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Novel applications of Molecular Biology to aid in environmental bioremediation.

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Abstract Bioremediation of environmental heavy metal contamination presents a valuable tool for an effective and low-cost method of containing and minimizing the harm of heavy metal contaminations. However, many common bacteria used in this application such as *Acidothiobacillus ferroxidans do not have intrinsic rsistances to environmental heacvy metal. In this paper an attempt was made at using the traJ gene to trasnfer a plasmid containing antibiotic resistance and a mutated from of the rusticyanin protein as a test for future insertions of heavy metal resistance genes. While this was unsucessful important insights into the mechanistic requirements of conjugative gene trasnfer in A. Ferroxidans were identified.*

Keywords: *A. Ferroxidans*, traJ, Rusticyanin, ABSTE, Iron

1. Introduction

A. ferrooxidans is a ubiquitous microbe often studied in the field of bioleaching and bioremediation. Review articles published by Jung et al., (2022) make note of multiple attempts to confer a mercury resistance gene into *A. ferrooxidans*. However, this research was met with little success when conventional membrane manipulation-based methods of plasmid transfection such as electroporation and head shock were attempted. However alternative methods such as plasmid conjugation *i*nitially reported by Peng et al., (1994) have been identified. In this method suitable broad host range plasmid is transfected into *E.coli* and then transferred to *A.ferrooxidans.* This technique has previously been used in the overexpression of RUS operon genes such as (Liu et al., 2010) and Cyc2 (Liu et al., 2013) as such the overexpression of mutated version of the RUS gene may present a further opportunity to optimize A. ferrooxidans based bioremediation.

Therefore, this paper attempts to use the plasmid conjugation protocol described by Peng et al., (1994) to genetically modify A. ferrooxidans with the aim of accelerating the rate of iron oxidation therefore improving the rate at which It can leach metals from the environment. However as noted by Yarzábal et al., (2004) insertion of a gene into A. ferrooxidans via plasmid results in an increase of endogenous gene expression as well. Consequently, it is imperative to use a high strength inducible promoter. As such the tac promoter was placed upstream of the gene of interest to ensure credible expression. (Meng et al., 2013, Kernan et al., 2017).

Plasmid design is of great importance when aiming to overexpress a gene in A. ferrooxidans. Several papers including those published by Peng et al., (1994), Flores-Ríos et al., (2019), and Wang et al., (2012) note important regions on genes capable of successful conjugation. As opposed to naturally occurring conjugative plasmids such as those described by Ramsay et al., (2016). Instead, a minimal vector consisting of a puc-57kan backbone containing the required oriT and traJ gene was constructed. Puc-57kan was chosen due to the ability of kanamycin to function under low pH conditions. (Krause et al., 2017 Beaver et al., 2022).

2. Materials and Methods

Bacterial culture

Liquid media used for growth consisted of 30mmol Fe^{2+}, with added Acidophile basal salts (ABS) and Trace elements (TE) see tables 2 and 3 for composition, pH adjusted to 1.8 using $1molL^{-1}$ H_2SO_4. Solid media overlay plates were used as described by Peng et al, (1994). These plates were incubated at 30ºC until colonies formed. *A.ferrooxidans* bacteria were subcultured from existing laboratory stock .

Table 2.1 ABS media composition (50x concentrate)

Chemical	Concentration (g/L)
$(NH_4)_2SO_4$	7.5
$Na_2SO_4.10H_2O$	7.5
KCL	2.5
$MgS0_4.7H_2O$	25
KH_2PO_4	2.5
$Ca(NO_3)_2.4H_2O$	0.7

Table 2.2 Trace elements composition (1000x concentrate)

Chemical	Concentration (g/L)
$ZnSO_4.7H_2O$	10
$CuSO_4.5H_2O$	1
$MnSO_4.4H_2O$	1
$CoSO_4.7H_2O$	1
$Cr_2(SO_4)_3.15H_2O$	0.5
H_3BO_3	0.6

$NaMoO_4.2H_2O$	0.5
$NiSO_4.2H_2O$	1
Na_2SeO_4	1
$Na_2WO_4.2H_2O$	0.1
$NaVO_3$	0.1

Plasmids for the conjugative overexpression of the Rusticyanin protein and the met148leu Rusticyanin protein were synthesized by commercial plasmid synthesis services (Genscript). The Rusticyanin protein nucleotide sequence was obtained from the NCBI database.

In addition to the sequence of Rusticyanin and mutated met148leu Rusticyanin these plasmids contained an upstream Shine-Dalgarno sequence, tac promoter, and downstream DDK tag. Following a stop codon in the previously described sequence, the required conjugative protein (traJ) and the oriT sequence were obtained via the Snapgene plasmid database from the pGA643 plasmid ("pGA643 Sequence and Map", 2022).

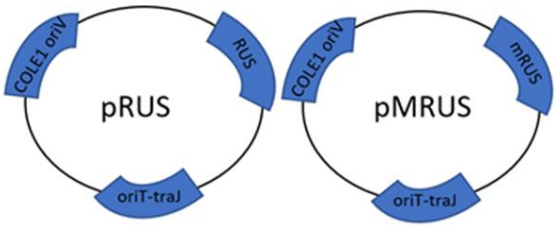

Figure 2.1 Final composition of the Rusticyanin (pRUS) and mutated Rusticyanin (pMRUS) mobilizable vector.

Conjugation of the mobilizable RUS and MRUS plasmids were performed as described by Peng et al., (1994).

3. Results

Conjugation of pRUS and pMRUS from *E.coli* into *A.ferrooxidans*

The results of this conjugation experiment were not successful. As can be seen in figures 3.1 and 3.2 ongoing pH and redox analysis of cultures seeded from the products of this conjugation reaction did not display any growth or development statistically significant from abiotic control media.

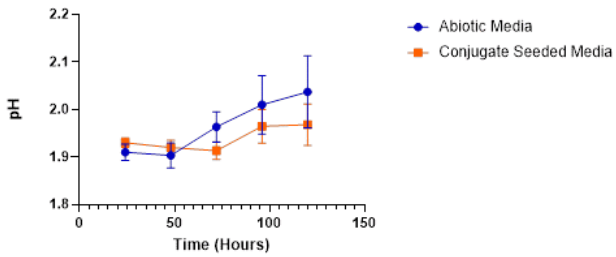

Figure 3.1 Comparison of pH progression in Abiotic (Blue) and Conjugate seeded ABSTE media (Orange)

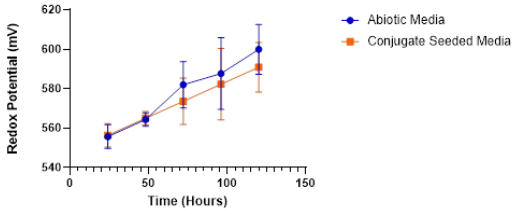

Figure 3.2 Comparison of redox potentials between abiotic and conjugate seeded samples of ABSTE media containing 30mmol of iron and 0.5g of elemental sulfur

4 Discussion

Proposed rationale for uncompleted plasmid conjugation

In this paper an attempt was made to undertake the genetic modification by plasmid conjugation of *A.ferrooxidans*. However as noted in figures 3.1 and 3.2 the liquid media seeded with the product of this reaction did not develop or grow in any way differentiable from that of abiotic control media. This indicates that the conjugation of plasmid into the A.ferrooxidans protein was unsuccessful as no growth was observed while under antibiotic pressure. This result somewhat contradicts the established research of Wang et al., (2012) and Meng et al., (2013) who note sucessful conjugation under these conditions. However there may be several reasons for this seemingly unsucessful plasmid conjugation. Primarily the oriV of the puc-57kan plasmid is not capable of replication otside of a narrow range of bacteria. As such a broad host range would allow the plasmid to remain inside the bacterium for a longer time as done by wang et al., (2012) and meng et al., (2013). Furthermore there are several plasmid backbones used in the literiture that present themselves as suitable alternatives such as RSF1010 and RP4 are as described by Ramsay et al., (2016). Due to the lack of success observed in the conjugation of Rusticyanin into *A.ferrooxidans*, the aim of evaluating the effectiveness of the mutated electron transport chain protein is unanswered. Whilst research has noted the protein has a superior redox potential (Hall et al., 1999, Kanbi et al., 2002) this evidence is still circumstantial and no in vivo testing in *A.ferrooxidans* has been attempted.

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