CRISPR-MEDIATED EDITION OF THE GS-GOGAT CYCLE IN *Bacillus subtilis*

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ABSTRACT

CRISPR-Cas technology is widely used as a tool for precise gene edition. We have used a one plasmid strategy developed to engineer a *Bacillus subtilis* strain with a potentially altered GS-GOGAT cycle. The GS-GOGAT cycle converts glutamate into glutamine and vice-versa. Glutamate is one of the most abundant molecules in *Bacillus subtilis* and a several number of derivative molecules can be produced from it. We constructed a CRISPR plasmid to delete the *rocG* gene, which encodes a glutamate dehydrogenase, and replace it by the *glnA* gene, which encodes a glutamine synthetase. The plasmid was successfully used to edit *B. subtilis*. In the next step, we will evaluate the effects of the edition in the pool of intracellular glutamate and glutamine.

Keywords: CRISPR-Cas. *Bacillus subtilis*. Genetic editing. Glutamate and glutamine.

1 INTRODUCTION

Precise gene edition technologies have improved in the last decade with the discovery of CRISPR editing tools [1]. As a versatile technique CRISPR-Cas has been widely used in recent years to engineer new strains to produce interesting compounds through metabolic engineering[2,3]. Briefly, derived from a bacterial immune system, it works using a guide RNA (gRNA) molecule to guide the Cas9 enzyme to a specific genome locus. There, Cas9 cleaves the double stranded DNA. Double strand repair is mediated by a donor DNA for homologous recombination [1].

In 2016, a one plasmid system was developed to improve and make easier the genetic edition in *B. subtilis*[4] via CRISPR-Cas. Here, we used this technique to edit the *B. subtilis* genome in an attempt to improve the glutamate and glutamine cellular pools. Glutamate is synthesized from 2-oxoglutarate originated in the TCA Cycle. The reaction is catalyzed by the glutamate synthetase and can be reversed by the glutamate dehydrogenase encoded by the *rocG* gene [5,6]. Flux balance analysis showed that deletion of *rocG* together with an increase in the glutamine synthetase (encoded by the *glnA)* can increase the cellular pool of glutamate [5]. Therefore, a pJOE8999 plasmid[4] was designed and constructed to delete the *rocG* gene and replace it with the *glnA* gene as a strategy to overproduce glutamate and glutamine in *B. subtilis*.

2 MATERIAL & METHODS

PLASMIDS AND STRAINS

We used the pJOE8999 plasmid[4] as a delivery edition system. It has a Cas9 gene controlled by a mannose-inducible promoter, replication origins for *E. coli* and *B. subtilis*, a kanamycin resistance gene, two *Bsa*I sites to insert the gRNA and two *Sfi*I sites to insert the donor DNA. For plasmid construction and propagation, we used *E. coli* TOP10 and *E. coli* JM109. As host for the edition we used *B. subtilis* KO7.

DESIGN OF THE gRNA

The gRNA with a PAM site in the NGG format was designed using the Integrated DNA Technologies (IDT) design tool online. The target gene sequence (*rocG*) was imputed in the software and all possible targets were generated. To choose the best gRNA, they were ranked based on target/off target effect, and specific secondary structure analyzed on the NUPACK software. Best gRNA was selected with high on target score and low probability to form secondary structures with the gRNA scaffold.

DNA ASSEMBLY

The gRNA sequence was assembled using complementary 20 nucleotides oligos with compatible overhangs for cloning into *Bsa*I digested pJOE8999 plasmid. The forward and reverse strands were mixed in equimolar concentration and incubated at 100ºC for 5 min and let to slowly cool down to room temperature. The *glnA* gene was synthesized by Twist Bioscience. The homologous sequences were PCR amplified from the genome of *B. subtilis*. All three parts were joined using Golden Gate Assembly [7]. Finally, *Sfi*I restriction sites were used to assemble the donor DNA and the pJOE8999 plasmid.

GENE EDITION PROTOCOL

Gene edition was carried out as described by ALTENBUCHNER [4]. Colonies that lost the plasmid after the protocol were screened for correct gene edition by colony PCR.

3 RESULTS & DISCUSSION

The generated gRNAs were tested using the NUPACK software. The selected sequence showed low probability of undesirable secondary structure formation. In the Figure 1-A, gRNA nucleotides labeled blue indicate a probability of hairpin formation lower than 30%. This region of structure corresponds to the 20 nucleotides target sequence of Cas9 in the genome. This part of gRNA interacts with the genome and because of that, the low chance of hairpin formation is highly desired. On the other hand, a high probability of secondary structure formation (more than 80%) is showed at the gRNA 3'-end. This region corresponds to the gRNA universal scaffold. This part interacts with Cas9 enzyme, and in this case the secondary structure is necessary.

The chosen gRNA was inserted into the pJOE8999 plasmid as well as the donor DNA. The plasmid pJOE8999_gRNA(*rocG*)_doador_*glnA* was generated and map of the resulting sequence is shown in Figure 1-B.

We successfully transformed *B. subtilis* with the plasmid. We expected to have a few numbers of positive colonies at the end of the editing protocol, but differently than expected, only one positive colony was recovered. The editing protocol ran into efficiency problems due to persistent plasmid retention. Figure 1-C shows the positive colony one compared with the negative control and other four negative screened colonies.

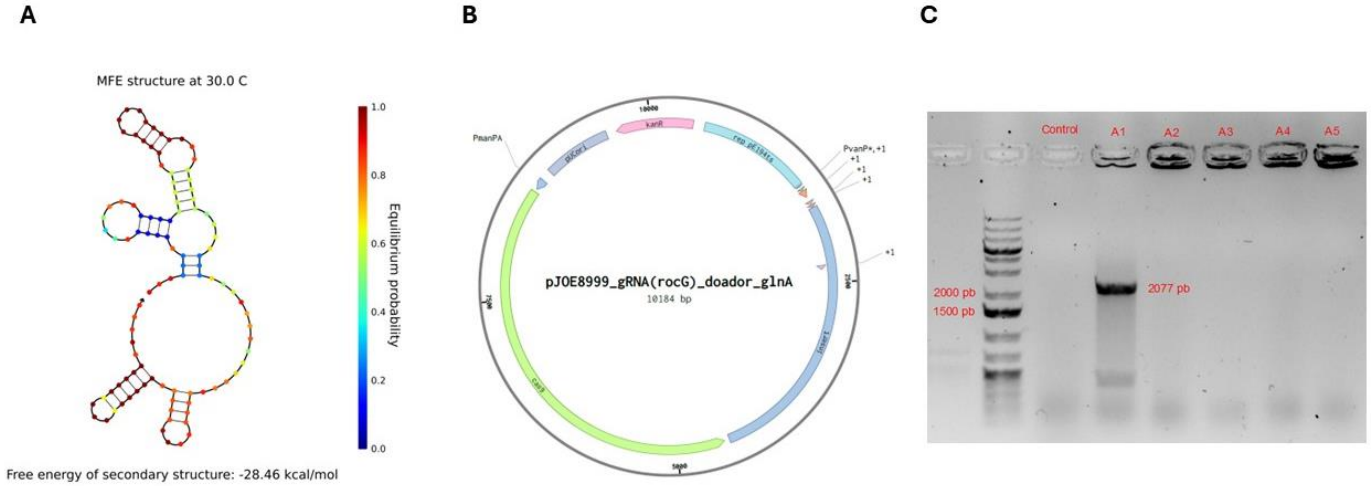

Figure 1. CRISPR-mediated gene edtion. **A.** gRNA(*rocG*) secondary structure predition. **B**. pJOE8999_gRNA(*rocG*)_donor_*glnA* plasmid map. **C.** Electrophoresis gel from colony PCR reactions showing the positive colony for *rocGΔ*::*glnA*(R62A) edition. The positive PCR should result in a 2077 pb band and a negative should generate no product.

As simulations showed that the chosen gRNA had a likely unproblematic secondary structure, we inferred that the efficiency problem was not due to the gRNA, but due the nature of the edition. Some papers in the literature have shown intrinsic problems to edit genes and metabolic networks associated with the GS-GOGAT cycle. We believe that the deletion of *rocG* gene negatively impacts the metabolism; therefore, viable edited cells are rare[8,9].

4 CONCLUSION

We successfully engineered the GS-GOGAT cycle in *B. subtilis*. The result shows the feasibility of the CRISPR technology to edit the genome of *B.s subtilis* in a one-week protocol. The next step is to evaluate the impact of the edition on the glutamate and glutamine accumulation in the cells.

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