Construction of *Escherichia coli* strains for higher plasmid production: Evaluation of *lon* gene knockout and implementation of a marker-free system

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Plasmid DNA (pDNA) vectors offer considerable benefits over viral systems in gene-based therapy and vaccination applications, including low immunogenicity and toxicity, and simple manufacture in large scale. However, also shows low transfection efficacy, and requires milligram scale of pharmaceutical-grade pDNA per patient, which implies extensive production efforts. In order to fulfill high pDNA production requirements, genetically engineered strains specially designed to achieve high pDNA yields are required. With this purpose, a Lon-protease deficient GALG20 strain was created, in this study. However, contrary to expected, GALG20*lon* and GALG20 strains achieved similar volumetric and specific plasmid production yields. In order to fully evaluate *lon* gene mutation potential in pDNA production, large-scale cultivations using a fed-batch strategy, with reduced amounts of amino acids, and testing different temperature conditions should be performed.

Despite all the advantages of using pDNA, some safety concerns were raised due to possible integration of sequences from plasmid into cellular DNA and possible spread to intestinal bacteria. Moreover, the presence of such sequences in plasmid backbone was associated with diminished transfection efficiency and additional metabolic burden to the host during manufacture. In an attempt to address this problem, the other goal of this project was the transference of a marker free system from JM108*murselect* strain into GALG20, in order to achieve higher pDNA yields than the ones verified in JM108*murselect*. It was not possible to transfer the system to GALG20 probably because JM108*murselect* is recA deficient.

**Keywords:** Plasmid DNA, Antibiotic resistance gene, Strain engineering, Lon protease, DNA vaccine, Plasmid selection

1 **Introduction**

Plasmid DNA (pDNA) has attracted much attention, in the field of pharmaceutical biotechnology, for DNA vaccines and gene therapy (GT) applications [1], [2]. That has been reflected in the increasing number of patents, and clinical trials in both therapies, with four licensed DNA vaccines for veterinary use [3], [4], [5]. pDNA vectors offer considerable benefits over viral systems, including low immunogenicity and toxicity, simplicity of use, [1], [6], [7] higher shelf stability [8], [9], ease to manufacture [1], [10], [11], improved safety [1], [6] and ability to deliver large and complex genetic loads [12]. Due to the fact that the efficiency and duration of expression of current pDNA-based products is very limited, considerable amounts of pharmaceutical grade pDNA per patient are required (milligram-scale) [13], [14]. To meet the demand for pDNA, research dedicated to the establishment of efficient and cost-effective manufacturing processes capable of delivering high amounts of high-quality pDNA, has been developed [15]. One of the areas of focus is the creation of *Escherichia coli* strains specially designed to achieve high pDNA yields. The main targets are central carbon metabolism genes, to increase flux toward nucleotide and amino acid precursor synthesis and reduction of by products, such as acetate [10], [12]; pDNA quality related genes, and genes involved in other cellular processes relevant for pDNA production [18], [19].

GALG20 (MG1655ΔendAΔrecAΔpgi) is a high pDNA production strain, constructed starting from MG1655 by knocking out the *pgi* gene in order to redirect carbon flux to the pentose phosphate pathway, enhance nucleotide
synthesis, and, consequently, increase pDNA production [19]. endA and recA genes were knocked out to improve plasmid yield and quality, by preventing pDNA degradation [16], and improving plasmid segregation stability [20] respectively. This strain produced 25-fold more pDNA (19.1 mg/g dry cell weight, DCW) than its parental strain, MG1655ΔendAΔrecA (0.8 mg/g DCW), in glucose. The high productivity, and the wild-type genetic background that is characteristic of this strain, makes it a suitable candidate [19] for pDNA production in large scale.

Although much safer than viral approaches, DNA vectors generally do encode antibiotic resistance genes in the plasmid backbone [21], for selection and maintenance of recombinant plasmids [20], [22]. However, these genes are responsible for the main drawbacks in pDNA performance [6], as they cause safety problems due to possible integration of plasmid DNA into cellular DNA or spread of antibiotic resistance genes to intestinal bacteria by horizontal gene transfer [13], [23], [24]. Also, resistance genes may cause inflammatory reactions in vivo, hamper transfection efficiency by increased pDNA size and disturbing sequences [25], [6], and cause an additional metabolic load for the host during manufacturing [6], [20]. Therefore, strategies have been developed to avoid or at least decrease the presence of bacterially active elements and to use alternative selection strategies [6], to further improve their safety profile [13]. Mairhofer et al. [13], [26] developed an antibiotic-resistance free system using a minimized plasmid that is devoid of any additional sequence elements on the plasmid backbone, and merely consists of the target expression cassette and the bacterial origin of replication, ColE1 [13]. The selection mechanism is based on RNA/RNA antisense interaction involving the naturally occurring RNAI, derived from the origin of replication of the plasmid, ColE1, and achieved by functionally linking it to a repressor protein, encoded on the host genome, that further controls the expression of an essential gene, murA [116]. In plasmid-free cells, the repressor gene is transcribed and mRNA thereof is translated to the repressor protein, which inhibits transcription of the essential gene by blocking its operator, which leads to cell growth inhibition in plasmid-free cells. Otherwise, in plasmid containing cells RNAI is deduced from plasmid replication control and translation of the repressor is inhibited with high efficiency by anti-sense hybridization of the RNAI to the RNAII-like sequence (RLS) fused to the mRNA of the repressor, allowing the expression of the essential gene, thus leading to cell survival [13] (Figure 1). Using this system a 2-fold increase in specific pDNA content and productivity, in comparison with the same strain using antibiotic resistance as a selection mark, was attained [13]. This huge increase could have been due to the decrease in metabolic load, to the decrease of plasmid size or to the increase in pDNA replication rate, but could also have been due to the lon gene mutation found in the used strain, JM108 [13], [27].

Figure 1- Schematic overview on the RNA-based plasmid maintenance system [13].

lon gene [28] encodes a cytosolic [29] ATP-dependent protease [30], and plays important roles in maintaining cellular functions by selectively eliminating misfolded and damaged proteins [31], [32], [33], preventing aggregation [32]; and by participating in regulatory circuits, controlling the amount and availability of specific substrates [29], [34]. It was verified that lon gene is not essential for viability in E. coli [34], [35], however lon mutants accumulate abnormal proteins, form mucoid colonies [29], [35] and long filaments [34], [35]. E. coli lon mutants also fail to adapt rapidly to a nutritional downshift, because they are unable to degrade free ribosomal proteins which would normally generate an amino acids pool required for the synthesis of adaptive enzymes [29]. The lon gene mutation in JM108 cells, used in Mairhofer et al. study [13], may have influenced pDNA production, by causing the occurrence of uncharged t-RNAs in consequence of amino acid starvation. The uncharged tRNAs are able to interact with regulatory RNAs of the ColE1-ori, which suggests that the increase in specific pDNA content and
productivity using this system could have influenced by lon gene mutation [13].

Considering this, one of the goals of the present study is the transference of the marker-free system from JM108murselect into the high productivity strain GALG20 and quantifying the resulting plasmid DNA yields, in order to verify if in GALG20 the system achieve higher yields than JM108murselect. This study also aims to study the role of the lon gene in plasmid production and the possibility of using this mutation in the future as a target for cell engineering for plasmid production.

2 Materials and methods

2.1 Chemical and other reagents

For electrophoresis gel it was used Seakem LE agarose (Lonza ®), and the DNA ladder used was the 10 kb DNA Ladder III from Nzytech. The L-arabinosone (20%) solution was prepared from L-arabinose (Merck ®).

For phage P1 procedure were prepared the following solutions: 0.1 M calcium chloride (CaCl₂) (Merck ®), 0.1 M Sodium Citrate (Merck ®) and 10mM Magnesium sulphate (MgSO₄) (Merck ®). For cell cultivation studies, semi-defined medium was used, prepared from yeast extract (BD ®), Bacto Peptone (BD ®), (NH₄)₂SO₄ (Panreac ®), K₂HPO₄ (Panreac ®), KH₂PO₄ (Panreac ®), thiamine (Sigma Aldrich ®), MgSO₄ (Sigma Aldrich ®), Glucose (Panreac ®). The antibiotics used: Ampicillin and Kanamycin are from Calbiotech, while anhydrous-tetracycline and Chloramphenicol are from Sigma Aldrich.

2.2 Bacterial Strains

E. coli strain DH5α was used to replicate plasmids pKD13, pCP20, pkD46 and pVAX1-GFP. MG1655Δpgi was constructed by Geisa Gonçalves, former PhD student at IST. This strain was used as a starting point for the construction of the strains GALG20 and GALG20Δlon, during this study. endA gene deletion in MG1655Δpgi was performed resorting to phage P1 protocol, and using JW2912-1 [ΔendA720::kan], from Keio collection, as donor strain [36]. Phage lysate used to infect cells, in the attempts to construct GALG20murselectΔlon and GALG20murselectΔlon, was produced from E. coli K-12 strain JM108murselect [13], and was donated by Jürgen Mairhofer from the Department of Biotechnology at the University of Natural Resources and Applied Life Sciences (Vienna, Austria). Deletions of recA gene, in the constructed strains, was carried out with P1 transduction, using as donor the strain JW2669-1 [ΔrecA774::kan], from the Keio collection [36].

2.3 Plasmids

For gene knockouts, three plasmids were used: pKD13, pCP20 and pkD46. The pKD13 plasmid, carries kanamycin resistance gene flanked by FRT (FLP recognition target) sites [37], and is used to make an insertion cassette containing kanamycin resistance (Kanr) gene, surrounded by FRT sites. The pKD46 [38] plasmid comprises an Ampicillin resistance gene, the Red recombinase genes (β, γ, and exo) from phage I [39], and is temperature-sensitive. Plasmid pCP20 carries Chloramphenicol and ampicillin-resistance genes and carries the FLP recombinase. This plasmid shows temperature-sensitive replication and thermal induction of FLP synthesis [39]. It is used for the removal of the kanamycin cassette [39]. These constructions were performed at MIT and provided by Geisa Gonçalves, former PhD student at IST [19]. pVAX1-GFP was transformed into GALG20Δlon and GALG20 strains, to explore their plasmid production potential. This plasmid contains a kanamycin resistance gene for selection in E. coli, and a pMB1 origin (pUC-derived) [12].

These plasmids were replicated in cells grown until middle exponential phase, according to the conditions described in section 2.4 and then harvested by centrifugation (3200g, 10 min, 4°C). The purification was made using the High Pure Plasmid Isolation Kit (Roche®). The plasmids were eluted in 100 µL of elution buffer (Tris-HCl Buffer, pH 8.5). Purified plasmids were used directly after purification or stored at -20°C. The plasmid concentration was determined using Nanodrop Spectrophotometer (Nanovue Plus, GE Healthcare®), and plasmid quality was assessed by gel electrophoresis (agarose 1% in TAE buffer 1x).

2.4 Growth Media and conditions

E. coli DH5α cells were grown in liquid LB medium (Luria Broth, 20 g/L), from Sigma Aldrich, for plasmid replication. DH5α cells harboring pKD13 were grown in medium supplemented with 30 µg/mL of kanamycin at 37°C and 250 rpm, cells with pKD46 were grown in 100 µg/mL of ampicillin at 30°C and 250 rpm, whereas cells with pCP20 were grown in 50 µg/mL of Chloramphenicol at 30°C and 250 rpm. For gene knockout and phage P1 procedures E. coli strains were also grown in LB medium with the appropriate antibiotic, at 37°C and 250 rpm, except when specified otherwise.

When necessary, cells were grown in LB agar (2%) (Sigma Aldrich) plates, with the appropriate antibiotic, at 37°C, and then stored at 4°C. Mutants were stored at -80°C in glycerol 15% (v/v) final concentration.

2.5 Gene knockouts

lon gene Knockout

lon gene knockout was performed using Datsenko and Wanner method [39]. The basic strategy is to replace a chromosomal sequence with a cassette containing a selectable antibiotic resistance gene that is generated by PCR, by using hybrid primers with homology extensions (H1 and H2) to regions adjacent to the gene to be inactivated and to the template plasmid carrying antibiotic resistance gene that is flanked by
FRT (FLP recognition target). The replacement of the chromosomal sequence is accomplished by Red-mediated recombination in the flanking homologies H1 and H2. After selection, the resistance gene can also be eliminated by using a helper plasmid expressing the FLP recombinase, which acts on the directly repeated FRT sites flanking the resistance gene [39].

The first step is to make the linear insertion cassette. The insertion cassette is composed by the selectable marker for kanR, surrounded by FRT sites, to facilitate later excision of the cassette, and regions adjacent to the gene to be inactivated. PCR amplification was used to construct this cassette. The DNA sequence of the lon gene was consulted in the EcoCyc database [27] and the drug resistance cassette used was the kanamycin gene from plasmid pKD13 [37]. The primers used contained a homology sequence for lon gene, and a priming site coincident with the beginning/end of the Kanamycin resistance gene (Table 1).

### Table 1 - Primers used in KanR cassette generation for lon gene knockout

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>bp</th>
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<tr>
<td>F</td>
<td>GACGTACATGTAAAATAGATGGCTG AAGCACAGTCGGTGCA TCTGATTACCTGGCCGGAAAAGtgagctgt ggaagctcttc</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>GCACCTGAATCTTCAAGTACCGAA CGGCAGACGTTATAT CAGGCCAGCACCTCCCTATcgcgcagctgcagttctgcagtt</td>
<td>77</td>
</tr>
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</table>

After designing the primers, a PCR with the chimeric primers and the plasmid pKD13 was performed, in order to make the linear recombination cassette. Latter, the PCR product, was run in a 1% agarose gel with molecular weight markers (Ladder III from Nzytech) to confirm its size. If the size was in agreement with the expected, the PCR product was extracted, to make purified extract, with the QIAquick Gel Extraction Kit (QIAGEN®).

Then, the linear drug-resistant cassette made by PCR was transformed into recombination-competent cells (E.coli K-12 MG1655ΔendAΔpgi), by electroporation. Previously, the host strain had been, transformed with the plasmid pKD46, in order to express a RED recombinase, that enables the integration of the cassette (removing wild-type sequence between the regions of homology). To induce recombination, cells had to be grown in LB supplemented with L-arabinose, before electrotropansformation with the cassette. LB plates with kanamycin (30 μg/mL) were used to select successful recombinants and colony PCR was used to verify integration in the correct location.

In case of integration in the right location, verified by agarose gel electrophoresis, positive clones were transformed with pCP20, and grown at 43°C to induce FLP recombinase that removes the kanR cassette and cure all helper plasmids used. After this step, the selected colony were re-streaked on LB plates supplemented with ampicillin, kanamycin and chloramphenicol; and grown at 30°C overnight to confirm that all helper plasmids and kanR cassette were fully cured. In the end, a second colony PCR to the region surrounding the modification should be performed to ensure the entire kanR cassette removal.

### Knockout of endA and recA genes by P1 transduction

The endA knockout in MG1655Δpgi and the recA knockout in MG1655ΔpgiΔendAΔlon and in MG1655ΔpgiΔendA were performed using P1 transduction protocol. In order to do that, JW2912-1 [ΔendA720::kan] and JW2669-1 [ΔrecA774::kan] strains from Keio collection [36], were used as donor strains for endA and recA gene knockout, respectively. The donor strains contain a kanR cassette disrupting endA and recA genes, providing selectable markers for the knockout of the genes. recA was the last gene to be deleted, once RecA Recombinase is required for the homologous recombination needed to integrate the donor DNA [40]. P1 transduction protocol can be divided in 3 important stages: preparation of donor phage stock, preparation of recipient cells and transduction. For the preparation of donor phage stock, 30 μL of donor cells grown in LB supplemented with (30 μg/mL) kanamycin (37°C, overnight), were used to inoculate 5 mL LB medium supplemented with 0.1% glucose, 250 μL of CaCl2 and kanamycin. After incubation at 37°C until OD600 = 0.15-0.25, 5 - 10 μL of P1 phage stock (ATCC #25404-B1) were added. The donor cells continued incubating with phage P1, overnight. During that time, phage should infect and lysate donor cells, leading to clear up of the culture medium. Then, 100 μL of chloroform were added, followed by vortex of the culture for 1 minute, to mix. Chloroform function is to kill the remaining live donor cells as well as help in the precipitation of cell debris. Having in mind that the phage should be in the aqueous phase of the culture, 1 mL of this phase was recovered and centrifuged at maximum speed for 2 minutes. Again, the supernatant was recovered and 50 μL of chloroform were added, followed by vortex, to dissolve chloroform into the aqueous phase. Another centrifugation at maximum speed for 2 minutes was performed. At this stage the supernatant is mainly composed by the donor phage stock that was later used to infect recipient cells.

For the preparation of recipient cells, 5mL of culture media, where cells were grown in LB at 37°C, overnight, were inoculated in 50 mL of LB, and incubated at 37°C until the OD600 was approximately 0.8-1.2, which corresponds to mid-exponential phase cells. At that time cells were centrifuged at 3200 g, 4°C during 15 minutes. After discarding the supernatant, cell pellet was resuspended in 300 μL of 10 mM MgSO4 and 0.1 M CaCl2, and then placed in ice (4°C) in 100 μL aliquots. This procedure was performed simultaneously with the preparation of donor phage stock.

The last step is the transduction, where the recipient cells were infected with donor phage stock. Recipient cells were incubated
at 30°C for 30 min after the addition of 0, 30, 50, 70 and 120 μL of donor phage stock. During this time, P1 phage adsorbed and infected the recipient cells. The sample with 0 μL of phage was used as control. To stop the infection process 100 μL of 0.1 M sodium citrate (pH 7.0) were added to each aliquot. Then cells were centrifuged at 5200 g for 1 min and the supernatant was discarded. Cell pellet was resuspended in 100 μL of 0.1 M sodium citrate, again centrifuged at 5200 g for 1 min, and the supernatant was discarded. One more sodium citrate wash was performed, before the last resuspension of the pellet in 100 μL of 0.1 M sodium citrate, followed by plating of the entire mixture on LB plates with kanamycin. Donor phage stock was directly plated on LB plate with Kanamycin, as a negative control. Plates were incubated at 37°C overnight, and in the next day colonies were re-streaked from the non-control plates onto fresh plates because the original plates were covered with bacteriophage. Confirmation of Kanamycin resistance cassette insertion in place of the gene was performed by colony PCR.

In case of kanR gene integration in the right location, positive clones were transformed with pCP20 to remove the kanR cassette. The selection and kanR cassette removal was performed in the same way described in section 2.5.1, after pCP20 transformation.

2.6 Transference of murselect trait by phage P1 transduction

In order to insert the murselect trait [26], [13] in MG1655ΔpgiΔendA and MG1655ΔpgiΔendAΔlon the phage P1 transduction method was used. The protocol followed was similar to the one used for knockout of endA and recA gene, described in section 2.5.2. However the donor phage stock preparation step, was not performed, once the P1 lysate carrying the murselect trait was sent from DBT, Austria. Therefore, the protocol was initiated in the preparation of recipient cells step. In this system after transduction, and sodium citrate washes the selection was made by incubation in plates containing Chloramphenicol (10 μg/mL) and anhydro-tetracycline (20 ng/μL).

2.7 Shake flask cultivation

For the cell cultivation studies, seed banks of GALG20Δlon and GALG20 transformed with pVAX1-GFP were used to inoculate semi-defined medium with Glucose, 20 g/L [19]; trace element solution, 1 mL/L [41]) and supplemented with 30 μg/mL of kanamycin. Cells were grown overnight at 37°C and 250 rpm, and then, used to inoculate batch cultures to an OD₆₀₀nm of 0.1. Cultures were grown in shake flasks with 50 mL of semi-defined medium with initial pH of 7.1, 37°C and agitation of 250 rpm. Cell cultures were grown during 24 hour, and monitored on an hourly basis for the first 12 hours and at the end of the 24 hours, for optical density measurements. At 12 hours were collected samples for plasmid DNA quantification. Cells were recovered and then plasmid was purified using High Pure Plasmid Isolation Kit (Roche®). Plasmid concentration was determined using Nanodrop Spectrophotometer, as described in more detail in Section 2.3.

3 Results and discussion

3.1 endA gene knockout by phage P1 transduction

In order to construct the desired strains: GALG20, GALG20Δlon, GALG20murselect and, GALG20murselect Δlon, the strain MG1655Δpgi was used as a starting point. endA gene knockout was performed by phage P1 transduction, as described in section 2.5.2. After infection of recipient cells, MG1655Δpgi, with phage donor stock, prepared from JW2912-1 [ΔendA720::kan] strain, a colony PCR was used to confirm the insertion of the kanR cassette in place of the gene. All colonies tested have shown a negative result for the insertion, or a double result exhibiting both the cassette inserted and the endA gene. It was decided to proceed to the next step of cassette removal, with a colony with double results. After pCP20 transformation, followed by selection, a second colony PCR was performed to verify cassette removal. When the gene is present the amplicon is expected to have 959 bp, in case of cassette insertion is expected to have 1510 bp, and finally a 350 bp PCR product in case of cassette removal. At the same time, the same colonies tested for endA gene knockout, were tested for pgI gene removal to assure that the colonies used to construct the desired strains carry the expected knockouts (Figure 2).

Figure 2- Agarose gel showing the colony PCR result for the kanR cassette removal for pgI gene (A), and for the endA gene (B). First lane corresponds to Ladder III, and the lanes A1, A2, A3 and A4 correspond to the test for endA gene, and lanes B1, B2 and B3 correspond to the test for pgI gene. Lanes with same number correspond to the same colony.

The three colonies tested (B1, B2 and B3) show a band with approximately 350 bp, which corresponds to the scar left by the removal of the cassette with the drug resistance gene. The concern in the previous step related to the presence of the endA gene and the kanR cassette at the same time, can be surpassed, once after the FLP
recombinase-mediated recombination step, a single band was obtained, with the expected result. Lanes A1, A2, A3 and A4 correspond to the result of the colony PCR used to test for lon gene knockout. The product of amplification appears between the 200 and 400 bp DNA-ladder bands, corresponding to the scar left by the removal of the cassette (approximately 300 bp).

As it shown in Figure 2, all of the tested colonies hold both knockouts, and the colony used for the next steps was colony 1, that was chosen arbitrarily among these colonies.

### 3.2 lon gene knockout by Datsenko and Wanner method

For lon gene knockout the Datsenko and Wanner method [39], was adapted (Section 2.5.1). The first step involves the PCR generation of a 1414 bp sequence composed by the homology regions to lon gene, the priming sites and the FRT regions in each extremity of the kanR gene. This sequence replaces the lon gene by Red-mediated recombination. After PCR, the amplification of the cassette was verified in an agarose gel electrophoresis, and the fragment extracted from the gel, without UV or ethidium bromide exposure, was then purified.

After purification, the cassette was transformed into strain MG1655ΔpgiΔendA harboring pKD46 plasmid, expressing Red-recombinase. To check for the insertion of the kanR cassette, a colony PCR was performed. Colony, where kanR cassette insertion was verified, was transformed with pCP20 to remove the cassette. The colony PCR used to test for the lon gene knockout is represented in Figure 3.

The product of amplification should be a 2727 bp fragment, in case of lon gene amplification. In case of kanR cassette insertion the amplicon should have a size of 1526 bp. Finally, if the drug resistance cassette was removed, the size of the scar should be 303 bp PCR product, which corresponds to the expected outcome at this stage.

All the colonies tested seem to have lost the kanR cassette, as desired, with exception of colony represented in lane 3 that appears to have a low intensity band around the expected size for the kanR cassette, as can be seen in Figure 3. Colony represented in lane 1 was chosen to proceed for the next knockouts, but before that, the sequence amplified was sent for sequencing to STABvida. The result was positive for the gene knockout, as the colony PCR had shown.

### 3.3 recA gene knockout by phage P1 transduction

This projects intends to create the strains GALG20 (MG1655ΔpgiΔendAΔrecA) and GALG20Δlon. recA gene knockout should be the last to be performed, once it codes for a DNA strand exchange and recombination protein with protease and nuclease activity, in his absence, the recombination efficiency is severely diminished. The gene knockout was performed simultaneously in both strains.

**recA gene knockout in MG1655ΔendAΔpgi**

For the construction of the GALG20 strain, the cells used as recipient cells were MG1655 ΔpgiΔendA, specifically the cells stored at -80°C from colony correspondent to lane 1 in Figure 2.

After phage stock infection, the recipient cells were tested for the insertion of the kanR cassette. The colony PCR revealed colonies with two bands, coincident with the size of recA gene amplification and kanR cassette insertion. After several attempts, it was not possible to isolate colonies displaying only the kanR cassette insertion. Thus, it was decided to follow to next step of kanR cassette removal, and according to the result decide if it was necessary to return to this step, or if the strain was complete.

After transformation with pCP20, another colony PCR was performed in order to verify if the kanR cassette was removed. The six colonies tested did not growth in any antibiotic, after incubation in LB at 43 °C, and the agarose gel analysis is shown in Figure 4.

![Figure 3- Agarose gel obtained from the colony PCR used to verify the removal of the kanamycin cassette from the lon gene locus. In the first lane is Ladder III and the following lanes correspond to different colonies analyzed.](image-url)
All lanes exhibit a pronounced band around 1000 bp DNA ladder band, considering that the desired result would be a 978 bp product of amplification, the removal of the cassette was well achieved. The kanamycin cassette removal from recA locus originates strain MG1655 ΔpgiΔendAΔrecA, which corresponds to GALG20 genotype. This means that, at this time, one of the goal strains of this work was constructed.

3.4 murselect trait insertion

Considering the previous highlighted disadvantages of antibiotic resistance genes, in plasmid backbone, and also the benefits of using the marker free system, provided by the murselect trait described in Mairhofer et al. study [13], the construction of GALG20murselect and GALG20murselectΔlon were also goals of this project. For the construction of these last two strains, murselect trait (cassette with marker free system) has to be inserted in MG1655ΔpgiΔendA, and in MG1655ΔpgiΔendAΔlon, once recA knockout should be the last knockout to be performed, as explained above.

After infection of recipient cells MG1655ΔpgiΔendA, and MG1655ΔpgiΔendAΔlon with phage stock, and subsequent selection for cells containing murselect cassette inserted on the chromosome, colonies were tested for the insertion of the murselect trait in murA locus, by colony PCR. However, all colonies tested resulted negative for murselect trait insertion, despite all efforts. The fact that JM108murselect is a recA minus strain was pointed as one of the possible reasons for the unsuccessful insertion of the cassette, once phage P1 replicate poorly in recA minus strains. In addition, the large size of the murselect trait can be an obstacle to recombination.

Considering that was not possible to confirm the murselect trait insertion, it was not possible to proceed for the GALG20murselect and GALG20murselectΔlon strains construction. As well, the impact of the marker free system implementation in GALG20 in plasmid production was not accessed.

3.5 Cultivation studies in shake flask

After the construction of GALG20 and GAG20Δlon strains, cells were transformed with pVAX1-GFP plasmid and then
grown in shake flask, to explore the potential of the lon gene knockout strain GALG20 for plasmid DNA production and cell growth rate, in comparison to GALG20. Shake flask cultivations were performed in a batch fermentation strategy.

GALG20 and GALG20Δlon, in shake flask cultivations, during 24 hour, in semi-defined medium supplemented with 20 g/L of glucose, show similar growth curves. However, GALG20 growth rate (1.07 ± 0.06 h⁻¹) is higher than GALG20Δlon (0.94 ± 0.05 h⁻¹). The slight reduced growth rate in GALG20Δlon in comparison with GALG20 can be explained by the adverse effects that a lon mutation can cause in cells. In GALG20Δlon, lon mutation prevents the cellular response to amino acid starvation, making hard for the cells to adapt in the end of the exponential phase, when nutrients become scarce. In addition, Lon protease is involved in several other cellular responses, so its absence may cause imbalance in some cellular pathways, delaying growth rate [29], [34].

The growth rate achieved by GALG20 in this study is lower than previously reported. Gonçalves et al. reported a growth rate of 0.77 h⁻¹. Such difference was not expected, once the cultivation conditions were the same. The use of different lots or different brands of some products used in media composition may have influenced growth rate. In addition, small differences in growth conditions may have a big impact in growth rate, like dissolved oxygen or pH that are difficult to control in shake flask cultivation [42].

Plasmid DNA production

In order to compare the plDNA production potential of GALG20Δlon with GALG20, at hour 12 of shake flask cultivation, samples were collected. Cells were recovered and the plasmid was purified. Both the volumetric and specific yield were similar in both strains.

Table 2 - Comparison of plasmid DNA specific and volumetric yield between GALG20 and GALG20Δlon. Samples were collected at 12 hours of shake flask cultivation at 37°C, in semi-defined media supplemented with 20 g/L of glucose.

<table>
<thead>
<tr>
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<th>GALG20</th>
<th>GALG20Δlon</th>
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<tr>
<td><strong>Volumetric</strong></td>
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<td></td>
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<tr>
<td><strong>yield (mg/L)</strong></td>
<td>16.8 ± 4.79</td>
<td>17.3 ± 3.77</td>
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<tr>
<td><strong>Specific Yield</strong></td>
<td></td>
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<tr>
<td>(mg/g DCW)</td>
<td>5.9 ± 2.11</td>
<td>6.1 ± 1.54</td>
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</table>

It would be expected a higher plasmid yield in GALG20Δlon in comparison to GALG20, due to the occurrence of uncharged-tRNAs in Lon deficient cells, as consequence of amino acid starvation, that are able to interact with regulatory RNAs of the origin of replication of pDNA. However, that was not verified, most likely because cellular response to amino acid starvation was not triggered, or at least not in a scale sufficient for the effects to be influencing the plasmid DNA yield. Considering the lack of reproducibility of shake flask experiments, large-scale experiments using a fed-batch strategy should be performed. This way the effect of Lon deficiency in plasmid production would be more accurately evaluated, as well as the influences of this mutation in growth rate. Also, could be of interest perform GALG20Δlon cultivation experiments using a calculated amount of amino acids in a way that could trigger cellular response to amino acid starvation, without impairing cell viability. Lon protease is involved in heat shock response too [35], so, experiments testing different temperatures could be of interest.

Gonçalves et al. [19] reported a specific yield of 19.1 mg/g DCW, and a volumetric yield of 140.8 mg/L, in GALG20 strain, in media supplemented with 20 g/L of glucose. The imparity between the yields obtained by Gonçalves et al. and the ones obtained in this study are probably related to the method used for recovery and purification of the plasmid, and with the quantification method. The method used by Gonçalves et al. uses the crude lysates, which means that less plasmid is loss.

Some of the factors previously suggested to explain the different growth rate between the two studies, also apply to the plasmid production yields, like the lot-to-lot variations, and different brands of some of the compounds used in the cultivation medium. In addition, parameters like pH and dissolved oxygen are difficult to control in shake flask cultivations, which reduces the reproducibility of cultivations.

Despite the dissemblance between both studies, the results obtained in this study show consistency among them, which allows the comparison between GALG20 and GALG20Δlon.

4 Conclusions and Future work

In this study a Lon-deficient GALG20 strain was created to assess the effect of lon gene knockout in plasmid production. The results obtained were compared with the GALG20 reference strain. It was verified that GALG20Δlon strain growth rate was lower than the GALG20.
Considering the important roles played by Lon protease in several cellular responses [29], the decreased growth rate displayed by the lon gene mutated strain is in accordance with the expected results.

Regarding plasmid DNA production GALG20Δlon shown similar specific and volumetric yields in comparison to GALG20. The expected result would be a higher plasmid DNA production in GALG20Δlon, resulting from the interaction between uncharged-tRNAs and the origin of replication of the plasmid. However, in order to fully evaluate the lon gene mutation potential in pDNA production, large-scale cultivations using a fed-batch strategy, with reduced amounts of amino acids, and testing different temperature conditions should be performed.

The second goal of this study was the transference of the marker-free system developed by Mairhofer et al. [13] from JM108murselect strain into the high productivity strain GALG20. However, this goal was not achieved despite all attempts, probably due to the fact that JM108murselect strain (used as donor strain in phage P1 protocol for cassette insertion) is recA minus, and phage P1 replicate poorly in these strains. In order to surpass this problem it could be of use repeat the procedure using a JM108murselect strain harboring a plasmid encoding recA gene, to ease the recombination step. Given that the marker free system developed by Mairhofer et al. [6], [13] shows so many advantages in comparison to the use of antibiotic resistance genes, and to the existing alternative systems, for plasmid containing cells selection, more efforts should be taken to implement this system in a high yield plasmid production strain combined with an optimized fermentation strategy.

New methods combining high plasmid DNA yield with antibiotic-free maintenance systems are urgent. The interest in these systems has increased in last few years, as the disadvantages of antibiotic resistance genes were pointed out, and as the advantages of these systems were highlighted. However more studies, and more interest from companies and researchers to implement these emerging technologies are necessary.

5 References


