Part II- SKP integration into bacterial chromosome :

Introduction:

Our purpose was to produce antibodies through our transportable factory "The BioMaker Factory". As many eukaryotic proteins it depends a lot of various post-traductional modification and a precise tridimensional architecture. In this way, it's appears important for us to coexpress our recombinant protein with chaperones. However, WHO legislations demand a total safety of sanitary product exempt from any dangerous substance. So il was a necessity for us to avoid usage of antibiotics during cell growth. The direct insertion of chaperone gene within the bacterial genome appeared to be the best way to ensure a stable expression of additional chaperone proteins into the genome of our designed bacteria allowing a proper folding of eukaryotic therapeutical proteins.

Method:

To do so, we used pGRG25 integration vector obtained from Addgene in transformed DH5α *E. coli*. This vector was designed by McKenzie et al to insert DNA sequence in bacterial chromosome without inducing drug resistance of the host. This system uses Tn7 to insert transgenes at a defined neutral site in the chromosome (*attTn7*). The site is highly conserved and is known to work as a Tn7 attachment site in *E. coli* and its relatives. The *attTn7* sequence is conserved in most (all) bacteria.

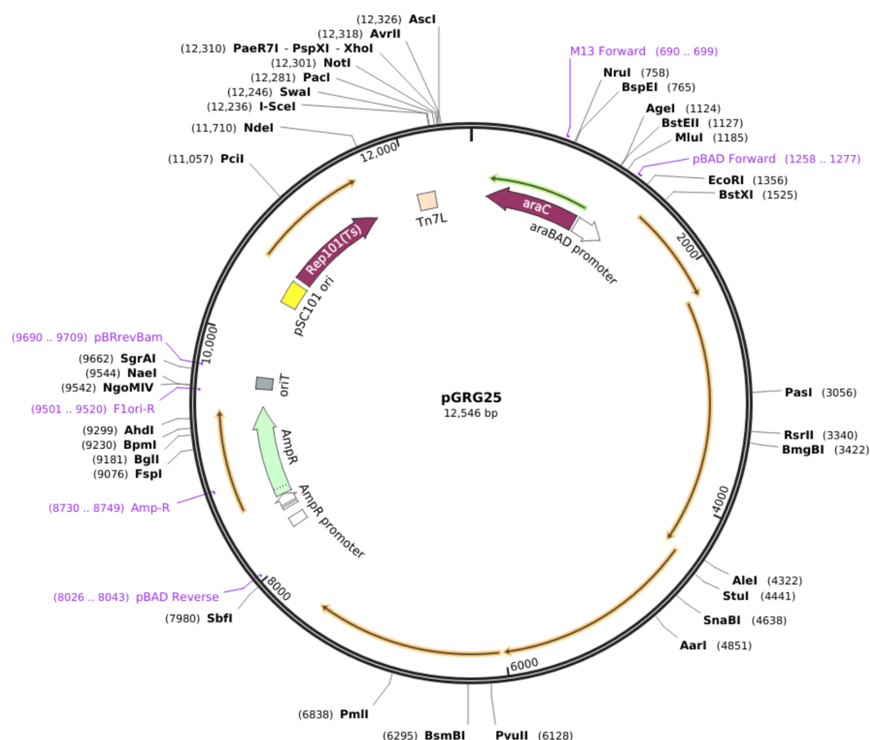

Figure 3: Schematic pGRG25 map

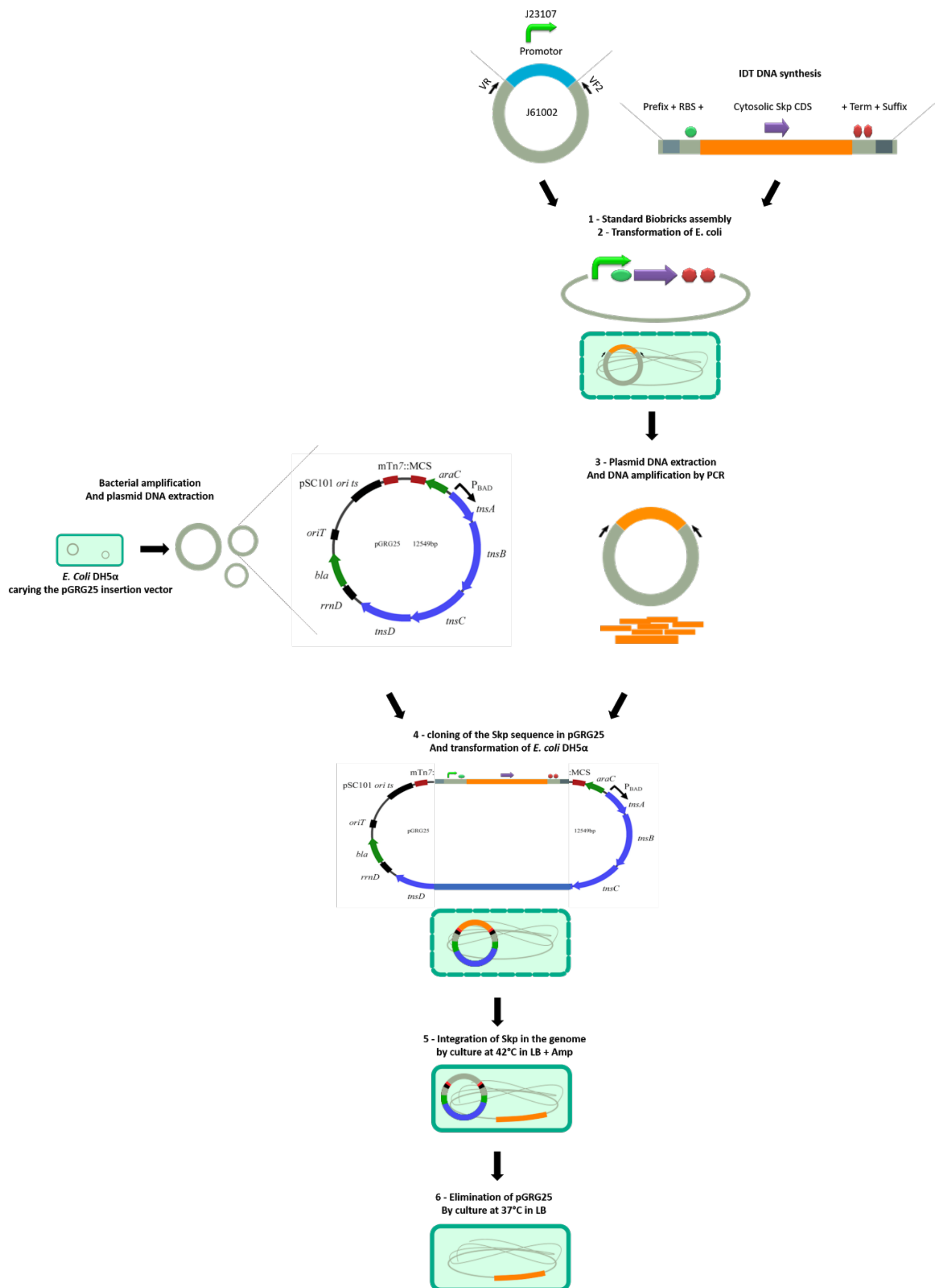

Figure 4 Schematic representation of our global strategy for the insertion of Skp within the bacterial genome of *E. coli* DH5α

<u>Construction of cytosolic Skp gene</u>

We had synthesized a cytosolic form of Skp under the control of T7 promoter and ended by a T7 terminator by IDT.

Name - SKP CHAPERONNE **Customer Name -** iGEM UPMC PARIS 2017 iGEM gBlocks® Gene Fragments 695 base pairs

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5'- GTT TCT TCG AAT TCG CGG CCG CTT CTA GAG TAA TAC GAC TCA CTA TAG GTC ACA CAG AAA TTC ATT AAA
GAG GAG AAA GGT ACC CCA TGG CTG ACA AAA TTG CAA TCG TCA ACA TGG GCA GCC TGT TCC AGC AGG TAG CGC
AGA AAA CCG GTG TTT CTA ACA CGC TGG AAA ATG AGT TCA AAG GCC GTG CCA GCG AAC TGC AAC GTA TGG AAA
CCG ATC TGC AAG CTA AAA TGA AAA AGC TGC AAT CCA TGA AAG CGG GCA GCG ATC GCA CTA AGC TGG AAA AAG
ACG TGA TGG CTC AGC GCC AGA CTT TTG CTC AGA AAG CGC AGG CTT TTG AGC AGG ATC GCG CAC GTC GTT CCA
ACG AAG AAC GCG GCA AAC TGG TTA CTC GTA TCC AGA CTG CTG TGA AAT CCG TTG CCA ACA GCC AGG ATA TCG
ATC TGG TTG TTG ATG CAA ACG CCG TTG CTT ACA ACA GCA GCG ATG TAA AAG ACA TCA CTG CCG ACG TAC TGA
AAC AGG TTA AAT AAG CGC TCC GCG CAG CAT TCC GCC GCC CGG TCC CGG TAT ACC GCG ACC GCT ATC CGG GAC
CAG GCA TCA AAT AAA ACG AAA GGC TCA GTC GAA AGA CTG GGC CTT TCG TTT TAT CTG TTG TTT GTC GGT GAA
CGC TCT CTA CTA GTA GCG GCC GCT GCA GGA AGA ACC CTA GGC TGC TGC CA -3'
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<u>Figure 5 : Sequence synthesized by IDT. Elements from 5’ to 3’ : iGEM Prefix, T7 promotor, RBS, cytosolic Skp CDS, T7 terminator, iGEM Suffix.</u>

We amplified by Polymerisation Chain Reaction the Skp CDS preceded by its RBS and ended by the T7 promoter with the following primers, introducing XbaI restriction site and SpeI NotI restrictions sites.
Primer forward : 5’GCGGCCGCTACTAGTATTATTTAACCTG3’
Primer Reverse : 5’CTTCTAGAGCCATGGCTGACAAAA3’
TM :67,3°C

We used the Biobrick Assembly kit to place Skp under the control of the promoter from the iGEM distribution kit 2017 BBa_J2310 we used this new construct to transformed *E. coli* competent DH5α.

<u>Cloning of Skp in pGRG25 :</u>

Culture of these strain was made at 30°C. Indeed, the plasmid backbone is the easily curable temperature sensitive mutant of pSC101, carrying the pSC101 temperature sensitive origin, which must be grown at 30-32°C to allow replication. So in order to extract plasmids, we inoculate these bacterial cells overnight in culture with ampicillin. We inserted the Skp sequence into the NotI restriction site located on the MCS of pGRG25 and then we transformed *E. coli* competent DH5α with the cloned vector.

<u>Integration of the cytoplasmic form of Skp in *E. coli* DH5α genome</u>

Cultures of the transformed cloned cells was made overnight without antibiotics at 32°C (this step allows for some loss of plasmid) and then at 42°C to block replication of the plasmid. Colonies were streaked once on LB at 42°C to ensure the complete loss of the plasmid and DNA integration into the genome.

After this step of integration and before characterization of the effect of SKP in protein synthesis we made verifications:
A simple checking was to pick the next day 3 colonies to put them in culture in LB+/- ampicillin.

Verification of the genomic insertion of cytosolic Skp

We check out the proper insertion of our Skp by performing a PCR on colonies allowing the specific amplification of cytosolic Skp with the following primers:

Primer forward : 5'GCGGCCGCTACTAGTATTATTTAACCTG3'
Primer Reverse : 5'CTTCTAGAGCCATGGCTGACAAAA3'
TM :67,3°C

Transposition was verified by the absence by PCR amplifying sequences which flank the *attTn7* site with the following primers.
Primer Foward 5'GATGCTGGTGGCGAAGCTGT3' and 5'GATGACGGTTTGTCACATGGA3'

Then, we purified DNA from PCR and we visualized PCR product on an agarose gel electrophoresis (1.5%).

Characterization of Skp insertion

In order to assess the effect related to the SKP chromosomique intégration, we transformed our engineered *E. coli* with our composite part BBa_I13500-BBa_I13507 created for the promoter caracterisation under the BBa_J231107 promoter and then we quantified the production of GFP and RFP.

Results

Verification of the genomic insertion of cytosolic Skp

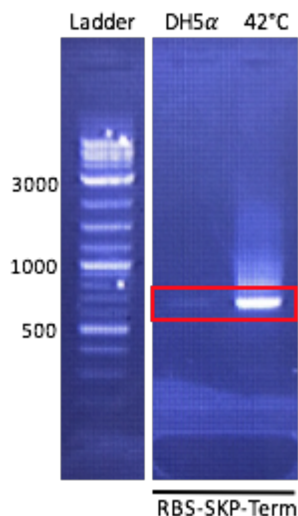

Figure 6 Agarose gel electrophoresis (1.5%) after migration of PCR products for the verification of the insertion of cytosolic Skp in the genome of *E. coli* DH5α
After amplification by polymerisation chain reaction of RBS-SKP-Terminateur sequence, which represent a fragment of 622pB we realized a comparison between non integrated DH5α cells and bacterial cells with DNA insertion (at 42°C). Here on these electrophoresis gel we can see that there is a band with a very high level of intensity and some SMIRS. We made a mistake

between preparation of PCR and used primers 10 times more concentrated but this observation confirm the fact that we can improve the level of SKP production directly into the genome.

Characterization of the SKP insertion:

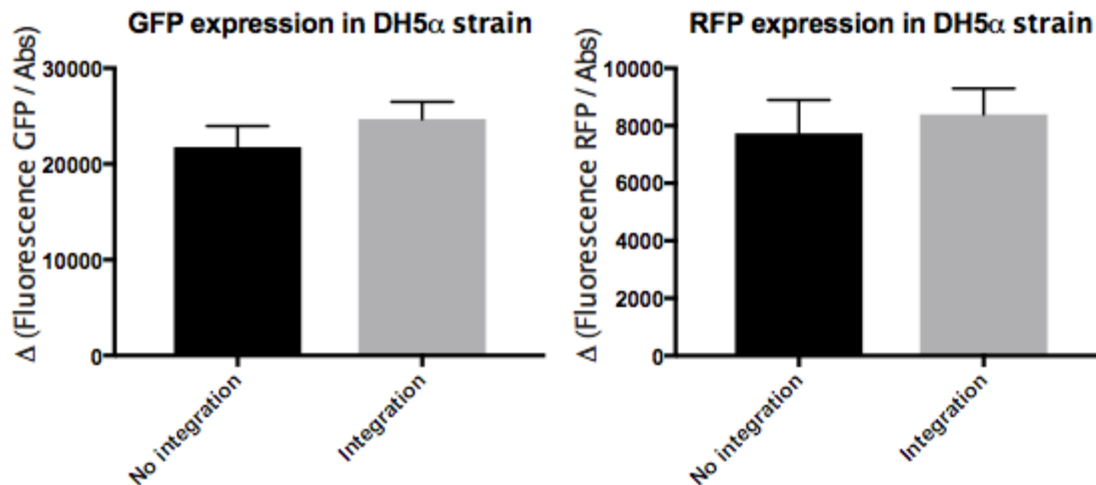

Figure 7 Quantification of GFP fluorescence in the context of SKP integration into the genome, under the control of Anderson promoters BBa_J23107.

Promoter strength was assessed through the quantification of specific fluorescence from GFP (A) and RFP (B) normalized with OD600 to obtain relative fluorescent units (RFU).

After transformation we compared the level of GFP and RFP expression between DH5α with and without SKP integration. Firstly, we can find with the same condition of transformation a same range of fluorescence for both reporters compared to the characterisation of Anderson's promoters. Here we can see that the level of GFP expression (around 20000U) is not significantly change when we introduce SKP into the genome, even if there is a very weak increase in the emission level of GFP and RFP. Our supposition is that GFP is a protein which is relatively simple, which don't need chaperon proteins or an high level of post traductional modifications, and we didn't had the time to realize other tests with more complex proteins