Knockout of a secondary alcohol dehydrogenase gene in *Nocardia cholesterolicum* NRRL 5767 by CRISPR-Cas9 technology

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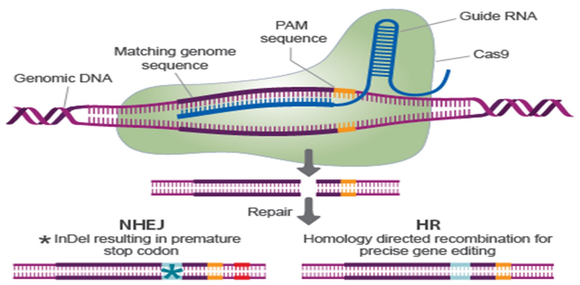
Abstract

In literature, *Nocardia cholesterolicum* NRRL 5767 (NC NRRL5767) is well-known for its ability to transform ~95% of added oleic acid, an abundant agricultural commodity, to value-added product of 10-hydroxystearic acid (10-HSA). A small amount of unwanted 10-ketostearic acid (10-KSA) was also produced. This microbe also transforms ~80% of added linoleic acid to 10-hydroxy-12(Z)-octadecenoic acid (10-OH-12-OD) (an isomer of ricinoleic acid) with minor 10-oxo-12(Z)-octadecenoic acid (10-oxo-12-OD). The conversion of oleic acid to 10-HSA and then to 10-KSA (or linoleic acid to 10-OH-12-OD and then to 10-oxo-12-OD) is catalyzed by oleate hydratase and secondary alcohol dehydrogenase (2º-ADH), respectively. The objective of this project was to knockout the *2º-ADH* gene in NC NRRL5767 so that the sole biotransformation product from oleic acid would be 10-HSA. Here, we report construction of CRISPR/Cas9/sgRNA chimeric plasmid that specifically target 5' coding region of the *2º-ADH* gene by Golden Gate Assembly. The construct was confirmed by DNA sequencing and transformed into NC NRRL 5767 via electroporation. The transformants were selected by apramycin resistance and screened for the presence of the target insert (crRNA) by PCR. The ability of the selected transformants to transform oleic acid to 10-HSA was screened by TLC and further confirmed by GC-MS. Our results showed that two of the transformants produced only 10-HSA with no detectable 10-KSA from oleic acid suggesting successful knockout of the 2º-ADH gene. Final confirmation came from the isolation of genomic DNA from these two transformants and the wild type NC NRRL5767 (used as DNA template) and using 17 primers (locate at different positions along the *2º-ADH* gene and the 5' upstream of this gene) for PCR. To our best knowledge, this is the first report to knockout the target gene in Nocardia species by CRISPR-Cas9 technology.

CRISPR/Cas9 Genome Editing Mechanism

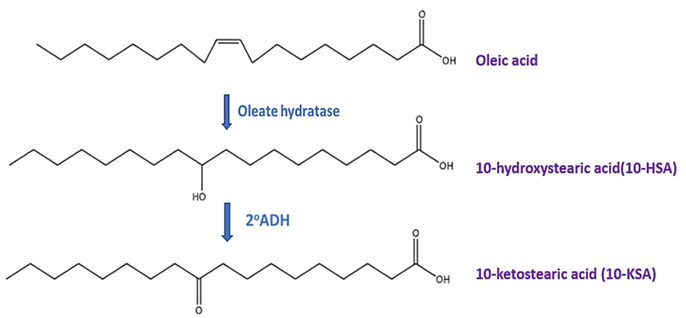
CRISPR-Cas9 is a genome editing tool that uses Cas9 endonuclease to cut target DNA on both strands guided by single-guide RNA (sgRNA) to match the DNA target sequence. The double stranded break (DSB) resulting from the cleavage is repaired by the error prone but efficient Non-Homologous End Joining (NHEJ).



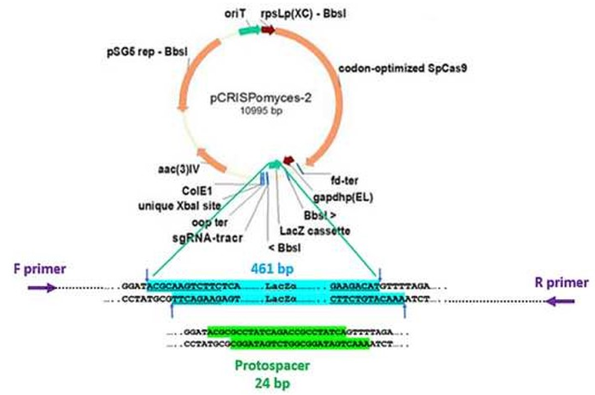
http://www.transomic.com/getattachment/0c3a5b99-05eb-43ee-a253-892eaf34b018/transEdit.aspx?maxsidesize=800

2º-ADH is the target for gene knockout

The 10-HSA is an industrial valuable product. Knocking out the 2º-ADH gene would enhance the production of the 10-HAS from oleic acid.

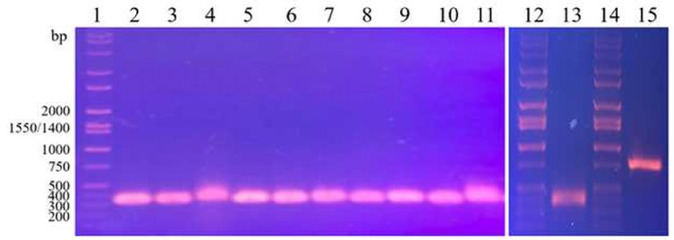


Construction of CRISPR/Cas9/sgRNA that target the *2º-ADH* gene



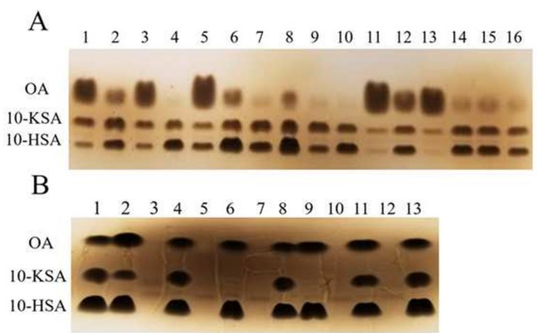
The BbsI fragment (blue, 461 bp) is replaced by the 24 bp protospacer in the pCRISPomyces-2/sgRNA construct. Location of the forward primer (92 nucleotides upstream of the left BbsI site) and reverse primer (238 nucleotides downstream of the right BbsI site) is shown by purple arrows. These primers were used in DNA sequencing and colony PCR.

Screening of *N. cholesterolicum* NRRL5767 transformants by colony PCR



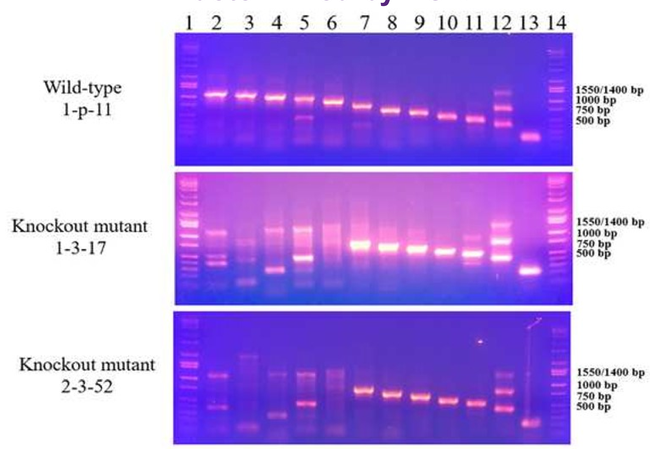
Lanes 1, 12, 14: Hi-Lo DNA marker; lanes 2-11: selected apramycin resistant *NC* NRRL5767 transformants were the sources of DNA template; lane 13: purified pCRISPomyces-2/sgRNA was used as DNA template; lane 15: purified pCRISPomyces-2 was used as DNA template.

TLC analysis of bioconversion products of *N. cholesterolicum* NRRL 5767 transformants



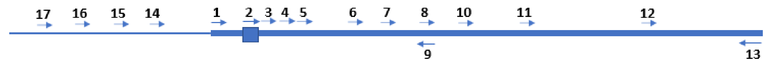
Panel A. lane 8: fatty acid marker; lanes 1-7 & 9-16: individual transformants with OA as substrate. Panel B. lanes 1, 8, & 13: fatty acid marker; lanes 2 & 3 (*NC* NRRL5767 with and without OA, respectively), lanes 4 & 5 (transformant, 1-3-3, with and without OA), lanes 6 & 7 (knockout mutant, 1-3-17, with and without OA), lanes 9 & 10 (knockout mutant, 2-3-52, with and without OA), lanes 11 & 12 (*NC* NRRL5767 transformed with parent pCRISPomyces-2 (clone 1-p-11) with and without OA).

On-target mutation of the *2º-ADH* gene determined by PCR



Lanes 1 and 14 (Hi-Lo DNA marker), lanes 2-5 (primers #17-#14 paired with primer #13,respectively), lane 6 (primer #1 paired with primer #13), lanes 7-13 (primers #5 - #11 paired with primer #13, respectively).

Location of the PCR primers



Summary

1. The desired CRISPR/Cas9/sgRNA chimeric plasmid that specifically target 5' coding region of the 2º-*ADH* gene was constructed by Golden Gate Assembly and confirmed by DNA sequencing.
2. The construct was transformed into NC NRRL 5767 via electroporation. The transformants were selected by apramycin resistance, screened for the presence of the target insert (crRNA) by PCR, and screen for 2º-ADH knockout mutants.
3. We have demonstrated that mutants 1-3-17 and 2-3-52 lack 2º-ADH enzyme activity through bioconversion screening.
4. Mutation of the knockout clones occur on target and at the 5' upstream as demonstrated by PCR
5. The knockout mutants offer improvements in that they convert added oleic acid to solely 10-HSA, thus eliminating downstream separation of 10-HSA from 10-KSA.

Acknowledgements

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Selected References

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