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Metal bioremediation by *CrMTP4* over-expressing *Chlamydomonas reinhardtii* in comparison to natural wastewater-tolerant microalgae strains

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ABSTRACT

Metal pollution in freshwater bodies is a long-standing challenge with large expense required to clean-up pollutants such as Cd. There is widespread interest in the potentially low-cost and sustainable use of biological material to perform bioremediation, such as the use of microalgae. Efficient metal bioremediation capacity requires both the ability to tolerate metal stress and metal accumulation. Here, the role of a *Chlamydomonas reinhardtii* metal tolerance protein (MTP) was examined for enhanced Cd tolerance and uptake. The *CrMTP4* gene is a member of the Mn-CDF clade of the cation diffusion facilitator family of metal transporters but is able to provide tolerance and sequestration for Mn and Cd, but not other metals, when expressed in yeast. Over-expression of *CrMTP4* in *C. reinhardtii* yielded a significant increase in tolerance to Cd toxicity and increased Cd accumulation although tolerance to Mn was not increased. In comparison, the metal tolerance of three chlorophyte microalgae strains (*Chlorella luteoviridis*, *Parachlorella hussii*, and *Parachlorella kessleri*) that had previously been adapted to wastewater growth was examined. In comparison to wild type *C. reinhardtii*, all three natural strains showed significantly increased tolerance to Cd, Cu, Al and Zn, and furthermore their Cd tolerance and uptake was greater than that of the *CrMTP4* over-expression strains. Despite *CrMTP4* gene over-expression being a successful strategy to enhance the Cd bioremediation potential of a metal-sensitive microalga, a single gene manipulation cannot compete with naturally adapted strain mechanisms that are likely to be multigenic and due in part to oxidative stress tolerance.

1. Introduction

The potential of using plants and algae for metal bioremediation has led to widespread evaluation of natural species that have innate or adapted ability for metal tolerance and accumulation [1–3]. Furthermore, the genetic engineering of photosynthetic organisms for increased tolerance, uptake, sequestration, transport and chelation of metals has been explored [4]. Macroalgae and unicellular microalgae are particularly attractive for the bioremediation of aquatic environments and wastewaters and a number of natural strains have been identified that are tolerant to a range of metals and show high metal removal abilities [2]. The mechanisms of metal tolerance and accumulation in microalgae appear to be varied, due in part to different responses to different metals, and include metal binding to the cell wall and secreted extracellular polysaccharides, intracellular metal binding peptides and proteins such as phytochelatins, glutathione abundance, oxidative stress tolerance, and metal transporter activity [5–9]. Many of these mechanisms are understood genetically and so there is potential to genetically enhance some of these algal characteristics. However, in contrast to higher plants, so far there have been very few examples of genetic engineering of microalgae to increase metal tolerance and accumulation.

Expression of a chicken class II metallothionein gene in the model green microalga *Chlamydomonas reinhardtii* led to increased cell growth in the presence of 40 μM Cd and increased Cd binding and removal efficiency relative to wild type, likely due to direct Cd chelation and sequestration by the metallothionein protein [10]. Chelation of some metals occurs *via* histidine binding and this has been recently exploited in *C. reinhardtii* by over-expression of the *HISN3* gene, which encodes the enzyme in the fourth step of the histidine biosynthesis pathway. The transgenic microalgae showed an approximately 50% increase in histidine concentration under Ni exposure conditions and increased

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tolerance to Ni stress [11]. Furthermore, the accumulation of metals including Ni, as well as Zn, Cu and Mn, was increased in the *HISN3* over-expressing *C. reinhardtii* compared to wild type. In addition to metal binding, metal tolerance can be achieved *via* anti-oxidant or osmotic protectant activities. Proline accumulation has been linked to the tolerance of a variety of abiotic stresses, and increased accumulation of proline in transgenic *C. reinhardtii* expressing a mothbean Δ1-pyrroline-5-carboxylate synthetase (*P5CS*) gene mediated increased Cd tolerance, possibly due to enhanced anti-oxidant activity and induction of Cd-binding phytochelatin synthesis [12]. All of these examples of microalgal engineering have evaluated improvements to metal chelation and oxidative stress response but as yet there are no examples of metal transporter over-expression in microalgae.

At a cellular level, the manipulation of metal transporter proteins has the potential to enhance metal accumulation into a cell, if metal uptake transporters are over-expressed, or to increase metal tolerance and internal storage, if organelle metal uptake transporters are over-expressed. There are a number of examples in higher plants where this latter strategy has been examined, particularly for tonoplast-localised transporters that perform vacuolar metal sequestration [13–16]. One class of metal transporter involved in metal sequestration and internal metal transport are the Cation Diffusion Facilitators (CDF) that are also called Metal Tolerance Proteins (MTP) in plants and algae [7,17]. Members of this family transport metal ions like Zn^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+} and Fe^{2+}, and can be phylogenetically classified within a Zn-CDF, Mn-CDF or Fe/Zn-CDF clade [18]. Many MTPs are vacuolar proteins such as ShMTP8 from *Stylosanthes hamata* that is responsible for vacuolar Mn^{2+} sequestration [14], AtMTP3 from *Arabidopsis thaliana* that can mediate Zn^{2+} and Co^{2+} sequestration [19], or CsMTP1 from *Cucumis sativus* that transports Zn^{2+} and Cd^{2+} [20]. Others are localised in the secretory pathway, such as the Mn^{2+} transporting AtMTP11 [21], or at the plasma membrane, such as the Mn^{2+} and Cd^{2+} transporting CsMTP9 [22]. In many of these studies, increased expression of an MTP gene often in yeast, led to enhanced cellular metal accumulation and tolerance to high metal concentration, indicating that MTP genes are potential targets for bioremediation studies.

Five MTP genes have been predicted in the *C. reinhardtii* genome [7] and one of these, *CrMTP1* was shown to be transcriptionally induced under Zn deficiency conditions [23] while *CrMTP2* and *CrMTP4* are induced by Mn deficiency [24]. However, none of the microalgae MTP genes have yet been directly functionally characterized or evaluated as a target for genetic manipulation. Here we describe the cloning and characterization of *CrMTP4* and the evaluation of *C. reinhardtii* lines over-expressing this gene with regard to Mn and Cd tolerance and transport ability. In addition, these transgenic strains were compared with natural strains of chlorophyte microalgae that had been previously obtained from a metal-containing municipal wastewater environment; *Chlorella luteoviridis* and *Parachlorella hussii* [25], or had been acclimated under laboratory conditions to tolerate wastewater; as with *Parachlorella kessleri* [26]. All three strains had been found to tolerate wastewater conditions in part due to increased oxidative stress tolerance, but the specific abilities of these strains to tolerate and accumulate metals has not been previously examined.

2. Materials and methods

2.1. Microalgae strains and growth conditions

C. reinhardtii wild type strain CC125 was obtained from the Chlamydomonas Resource Center. *P. kessleri* (CCAP 211/11G) was originally obtained from the UK Culture Collection of Algae and Protozoa (CCAP), Oban, Scotland, UK and was subsequently acclimated for growth in municipal secondary-treated wastewater conditions as described previously [26]. *C. luteoviridis* and *P. hussii* were previously obtained from a municipal wastewater secondary treatment pond as described previously [25]. *C. reinhardtii* strains over-expressing *CrMTP4* were generated as described below. Strains were grown photo-heterotrophically in batch culture in Tris–acetate–phosphate (TAP) medium at pH 7 [27] in 200 ml glass flasks on an orbital shaker rotating at 2 Hz or in 50 ml Nunc flasks, at 25 °C under cool-white fluorescent lights (150 μmol m^{-2} s^{-1}) with a 16 h:8 h light:dark regime. For metal tolerance and accumulation experiments strains were grown in TAP media supplemented with various concentrations of $Al_2(SO_4)_3$, $CdCl_2$, $CuSO_4$, $MnCl_2$, $ZnSO_4$ as indicated in the Results section. All cultures were inoculated with the same starting cell density as determined by cell counting to give an initial cell count of ~65×10^3 cells ml^{-1}.

2.2. MTP4 cloning and bioinformatic analysis

C. reinhardtii MTP4 (Cre03.g160550) sequence and gene model information was obtained from Phytozome v.9.1 using v.5.3 of the *C. reinhardtii* genome annotations. Phylogenetic relationship at the amino acid level was performed using full length sequences, as described previously [28]. The genome ID numbers or accession numbers for the sequences used are: AtMTP1 (At2g46800), AtMTP3 (At3g58810), AtMTP6 (At2g47830), AtMTP7 (At1g51610), AtMTP8 (At3g58060), AtMTP11 (At2g39450), CsMTP1 (EF684941), CsMTP9 (AFJ24702), OsMTP1 (Os05g03780), OsMTP8.1 (Os03g12530), PtrMTP11.1 (EF453693), ShMTP8 (AY181256), ScZRC1 (YMR243C), ScCOT1 (YOR316C), ScMSC2 (YDR205W), ScMMT1 (YMR177W). RNA was isolated from exponential growing *C. reinhardtii* CC125 cells using TRIzol reagent (Life Technologies) and further purified by phenol/chloroform extraction and precipitation with isopropanol. The full length *CrMTP4* cDNA (1617 bp) was amplified by RT-PCR using 1 μg of DNase-treated RNA using Superscript III reverse transcriptase (Life Technologies) and an oligo(dT) primer, then KAPA HiFi DNA polymerase (Kapa Biosystems) and gene-specific primers MTP4XbaIF (5′-AAA TCT AGA ATG TCG CAA CTA ACG CGC GAA G-3′; *Xba*I restriction enzyme site underlined) and MTP4SacIR (5′-AAA GAG CTC TCA CAG CAG ATT GAG AGC CTC GCT G-3′; *Sac*I restriction enzyme site underlined). Genomic DNA was isolated from *C. reinhardtii* CC125 as described previously [29]. A *CrMTP4* genomic DNA fragment spanning the exon and intron regions (3067 bp) was amplified using KAPA HiFi DNA polymerase and the MTP4XbaIF/MTP4SacIR primers. For all PCR amplification conditions, an annealing temperature of 60 °C and 35 amplification cycles were used. Following amplification, the PCR products were cloned into pGEM-T Easy plasmid (Promega) for propagation and sequencing (GATC Biotech) to confirm sequence fidelity. *CrMTP4* cDNA was sub-cloned into the *Xba*I and *Sac*I sites of the yeast expression plasmid piUGpd [30] to allow expression under control of the constitutive yeast GAPDH promoter and selection of the *URA3* gene. *CrMTP4* genomic DNA was sub-cloned into the *Eco*RI site of the Gateway entry plasmid pENTR1A (Life Technologies) for subsequent recombination using an LR Clonase reaction (Life Technologies) into the destination plasmid pH2GW7 [31] to allow expression of *CrMTP4* in *C. reinhardtii* under control of the constitutive cauliflower mosaic virus 35S promoter and selection of the *Aph7* gene.

2.3. Yeast heterologous expression and metal tolerance analysis

Yeast (*S. cerevisiae*) strains *pmr1* (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *pmr1*::*kanMX4*) (Euroscarf, Frankfurt, Germany) and the corresponding wild type strain BY4741 (*MATa; his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) (Euroscarf) were each transformed using the lithium acetate-polyethylene glycol method with *CrMTP4*-piUGpd plasmid or empty piUGpd plasmid and grown at 30 °C in synthetic defined medium minus uracil (SD – Ura) as described previously [32]. Expression of the *CrMTP4* cDNA in yeast was confirmed by RT-PCR using the internal *CrMTP4* primers MTP4F (5′-ACA TGT GTG TGC GGG AGT CG-3′) and MTP4R (5′-CTT GTG CCG GTG CAG GGA CC-3′) and RNA extracted from yeast using TRIzol reagent, then RT-PCR was performed as described above. PCR products were examined on a 1% agarose gel stained with SafeView (NBS Biologicals). Metal tolerance assays were