**Increasing plasmid copy number of pTRKH3 in *Lactococcus lactis* for biopharmaceutical-grade pDNA production**

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**Highlights**

* pTRKH3-b reached 215 copies in *L. lactis*, a 3.5-fold increase from the wild-type.
* pTRKH3-b had one additional RBS sequence and two additional start codons.
* pTRKH3-b encodes a mRNA secondary structure with the most negative ΔG value.
* pTRKH3-b showed an intermediate amount of transcriptional repressors.

**1. Introduction**

Pharmaceutical-grade plasmid DNA (pDNA) production in high quantities and in a cost-effective manner is a key point for several biotechnological and pharmaceutical applications, such as the production of DNA vaccines1 and recombinant proteins and also for the use of food-grade bacteria in live mucosal vaccination.

Traditionally, pDNA is produced in *Escherichia coli*, but the use of this host shows a major drawback because lipopolysaccharides (LPS) may co-purify with pDNA, which raises several safety concerns and increase the manufacture cost2.

Lactic acid bacteria (LAB), particularly *Lactococcus lactis* are a safer alternative host for pDNA manufacturing since they are naturally LPS-free and have a food-grade and Generally Recognized As Safe status3. In order to establish a cost effective *L. lactis*-based pDNA manufacturing platform it is imperative to engineer new high-copy number plasmids, since the highest copy number reported for the commonly used pAMβ1 replicon is around 100 copies, which is much lower than the 500-700 copies reported for *E. coli* pUC vectors4.

**2. Methods**

In order to achieve different plasmid copy number (PCNs) in *L. lactis* LMG19460 cells, the RBS sequence of the *repDE* promoter of the pAMβ1 replication origin of the plasmid pTRKH3 was modified by site-directed mutagenesis.

*L. lactis* LMG19460 cells harbouring the non-modified pTRKH3 and the plasmids with the designed mutations were grown under previously optimized conditions. The PCN per cell was determined by real-time quantitative PCR and the data was analysed using a relative quantification method5. The translation initiation rates and consequently the predicted protein expression levels of each designed RBS sequences were evaluated using the RBS Calculator (https://salislab.net/software/, Salis Lab, Penn State University)6,7. The mRNA of the *repD* gene, along with the mRNA of its transcriptional repressors (*copF* and *CT-RNA*), were quantified by real-time qPCR, using the same method described above.

**3. Results and discussion**

The specific growth rates of cells containing the modified plasmids were not significantly (p<0.05) affected, suggesting that the modifications in the *repDE* RBS sequence of pTRKH3 do not influence negatively the overall cell metabolism.

With the exception of pTRKH3-a and pTRKH3-e, the average PCN of the modified plasmids at 10.5h of growth (i.e. late exponential/early stationary phase) were statistically higher (p<0.05 for pTRKH3-c and p<0.01 for pTRKH3-b, pTRKH3-d and pTRKH3-f) than the PCN obtained for the wild-type pTRKH3. The highest average PCN value was obtained for pTRKH3-b (215±38), representing a 3.5 fold increase over the wild-type plasmid (62±5) and much higher than the maximum of ~100 copies obtained for the pAMβ1 replicon in previous studies.

The pTRKH3-b plasmid has neither the most similar RBS to the 16S rRNA sequence nor the strongest RBS predicted by the Anderson library, but instead it was detected an unexpected 77 bp insertion, which led to the creation of an additional RBS sequence and two additional relevant start codons. Without that insertion, the mutant lost the ability to replicate with such a high PCN.

The *in silico* mRNA secondary structures analysis, using RNA folding software tools, predicted the pTRKH3-b mutant as the one having the most negative ΔG value, when compared with the remaining mutants and the parental plasmid, increasing the likelihood that the ribosome will bind to the mRNA and initiate translation of the replication initiation protein, leading ultimately to an increase in the PCN.

A principal component analysis performed with the mRNA quantification results showed that pTRKH3-b had an intermediate amount of CopF and CT-RNA repressors, which seems to allow the perfect balance between transcription initiation and its repression.

**4. Conclusions**

pTRKH3-b is a very promising high copy number shuttle plasmid that will contribute to bring LAB to the same level as *E. coli* as pDNA producers, with the additional advantage of being endotoxin free, which turns the process more profitable.

The main possible applications for this new plasmid is to use *L. lactis* as efficient producers of pharmaceutical-grade DNA vaccines and recombinant proteins, and as live mucosal vaccination vectors.

Recently, an effort is being made in our research group in order to engineer knockout *L. lactis* strains that could improve even more the quality and quantity of the pDNA produced.

**References**

1. E.F. Fynan, S. Lu, H.L. Robinson, *Hum. Gene Ther.* *29*(9) (2018) 966–970.
2. D. Dixon, R. Darveau, *J. Dent. Res.* *84*(7) (2005) 584–595.
3. G. Gram, A. Fomsgaard, M. Thorn, S. Madsen, J. Glenting, *Genet. Vaccines Ther.* *5(*3) (2007).
4. J. Sambrook, E.F. Fritsch, T. Maniatis,Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, Plainview, NY, 1989.
5. M. Skulj, V. Okrslar, S. Jalen, S. Jevsevar, et al., *Microb. Cell Fact. 7*(1) (2008) 6.
6. A.E. Borujeni, A.S. Channarasappa, H.M. Salis, *Nucleic Acids Res.* *42*(4) (2013) 2646–2659.
7. H.M. Salis, E.A. Mirsky, C.A. Voigt, *Nat. Biotechnol. 27*(10) (2009) 946–950.