi:

10.1038/ng.343. Epub 2009 May 3. PMID: 19412179.

Mehrotra S, McKim KS. Temporal analysis of meiotic DNA double-strand break formation and repair in Drosophila females. PLoS Genet. 2006 Nov 24;2(11):e200. doi: 10.1371/journal.pgen.0020200. Epub 2006 Oct 10. PMID: 17166055; PMCID: PMC1657055.

Meyer AJ, Segall-Shapiro TH, Glassey E, Zhang J, Voigt CA. Escherichia coli "Marionette" strains with 12 highly optimized small-molecule sensors. Nat Chem Biol. 2019 Feb;15(2):196-204. doi: 10.1038/s41589-018-0168-3. Epub 2018 Nov 26. PMID: 30478458.

Neale MJ, Pan J, Keeney S. Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. Nature. 2005 Aug 18;436(7053):1053-7. doi: 10.1038/nature03872. PMID: 16107854; PMCID: PMC1262668.

Norrander J, Kempe T, Messing J. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene. 1983 Dec;26(1):101-6. doi: 10.1016/0378-1119(83)90040-9. PMID: 6323249.

Ohkura H. Meiosis: an overview of key differences from mitosis. Cold Spring Harb Perspect Biol. 2015 Jan 20;7(5):a015859. doi: 10.1101/cshperspect.a015859. PMID: 25605710; PMCID: PMC4448623.

Page SL, Hawley RS. Chromosome choreography: the meiotic ballet. Science. 2003 Aug 8;301(5634):785-9. doi: 10.1126/science.1086605. PMID: 12907787.

Pan J, Sasaki M, Kniewel R, Murakami H, Blitzblau HG, Tischfield SE, Zhu X, Neale MJ, Jasin M, Socci ND, Hochwagen A, Keeney S. A hierarchical combination of factors shapes the genome-wide topography of yeast meiotic recombination initiation. Cell. 2011 Mar 4;144(5):719-31. doi: 10.1016/j.cell.2011.02.009. PMID: 21376234; PMCID: PMC3063416.

Perkins EL, Sterling JF, Hashem VI, Resnick MA. Yeast and human genes that affect the Escherichia coli SOS response. Proc Natl Acad Sci U S A. 1999 Mar 2;96(5):2204-9. doi: 10.1073/pnas.96.5.2204. PMID: 10051619; PMCID: PMC26761.

Petes TD. Meiotic recombination hot spots and cold spots. Nat Rev Genet. 2001 May;2(5):360-9. doi: 10.1038/35072078. PMID: 11331902.

Robert T, Nore A, Brun C, Maffre C, Crimi B, Bourbon HM, de Massy B. The TopoVIB-Like protein family is required for meiotic DNA double-strand break formation. Science. 2016 Feb 26;351(6276):943-9. doi: 10.1126/science.aad5309. Erratum in: Science. 2016 May 6;352(6286). pii: aaf9649. doi: 10.1126/science.aaf9649. PMID: 26917764.

Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 1990;185:60-89. doi: 10.1016/0076-6879(90)85008-c. PMID: 2199796.

Székvölgyi L, Nicolas A. From meiosis to postmeiotic events: homologous recombination is obligatory but flexible. FEBS J. 2010 Feb;277(3):571-89. doi: 10.1111/j.1742-4658.2009.07502.x. Epub 2009 Dec 15. PMID: 20015080.

Trémolières F. Quand le miracle antibiotique vire au cauchemar [When the antibiotic miracle turns into a nightmare]. Med Sci (Paris). 2010 Nov;26(11):925-9. French. doi: 10.1051/medsci/20102611925. PMID: 21106173.

Tsukamoto Y, Ikeda H. Double-strand break repair mediated by DNA end-joining. Genes Cells. 1998 Mar;3(3):135-44. doi: 10.1046/j.1365-2443.1998.00180.x. PMID: 9619626.

Tsukamoto Y, Mitsuoka C, Terasawa M, Ogawa H, Ogawa T. Xrs2p regulates Mre11p translocation to the nucleus and plays a role in telomere elongation and meiotic recombination. Mol Biol Cell. 2005 Feb;16(2):597-608. doi: 10.1091/mbc.e04-09-0782. Epub 2004 Nov 17. PMID: 15548595; PMCID: PMC545897.

Wendorff TJ, Berger JM. Topoisomerase VI senses and exploits both DNA crossings and bends to facilitate strand passage. Elife. 2018 Mar 29;7:e31724. doi: 10.7554/eLife.31724. PMID: 29595473; PMCID: PMC5922973.

Williams RS, Moncalian G, Williams JS, Yamada Y, Limbo O, Shin DS, Groocock LM, Cahill D, Hitomi C, Guenther G, Moiani D, Carney JP, Russell P, Tainer JA. Mre11 dimers coordinate DNA end bridging and nuclease processing in double-strand-break repair. Cell. 2008 Oct 3;135(1):97-109. doi: 10.1016/j.cell.2008.08.017. PMID: 18854158; PMCID: PMC2681233.

Yadav VK, Claeys Bouuaert C. Mechanism and Control of Meiotic DNA Double-Strand Break Formation in *S. cerevisiae*. Front Cell Dev Biol. 2021 Mar 2;9:642737. doi: 10.3389/fcell.2021.642737. PMID: 33748134; PMCID: PMC7968521.

Yusa K, Zhou L, Li MA, Bradley A, Craig NL. A hyperactive piggyBac transposase for mammalian applications. Proc Natl Acad Sci U S A. 2011 Jan 25;108(4):1531-6. doi: 10.1073/pnas.1008322108. Epub 2011 Jan 4. PMID: 21205896; PMCID: PMC3029773.

Zhang FG, Zhang RR, Gao JM. The organization, regulation, and biological functions of the synaptonemal complex. Asian J Androl. 2021 Nov-Dec;23(6):580-589. doi: 10.4103/aja202153. PMID: 34528517; PMCID: PMC8577265.

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