Steps in development of a genetic engineering tool for Paenarthrobacter nicotinovorans ATCC 49919, a soil nicotine-degrading actinobacteria

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Paenarthrobacter nicotinovorans ATCC 49919 is a nicotine degrading microorganism with biotechnological potential to convert this alkaloid into compounds of industrial and pharmaceutical importance like 6-hydroxy-L-nicotine (6-HLN), methylamine, succinic acid, or γ-amino-butyric-acid. A genetic engineering tool based on the CRISPR system that would allow fast and easy editing of the *P. nicotinovorans* genome is key for increasing its applications. Hence, our aim is to develop such a tool and we focus on inactivating or reducing the expression of 6-HLN oxidase (*6hlnO*), a key enzyme that catabolizes the conversion of 6-HLN to 6-hydroxy-methylmyosmine. Two approaches have been employed, one based on the CRISPR-Cpf1 system that allows gene knock-out and one based on CRISPR/dCasi9 system that allows partial and controlled inactivation of gene transcription. For the first approach, CRISPR-Cpf1 genes from pJYS3-ΔcrtYf plasmid were isolated by PCR using the following primer set: For: TCCGACGTCGTCGACTTTGCTGTTTACAATTAATCATCGTGTGG; Rev:ACCACTAGTCCTAGGTTTTTGACAGCTAGCTCAGTCCT and cloned into the DraI linearized pART2 vector using Gibson Assembly. Positive recombinant plasmids were selected following digestions with SpeI and ApaLI. We are in the process of targeting the CRISPR-Cpf1 system for *6hlnO* by cloning a crRNA sequence and corresponding protospacers into the pART2-Cpf1. For the second approach, a 20 bp spacer targeting the gene of interest was obtained by annealing two 5' phosphorylated synthetic oligonucleotides (For: GAAAAAGTTGCAGCATCCAAAGCG and Rev: AAACCGCTTTGGATGCTGCAACTT) by incubating at 95°C for 3 minutes and then gradually cooling the mixture by 0.010°C every 10 seconds for 2 hours. The spacer was cloned using Golden-Gate assembly into pCasiART. Positive colonies have been selected by blue-white screening. At this stage, pCasiART-Δ6hlnO and pART2-Cpf1 plasmids were obtained, and tests are underway for evaluating the efficiency of these genetic engineering tools.