Cloning strategies iGEM Groningen 2020
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Introducing the NLP in the genome

NLP sequences

**NLP14a**

DNA: ATGGCACTGGATATTCTTGAAAGCGATGACTTTGGAGGCTTC

Protein: ALDILESDDFGGF

**NLP21e**

DNA: GCTGGAGGAAGACTTTTCAGAATGGTTGATCTTCCAGATGGAGATTTTCGTTCAGAA

Protein: AGGRLFRMVDPDGDFVPEG

**NLP15c**

DNA: TCATTCGATTCATTCACAGGATCAGGATTCACAGGACTTGA

Protein: SFDSFTGSGFTGLD

Selection of secretion signal

Because selecting the right signal peptide is a crucial step for ensuring abundant secretion, we suggest the following options:

1. **usp45 signal peptide (L. lactis)**
   ATGAAAAAAAGATTATCTCAGCTATTTTAATGTCTACAGTG
   ATACTTTCTGCTGCAGCCCCGTTGTCA
   GGTGTTCAGCT

2. **phrH (B. subtilis)**
   ATGCTATTAAGAAAAATGATGATGTTCTGCTGTTACTCTAGTTTTCGGAAGCATGTCGTTTCC
   ACCCTGACAAACTCCGGT

3. **phoD Tat type signal (B. subtilis)**
   ATGCCGAAGCAAGCAGTGCCTGTC
   AAATGGGAGGTCGCAAAGGATGAGCATTTCCGCAAAATCGTA
   AGAAAAAGGCACACTGAATGGCTAAACCAAGCTTTCGGCATTCCCGATCGTTCGGAAGCGGAGGCTTG
   AGCCAAAACAGTGTACTATTACGGTTTCAAAAACC

4. **amyE (B. megaterium)**
   ATGAAAAAGAAAAATGGACAGCTTTAGCACTAAACACTGCGCTGCTGCTGCTATTCAACAGGCG
   TGCAGCGC

5. **amyE (B. mycoides)**
   ATGTTTTGCAAAAACGTTCACCTTTCAATCGTGGGTGCTGCTGCTATTCAACAGGCG
   TGCAGCGC

*Sequences 3-5 have been identified using the signal peptide prediction server SignalIP*

Selection of promoter

To make sure that the NLP will have a high rate of transcription, we suggest the following promoters:

1. **PtaX**
   TTTGATTGATGGAAGAGATATGCTA

2. **P43**
   ATTTTACATTTTTAGAATGGCGGTGAAAAGACGCACGATATGAAAATAA

3. **Psigx**
4. \( \text{PgroES}^9 \)
\[
\text{ATCTTATCACTTGAAATTGGAAGGGAGATTCTTTATTATAAGAATTGTG}
\]
5. \( \text{Pdlt}^9 \)
\[
\text{TATGTATGGTTTCACACCGCGAATACCGGTTCCATATATTTAACAAGT}
\]

**Cloning strategy (figure 1)**

In order to create plasmid pYCR-gamyNLP, the backbone of pYCR, as well as the annealing products of the of gamy\_F and gamy\_R, will be digested with BsaI and ligated using T4 ligase to produce the pYCR\_gamy (CRISPR vector containing the gRNA). The NLP14a sequence will be synthetically ordered. The sequence will be PCR-amplified with the primers NLP14a\_Fw and NLP14a\_Rv. The resulting PCR product, as well as the pYCR\_gamy vector, will be digested with SfiI. The digested mixtures will be ligated using T4 ligase. The cloning strategy has been adapted from a study of Yi and colleagues.

For the constructs we used as an example the first secretion signal and the first suggested primers (see above). They can be exchanged with the alternative options in case they prove to be ineffective upon further examination.

**Safety mechanism: tryptophan auxotroph**

**Constructing the \( \text{trpE} \) knock-out strain (figure 2)**

In order to create plasmid pYCR-gtrpe, the backbone of pYCR, as well as the annealing product of the of gtrpe\_Fw and gtrpe\_Rv will be digested with BsaI and ligated using T4 ligase to produce the pYCR\_gamy (CRISPR vector containing the gRNA). For the repair fragment a 700 bp fragment upstream and downstream of the targeted gene was selected and PCR-amplified using primers trpe\_rep1/2\_Fw and trpe\_rep12\_rv. The resulting PCR product, as well as the pYCR\_gamy vector will be digested with SfiI. The digested mixtures will be ligated using T4 ligase. The cloning strategy has been adapted from a study of Yi and colleagues.

**Safety mechanism: toxin-antitoxin system**

**Cloning strategy for constructing the antitoxin plasmid (figure 3)**

The plasmid pAD-KPS12-Pman will used as a starting point for introducing the antitoxin gene (\( \text{ygcF} \)) in Bacillus mycoides. The vector will be cut with XbaI and SphI and ligated with the PCR amplified ygcF (using ygcF\_Fw and ygcF\_Rv as primers). In order to make the antitoxin expression inducible by solanine, we plan on replacing the mannose inducible promoter with a promoter induced by solanine. To accomplish this, we will use the primers Pman\_Fw and Pman\_Rv to cut the mannose inducible promoter and replace it with the newly discovered solanine-inducible promoter. The pAD-ygcF vector will be digested with EcoRI and XbaI, and ligated with the solanine promoter PCR product that will be digested as well with the up mentioned restriction enzymes. Moreover, we plan on cutting the ampicillin resistance gene from the plasmid using the primers Amp\_del\_fw and Amp\_del\_rv. Thus, the selection of the mutants will be solely based on the toxin-antitoxin system without using antibiotic resistance genes.

The cloning strategy has been adapted from a study of Yi and colleagues.
Fig. 1 Cloning strategy for introducing NLP14a sequence in the genome of Bacillus mycoides.
Fig. 2 Cloning strategy for knocking out the trpE gene of B. mycoides
Fig. 3 cloning strategy for constructing the plasmid that contains the antitoxin gene.
Cloning strategy for introducing the toxin gene ($yqcG$) in the genome

In order to create plasmid pYCR-gtrp-$yqcG$, the backbone of Pycr as well as the annealing product of the of grpe_Fw and grpe_Rv will be digested with Bsal and ligated using T4 ligase to produce the pYCR_gtrpe (CRISPR vector containing the gRNA). The $yqcG$ sequence will be synthetically ordered. The sequence will be PCR-amplified with the primers YqcG_Fw and YqcG_Rv. The resulting PCR product, as well as the pYCR_gamy vector will be digested with Sfil. The digested mixtures will be ligated using T4 ligase. The cloning strategy has been adapted from a study of Yi and colleagues.

The strategy targets $trpE$ in order to make it compatible with the tryptophan auxotroph option but of course other knock-in sites can be chosen.

Fig. 4 Cloning strategy for introducing the toxin gene ($yqcG$) in the genome of Bacillus mycoides.
Table 1 primers used in cloning strategies.

<table>
<thead>
<tr>
<th>Primer_name</th>
<th>Sequence (5'→ 3')</th>
<th>Restriction enzyme</th>
</tr>
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<tbody>
<tr>
<td>NLP14a_Fw</td>
<td>AATTCTTGTCAGCGCCCAACGAGGCCCATGAAAAAAGATTATCTTG</td>
<td>SfiI</td>
</tr>
<tr>
<td>NLP14a_Rv</td>
<td>TGTACAAGATCTAGAAAGGCCCTTATTGGCGTGATGATGTTGATG</td>
<td>SfiI</td>
</tr>
<tr>
<td>YqcF_Fw</td>
<td>GACTCTAGAATGGGTACCTACACAAGAAAAATTAAAG</td>
<td>XbaI</td>
</tr>
<tr>
<td>YqcF_Rv</td>
<td>ACTGATGCTTAAACACAGATCTCTACTATATC</td>
<td>SphI</td>
</tr>
<tr>
<td>Pman_Fw</td>
<td>GAGCGAATTTCGAGCGATTATTCCTTGGT</td>
<td>EcoRI</td>
</tr>
<tr>
<td>Pman_Rv</td>
<td>GAGCTCTAGATGTCTCTATACACCTGGAAATCC</td>
<td>XbaI</td>
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<tr>
<td>Amp_del_Fw</td>
<td>GGGCTCGAGGCTGACCAAGAATCTAGCTTATTGCCTTCAATCATCAT</td>
<td>AbsI</td>
</tr>
<tr>
<td>Amp_del_Rv</td>
<td>CCCCTCGAGGGCAGCAATTTCCCAGGAAGTG</td>
<td>AbsI</td>
</tr>
<tr>
<td>YqcG_Fw</td>
<td>AATTCTTGTCAGCGCCCAACGAGGCCATGAAAAAAGATTATCTTG</td>
<td>SfiI</td>
</tr>
<tr>
<td>YqcG_Rv</td>
<td>TGTACCAATCTAGAAAGGCCCTTATTGCCTTAAATCTCCAAGATATCATCC</td>
<td>SfiI</td>
</tr>
<tr>
<td>amy_Fw</td>
<td>AATTCTTGTCAGCGCCCAACGAGGCC</td>
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<tr>
<td>amy_Rv</td>
<td>TGTACCAATCTAGAAAGGCCCTTATTGCCTTAAATCTCCAAGATATCATCC</td>
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<tr>
<td>trpe_rep1-fw</td>
<td>AATTCTTGTCAGCGCCCAACGAGGCCCATGCAAAGAAAAATTTTTTTGGAAGG</td>
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<tr>
<td>trpe_rep1-rv</td>
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<tr>
<td>trpe_rep2-fw</td>
<td>AATTCTTGTCAGCGCCATAAGGCCTTATTGCCTTAAATCTCCAAGATATCATCC</td>
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<tr>
<td>trpe_rep2-rv</td>
<td>TGTACCAATCTAGAAAGGCCCTTATTGCCTTAAATCTCCAAGATATCATCC</td>
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Table 2 Oligos used in the cloning strategies

<table>
<thead>
<tr>
<th>name gRNA</th>
<th>sequence (5'→ 3')</th>
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</thead>
<tbody>
<tr>
<td>gTrpE_Fw</td>
<td>TACGTTATGTAGATGAGTGGAAG</td>
</tr>
<tr>
<td>gTrpE_Rv</td>
<td>AAACCTCACACATCACCTACAATA</td>
</tr>
<tr>
<td>gamy_Fw</td>
<td>TACGAGAAAGGCGTGTGAGTGAG</td>
</tr>
<tr>
<td>gamy_Rv</td>
<td>AAACCTCAAGAGGCCCTTCTT</td>
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Table 3 plasmids used in the cloning strategies

<table>
<thead>
<tr>
<th>plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYCR</td>
<td>PUC ori, spec R, rep pWV01ts, gRNA</td>
<td>Yi et al., 2018</td>
</tr>
<tr>
<td>pYCR-gamy-ssNLPhistag</td>
<td>pYCR backbone with gamy as gRNA and NLP +secretion signal+ HisTag as repair fragment</td>
<td>iGEM Groningen 2020</td>
</tr>
<tr>
<td>pYCR-gtrpe-yqcg</td>
<td>pYCR backbone with gtrpe as gRNA and amyE as repair fragment</td>
<td>iGEM Groningen 2020</td>
</tr>
<tr>
<td>pYCR-gtrpe-knockout</td>
<td>pYCR backbone with gtrpe as gRNA and amyE as repair fragment</td>
<td>iGEM Groningen 2020</td>
</tr>
<tr>
<td>pAD-yqCF</td>
<td>pAD backbone for YqCF expression</td>
<td>iGEM Groningen 2020</td>
</tr>
</tbody>
</table>
References


