Targeted Mutagenesis of Copper Tolerance Genes in *Frankia inefficax* EuI1c

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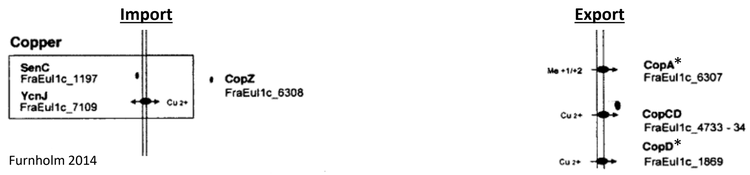
Abstract

When associated with bacteria in the genus *Frankia*, the adverse effects of heavy metal pollution on actinorhizal host plants are mitigated, which aids in phytoremediation performance and survival[1,2,3]. Previous studies have shown that the strain *Frankia inefficax* EuI1c can tolerate up to 5.0 mM of copper, and its resistance mechanisms are hypothesized to mimic those of heavy metal extremophiles[4]. To gain a better understanding of the genetic mechanisms underlying copper tolerance in *F. inefficax,* two genes that are hypothesized to contribute to this tolerance have been targeted for deletion.

Generation of *Frankia* mutants has historically been limited to non-targeted or transient transformants. Recently, a stable transformation method through conjugation was developed, allowing for CRISPR-Cas9 gene editing in *Frankia*[5]. Using this method, plasmid constructs are being generated to target two genes in *F. inefficax*: FraEuI1c_1869, a putative *copD* gene and FraEuI1c_6307, a putative *copA* gene. Generation of these mutant *F. inefficax* strains will allow for the downstream characterization of the mechanisms used by this strain to survive, and subsequently aid the health and survival of actinorhizal hosts, in soils degraded by excess concentrations of heavy metals.

Gene Selection



Locus Tag	FraEuI1c_1869
Original Gene Product Name	copper resistance D domain protein

Locus Tag	FraEuI1c_6307
Original Gene Product Name	copper-translocating P-type ATPase

JGI IMG

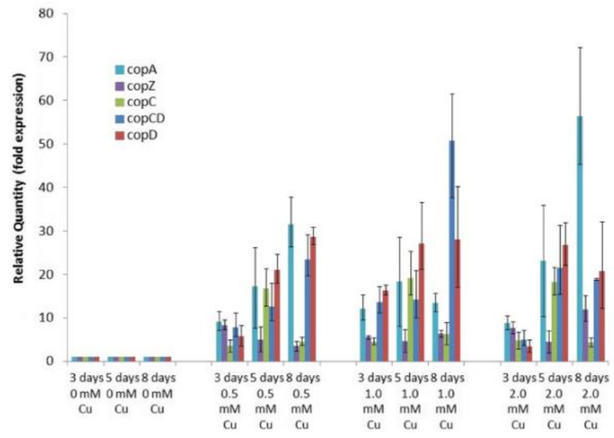
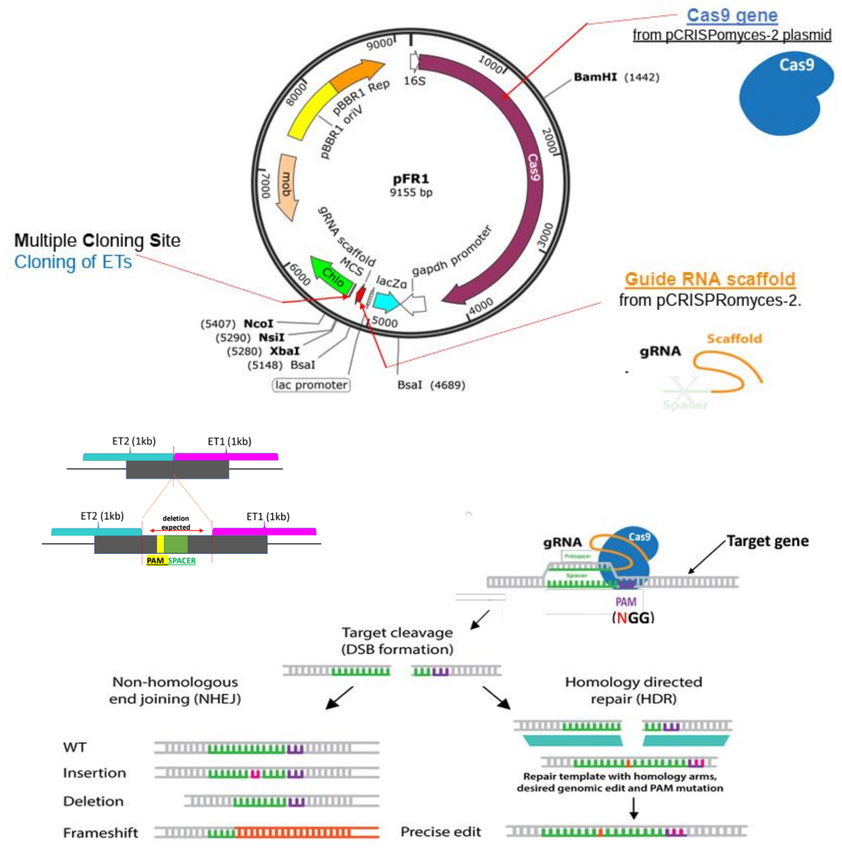


Figure 1. Relative gene expression (fold change) in response to Cu^{2+} exposure and time. Expression of copA (FraEuI1c_6307), copZ (FraEuI1c_6308), copC (FraEuI1c_4734), copCD (FraEuI1c_7109), and copD (FraEuI1c_1869) in response to Cu^{2+} stress. Cells were exposed to 0.5, 1, and 2 mM CuSO4 treatment for 3, 5, and 8 days. Experimental gene expression was normalized to the rpsA housekeeping gene and compared to the calibrator (control 0 mM treatment). Data are presented as the ratio (fold change) between the values obtained with Cu^{2+}-treated and untreated wild-type cells (Rehan et al. 2014).

CRISPR Plasmid System



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Plasmid Construction & Transformation

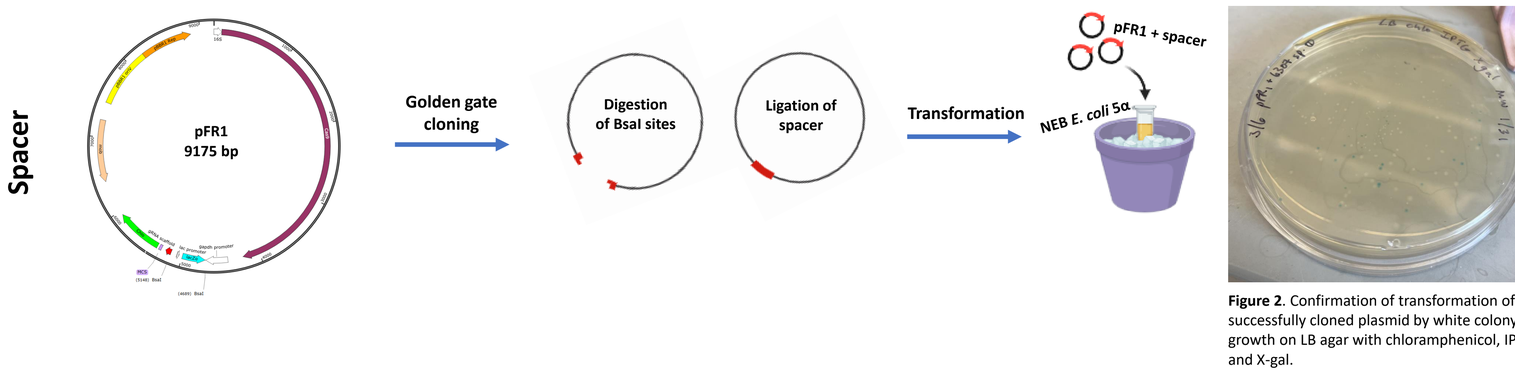


Figure 2. Confirmation of transformation of successfully cloned plasmid by white colony growth on LB agar with chloramphenicol, IPTG, and X-gal.

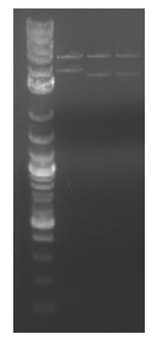


Figure 3. Confirmed insert of 1869 and 6307 spacers into pFR1 by digestion with NcoI-HF and BamHI-HF (NEB). Lane 1: ladder, lane 2: pFR1, lane 3: pFR1 sp. 1869, lane 4: pFR1 sp. 6307.

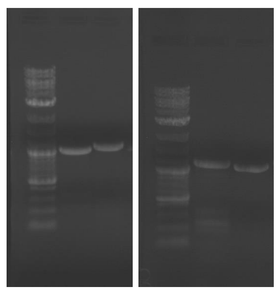
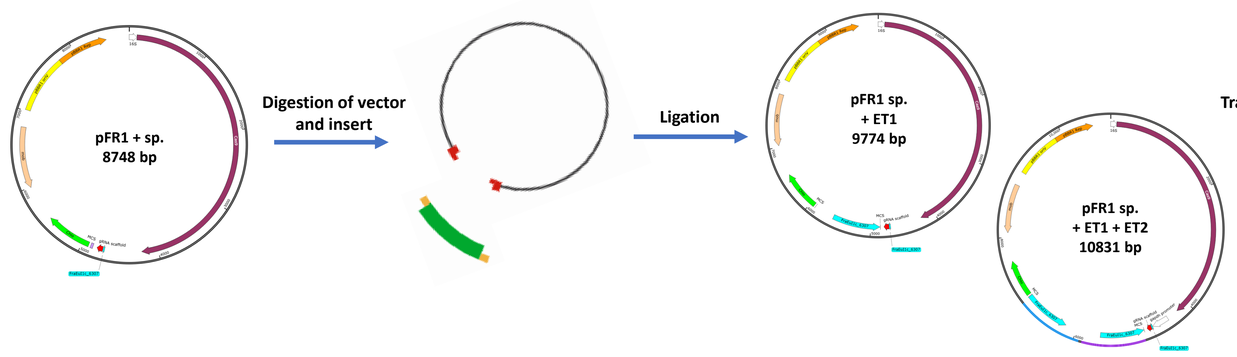


Figure 4. PCR amplification of editing template regions 1 (left) and 2 (right) from *F. inefficax* EuI1c gDNA.



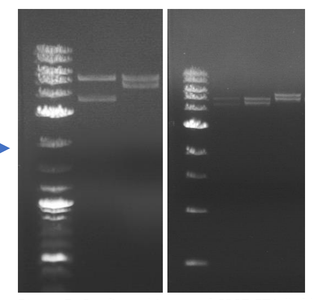
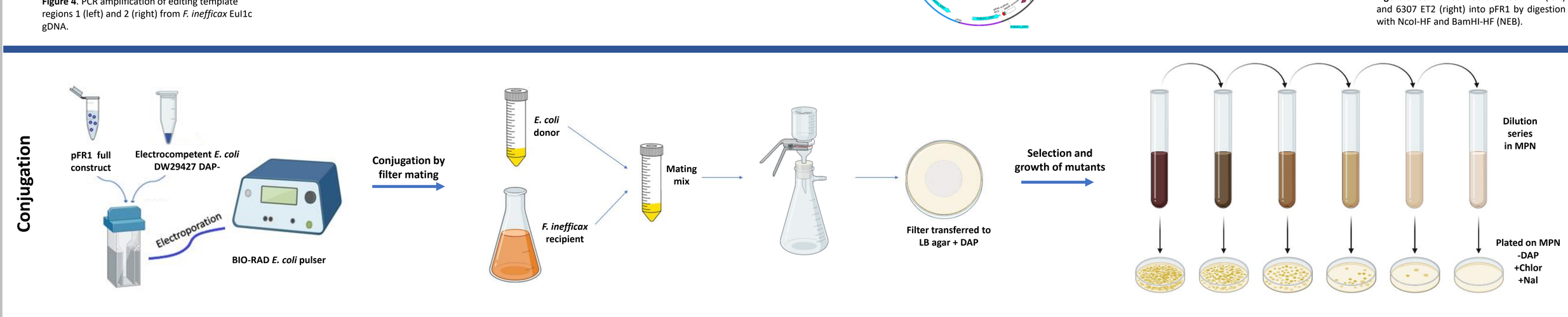


Figure 5. Confirmed insert of 6307 ET1 (left) and 6307 ET2 (right) into pFR1 by digestion with NcoI-HF and BamHI-HF (NEB).



Future Work

- Confirm successful uptake and retention of the plasmid through re-isolation from *F. inefficax*
- Perform PCR and whole genome sequencing to check the *F. inefficax* mutant strain genomes for successful deletion of each copper tolerance gene
- Compare the copper tolerance levels and mechanisms of each mutant strain compared to the wildtype via growth assay, qRT-PCR, and scanning electron microscopy
- Carry out plant inoculation experiments using *Hippophae rhamnoides* (sea buckthorn) to compare plant health and nodulation under copper stress when host plants are inoculated with the wildtype strain vs. each mutant strain

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References

1. Sayed, W. F. (2011). Improving Casuarina growth and symbiosis with Frankia under different soil and environmental conditions—Review. Folia Microbiologica, 56(1), 1–9. https://doi.org/10.1007/s12223-011- 0002-8
2. Karthikeyan, A., Deeparaj, B., and Nepolean, P. (2009). Reforestation in bauxite mine spoils with Casuarina Equisetifolia frost. And beneficial microbes. Forests, Trees and Livelihoods, 19. https://doi.org/10.1080/14728028.2009.9752661
3. Diagne, N., Arumugam, K., Ngom, M., Nambiar-Veetil, M., Franche, C., Narayanan, K. K., & Laplaze, L. (2013). Use of Frankia and Actinorhizal Plants for Degraded Lands Reclamation. BioMed Research International, 2013, e948258. https://doi.org/10.1155/2013/948258
4. Rehan, M., Furnholm, T., Finethy, R. H., Chu, F., El-Fadly, G., & Tisa, L. S. (2014). Copper tolerance in Frankia sp. Strain EuI1c involves surface binding and copper transport. Applied Microbiology and Biotechnology, 98(18), 8005–8015. https://doi.org/10.1007/s00253-014-5849-6
5. Pesce, C., Oshone, R., Hurst, S. G., Kleiner, V. A., & Tisa, L. S. (2019a). Stable Transformation of the Actinobacteria Frankia spp. Applied and Environmental Microbiology, 85(15), e00957-19. https://doi.org/10.1128/AEM.00957-19