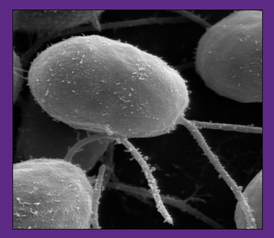
Cloning the Putative Zn-transport Gene crMTP1 for Heterologous Expression in Yeast



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Abstract

Zinc is a major micronutrient in the human body, binding to 10% of all human proteins, and is used for signaling and structure in cells.[1] Zinc deficiency may increase the risk of miscarriages in women and has been linked to diseases such as Alzheimer's.[1] This study investigates the zinc transporter crMTP1 from *Chlamydomonas reinhardtii* which is in the CDF family of zinc transporters. These proteins are the primary form of regulation in cells and cellular compartments. Placing the gene with a C-terminal FLAG-tag into the expression vector pGPD2 allows us to heterologously (in a different organism) express the protein in yeast. By isolating and investigating its cellular location and function we hope to better understand how this protein family works and regulates zinc metabolism in humans.

Zinc Transporters

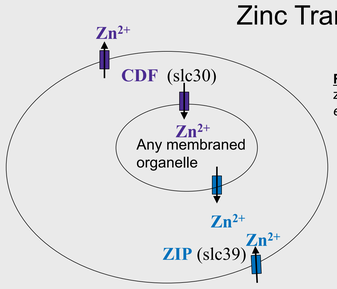


Figure 1: The two families of zinc transporters. ZIPs are responsible for zinc ion *influx* to the cytosol while CDFs are responsible for zinc ion *efflux* from the cytosol.[3]

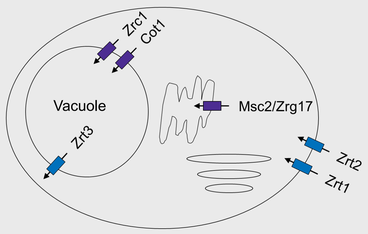


Figure 2: The two families of zinc transporters are also found in yeast and its organelles, like the vacuole.[3]

In-Fusion Base Insertion

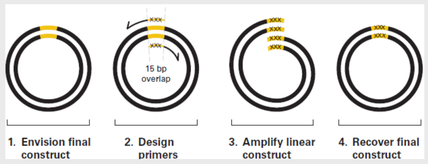
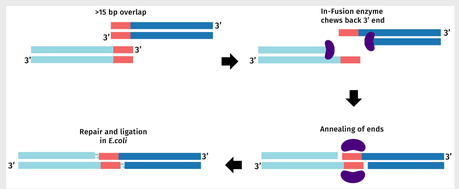


Figure 3: In-Fusion insertion is the overall procedure performed. The goal is to insert the FLAG-tag into the vector pGPD2/crMTP1. This is done by designing primers that will anneal to FLAG and the vector. Through PCR the construct can be amplified.

In-Fusion Seamless Cloning

Figure 4: In-Fusion® Cloning is a technique for seamless cloning of two or more DNA fragments. The specific procedure is described in this diagram.



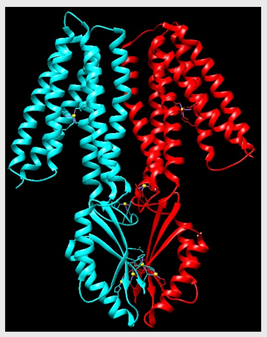


Figure 5: The CryoEM structure of the Yiip protein from *Shewanella oneidensis* bacteria is depicted. The yellow spheres are binded zinc ions.[2] This is a known CDF family zinc transporter.

Introduction

All organisms have mechanisms that allow them to regulate nutrients at the cellular and even subcellular level. Regulation of nutrients such as metal ions is essential in homeostasis and requires a delicate balance with several factors playing a role.

Chlamydomonas reinhardtii is a species of green algae with genes that code for apparent transport membrane proteins. One of these is crMTP1, which codes for a protein in the CDF family of zinc transporters responsible for the efflux of zinc from the cytosol. Research to date leaves many unanswered questions on where or how the protein functions. Using multiple cloning techniques, our goal is to construct a plasmid containing crMTP1 with a C-terminal FLAG tag that can express the protein heterologously in the yeast Saccharomyces cerevisiae, which can be used as a partial human cell model. The FLAG tag (DYKDDDDK) will aid in the purification of the protein and allow the location of the protein to be visualized within the yeast cells.

Methods

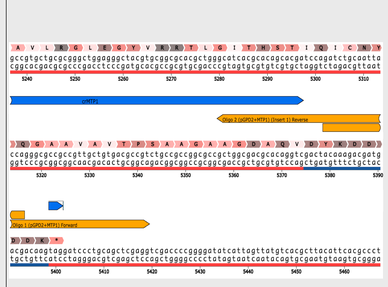
The pGPD2/crMTP1 vector has been previously clones by our group. This vector was used as the template, along with the Takara In-Fusion® Cloning Primer Design Tool, to design PCR primers for Takara In-Fusion® Cloning that added the FLAG tag to the protein. Based on these primer designs, a purposed map was drawn for each construct to depict the location of insertion and size of the new construct. Primers were ordered and different PCR reactions were performed in a thermocycler. Gels were run for all PCR products using some ladder to compare bands for size. All gels were stained with ethidium bromide and visualized using a BIORAD Chemidoc Imaging System.

Map and Features of pGPD2+crMTP1 Vectors

Figure 6A: Primers were designed for In-Fusion cloning for FLAG only. A map and features of the purposed plasmid is also shown with the corresponding primers.

(A)

Oligo Name	Sequence	Length (bp)	GC%	Tm (~°C)	Gene Specific Tm (~°C)
Oligo 1 (pGPD2+MTP1) Forward	ACAAAGACGATGACGACAAGTAGGATCCCTGCAGCTCGAGG	41	54	75.2	65.4
Oligo 2 (pGPD2+MTP1) (Insert 1) Reverse	CGTCATCGTCTTTGTAGTCGACCTGTGCGTCGCCAGC	37	59	76.2	66.5



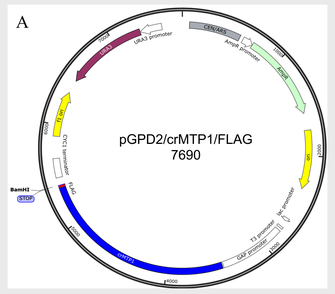
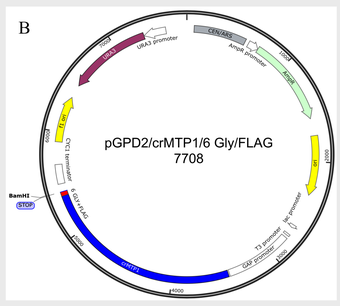
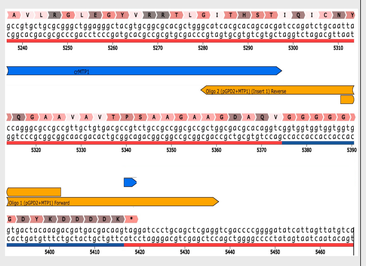


Figure 6B: Primers designed for In-Fusion cloning for 6 glycine amino acids with FLAG. A map and features of purposed plasmid is also shown with the corresponding primers.

(B)

Oligo Name	Sequence	Length (bp)	GC%	Tm (~°C)	Gene Specific Tm (~°C)
Oligo 1 (pGPD2+MTP1) Forward	GTGGTGACTACAAAGACGATGACGACAAGTAGGATCCCTGCAGCTCGAGG	50	54	75.2	65.4
Oligo 2 (pGPD2+MTP1) (Insert 1) Reverse	CTTTGTAGTCACCACCACCACCACCACCGACCTGTGCGTCGCCAGC	46	63	82.7	66.5

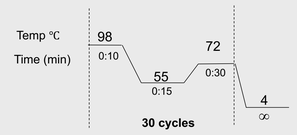


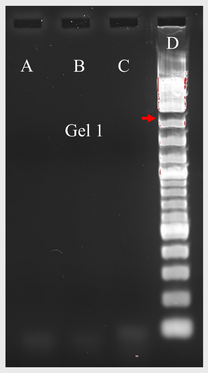


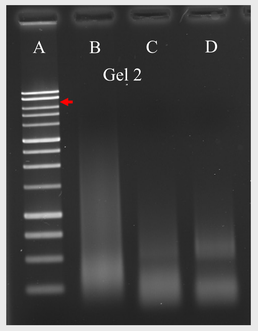
Gel Electrophoresis

Agarose gel electrophoresis was performed for the varying PCR parameters and inserts. **Gel 1** shows the varying products produced using a three-step cycling sequence and **Gel 2** shows the same inserts as Gel 1 but but with additional pre and post cycling steps. **Gel 3** shows only FLAG products under varying annealing times. All bands were expected to be greater than 7500bp based on the plasmid diagrams from the In-Fusion® primer design (Figures 6A and B). Red arrows indicate the expected size (7690-7708bp).

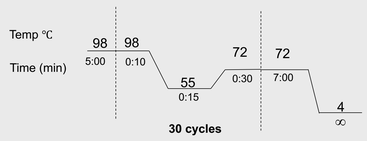
Gel 1: Lane A) FLAG only, B) 2 Gly/FLAG, C) 6 Gly/FLAG, D) NEB 1kb PLUS Log Ladder



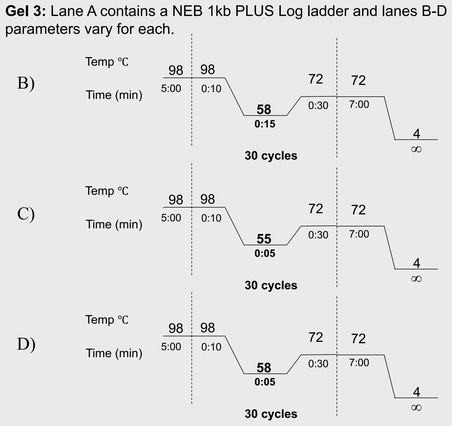


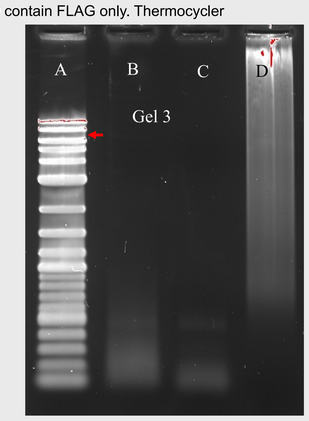


Gel 2: Lane A) NEB 1kb Ladder , B) FLAG, C) 2 Gly/FLAG, D) 6 Gly/FLAG.



Gel 3: Lane A contains a NEB 1kb PLUS Log ladder and lanes B-D contain FLAG only. Thermocycler parameters vary for each.





Conclusion

Although the design and construction of the purposed plasmids appear correct, the PCR reactions to add the FLAG tag to the crMTP1 gene were not currently successful according to the gel electrophoresis experiments. Multiple combinations of PCR reactions were performed but the products were still not showing the correct PCR products containing FLAG-tag. The bands on every gel should have been between 7000-8000bp, but were in fact smaller. Overall, more experiments need to be performed to modify the PCR conditions or create the new construct that will contain both crMTP1 with the C-terminal FLAG-tag.

References

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Acknowledgements

This work has been financially supported by:

SUNY Potsdam Department of Chemistry
Kilmer Undergraduate Research Apprenticeship (SUNY Potsdam)

National Science Foundation CCLI Award (DUE-0737460)