Development of tools for the widespread implementation of CRISPR-Cas technologies in Gram-negative bacteria

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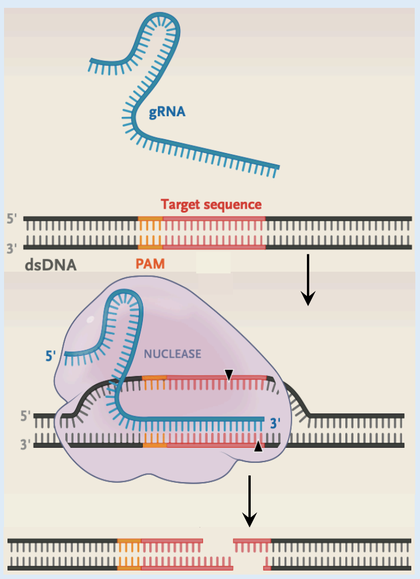
Introduction

CRISPR-Cas technology has made a huge impact due to its potential for editing, regulating and targeting genomes (**Fig1**) over the past years. It has been proven a very powerful and handy tool with a wide range of applications (1,2) It consists of a nuclease, like Cas12, guided by a single strand RNA molecule (gRNA) to its target sequence (which can be found within the genome, for example). Once the complex formed by the nuclease and the gRNA finds the target sequence, provokes a cleavage.



The aim of our project is to develop CRISPR-Cas-based genome editing tools of widespread use in Gram-negative bacteria and test their function in different members of the Rhizobial group. The importance of this group of organisms lies in the interactions (symbiosis) that they establish between themselves and different types of plants, such as legumes. These plants are well-known due to the nutritional relevance of their fruits.

Fig1. CRISPR-Cas12a system for genome engineering. Adapted from(3)

Results: Reporter Plasmid Construction

In the **Fig3** is depicted the Cas12 array construction which was used to develop the reporter plasmid. Firstly, the fragment comprised between EcoRI-SalI cleavaging sites was PCR-amplified (pAC-crRNA template). Then was enzymatically cleaved (*EcoR*I+*Sal*I) and introduced into previously cleaved as well pSEVA231-CRISPR plasmid. Subsequently, the same method was followed to amplify the fragments comprised between *Sal*I-*BamH*I (pAC-crRNA template) and *BamH*I-*Pst*I (pMPO1226 template) cleavaging sites and introduce them into previously cleaved pBBR1-mcs4 plasmid. Eventually, these fragments were introduced into previously cleaved (*Sal*I+*Pst*I) pSEVA231-CRISPR (which already hosted the first fragment of the construction), obtaining our reporter plasmid (**Fig2** left).

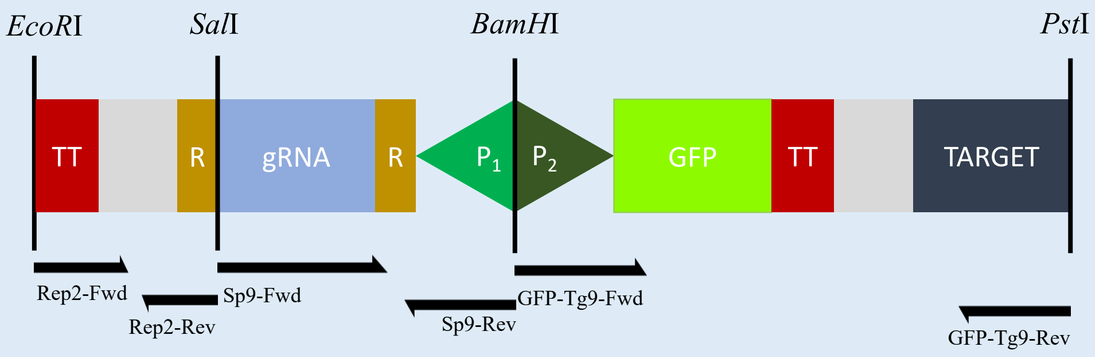


Fig3. Final CRISPR-Cas12 array construction. Primers used for each PCR-amplification are indicated with black arrows. Restriction enzymes cleavages sites are shown above the depicted CRISPR-Cas array. **P_1/P_2** (Promoters); **R** (repetitive sequences of Cas12a array); **TT** (transcriptional terminator sequences); **GFP** (Green Fluorescent Protein, reporter gene).

Experimental Design

The strategy designed consists on the construction of two broad host-range plasmids (4): one expressing the nuclease Cas12a (constructed by Sanz Marti), and a second one expressing a Cas12a gRNA, and containing the gRNA target sequence and GFP (Green Fluorescent Protein) as a reproter gene (**Fig2**). The ability of Cas12a to induce loss of this reporter plasmid (and the GFP reporter encoded therein) by cleavaging at the target sequence (Tg9) will be assessed, and the efficiency by which expression of different repair systems (NHEJ and HDR) enable persistance of the plasmid by introducing mutations at the cleaved target calculated.

When hosting both plasmids (**Fig2**) at the same time, colonies are expected to lose fluorescence due to the endonuclease activity of the Cas12a. Host cells will express Cas12a (FnCpf1), as well as the gRNA codified by the reporter plasmid. As a result, nuclease Cas12a and gRNA will assamble together creating a complex capable of recognising the target sequence (Tg9) for the mentioned gRNA. Afterwards, nuclease Cas12a will cut the plasmid at the cleavage site, inducing the reporter plasmid to be enzymatically digested, so as the host cell will lose its fluorescence (GFP is not expressed anymore).

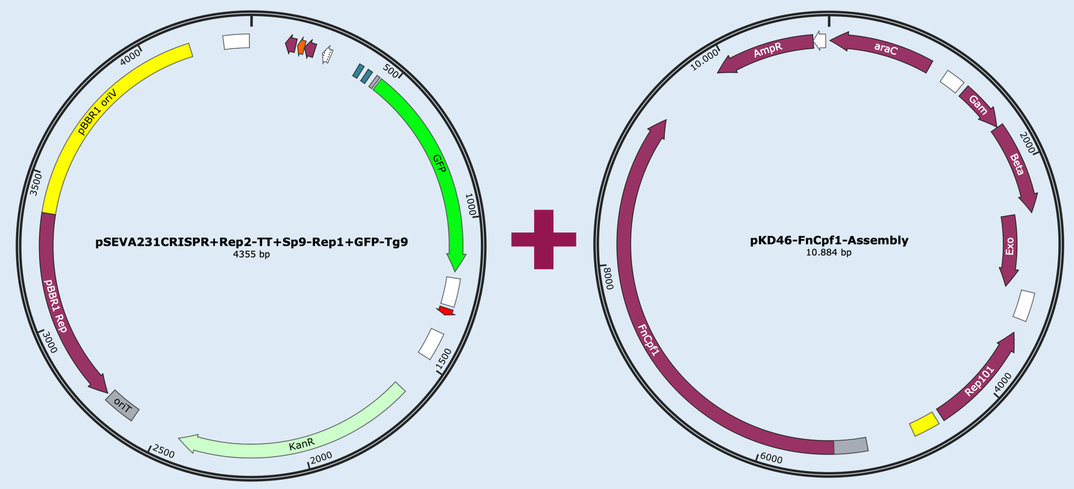


Fig2. Two broad host-range plasmids. Shown in the left the reporter plasmid construction, containing a Cas12 gRNA, the gRNA target sequence and GFP (Green Fluorescent Protein) as a reporter gene (Km^R). Short purple arrows represent repetitive sequences of the Cas12 array (Rep1 and Rep2), whereas short orange and red arrows represent a Cas12 gRNA sequence (Sp9) and its target (Tg9), respectively. Shown in the right, the structure of the plasmid for nuclease Cas12a (FnCpf1) expression (Ap^R), including Exo, Gam and Beta factors.

Prospects

1. Construction of a new DH5α *Escherichia coli* strain which hosts both the plasmid expressing the nuclease Cas12a and the reporter one (**Fig2**) simultaneously.
2. Assessment of fluorescence loss, which is directly related with loss of the reporter plasmid, in order to estimate the cleavaging efficiency of nuclease Cas12a.
3. Introduction of NHEJ and HDR repair systems into previous *Escherichia coli* strand to prove their efficiency enabling persistance of the plasmid.
4. Test this toolkit in different organisms within the Rhizobial group, such as *Shinorhizobium meliloti*, *Rhizobiium leguminosarum* and *Shinorhizobium fredii*.

References

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