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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.

Keywords: bioremediation, cadmium uptake, metal tolerance, manganese transport, *Chlamydomonas reinhardtii*, wastewater

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 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 characterised or evaluated as a

target for genetic manipulation. Here we describe the cloning and characterisation 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 x 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 (1,617 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′; XbaI restriction enzyme site underlined) and MTP4SacIR (5′- AAA GAG CTC TCA CAG CAG ATT GAG AGC CTC GCT G-3′; SacI 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 (3,067 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 XbaI and SacI 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 EcoRI 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; his3*Δ1; *leu2*Δ*0*; *met15*Δ*0*; *ura3*Δ*0*; *pmr1*::*kanMX4*) (Euroscarf, Frankfurt, Germany) and the corresponding wild type strain BY4741 (*MATa; his3*Δ1; *leu2*Δ*0*; *met15*Δ*0*; *ura3*Δ*0*) (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 performed essentially as described previously [33] on solid SD –Ura medium with or without 3 mM $MnCl_2$ or 100 µM $CdCl_2$ metal salts, or in liquid yeast-peptone-dextrose (YPD) medium with or without 100 µM $Al_2(SO_4)_3$, 75 µM $CdCl_2$, 1 mM $CoCl_2$, 1 mM $CuSO_4$, 5 mM $MnCl_2$, or 5 mM $ZnSO_4$ metal salts. Internal Cd and Mn content in yeast grown in liquid YPD containing 30 µM $CdCl_2$ or 2 mM $MnCl_2$, respectively, was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) as described previously [34].

2.4. C. reinhardtii nuclear genome transformation

The *CrMTP4gDNA*-pH2GW7 plasmid or empty pH2GW7 plasmid were transformed into *C. reinhardtii* CC125 using biolistic bombardment as described previously [29]. Lines were selected on TAP agar medium containing 10 µg ml^{-1} hygromycin B and further selected on fresh selection medium. Three of the lines named MTP4-OE1, MTP4-OE2 and MTP4-OE3 were studied further and were maintained in selection medium until gene expression

197 analysis and metal tolerance assays were performed. Semi-quantitative expression of
198 *CrMTP4* was determined in the MTP4-OE strains and the control (empty pH2GW7) strain at
199 day 3 of growth in TAP medium following cell harvest and snap-freezing in liquid N_2 then
200 RNA extraction using Trizol reagent and cDNA synthesis as described above. *CrMTP4*
201 cDNA expression was confirmed by RT-PCR using the internal MTP4F/MTP4R primers and
202 was compared relative to expression of the constitutive control transcript *CBLP* using
203 primers CBLPF (5′-CTT CTC GCC CAT GAC CAC-3′) and CBLPR (5′-CCC ACC AGG TTG
204 TTC TTC AG-3′). PCR amplification conditions were as described above except that 25
205 amplification cycles were used. PCR products were examined on a 1% agarose gel stained
206 with SafeView.
207
208 *2.5 Quantitative gene expression analysis*
209 *C. reinhardtii* CC125 was cultured in TAP medium then $CdCl_2$ (0.1 mM or 0.2 mM) or
210 $MnCl_2$ (0.5 mM or 1 mM) was added to day 3 cells and left to incubate for 8 h before cells
211 were harvested and frozen in liquid N_2. RNA was isolated and cDNA was produced as
212 described above. *CrMTP4* gene expression was determined by quantitative real-time PCR
213 (qPCR) using the internal MTP4F/MTP4R primers, a SYBR Green core qPCR kit
214 (Eurogentec) and a StepOnePlus machine (ThermoFisher) using the SYBR Green detection
215 program and normalised to *CBLP* gene expression. Reactions were run in triplicate and
216 qPCR analysis was performed as described previously [29].
217
218 *2.6. Microalgae analysis*
219 At regular intervals over 8 d, cultures were grown in TAP medium with or without
220 added metals and sampled to determine cell density by cell counting using a Nexcelom
221 Cellometer T4 (Nexcelom Biosciences) or total chlorophyll (chlorophyll a+b) concentration as
222 described previously [26]. Metal content in microalgae after 8 d growth in metal-containing
223 medium was performed following EDTA washing to remove external (cell wall) bound metals
224 by ICP-AES as described previously [35]. The *in vivo* production of reactive oxygen species

(ROS) following 0.4 mM Cd treatment was quantified using the fluorescent stain 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma Aldrich) as described previously [25]. Statistical analysis was performed by one-way or two-way ANOVA, as appropriate and Tukey post-hoc test using GraphPad Prism v.6.

3. Results

3.1. CrMTP4 is a Mn-CDF that can provide Mn and Cd tolerance and uptake in yeast

Previous analysis of the *C. reinhardtii* genome has identified five MTP genes that are members of the CDF family [7]. *CrMTP4* is suggested to be a Mn^{2+}-transporting CDF member but the transport specificity of this protein is unknown. Phylogenetic analysis of predicted amino acid sequence indicates that CrMTP4 does indeed fall within the Mn-CDF clade alongside other known Mn^{2+}-transporting MTPs including ShMTP8, AtMTP11, OsMTP8, and the Mn^{2+}/Cd^{2+} transporting CsMTP9, although it is distinctly clustered from the higher plant Mn-CDF proteins (Fig. 1a). To demonstrate expression of *CrMTP4* and to examine whether *CrMTP4* transcriptionally responds to Mn or Cd excess *in vivo*, day 3 *C. reinhardtii* CC125 cells were examined following 8 h treatment with Mn addition (up to 1 mM) or Cd addition (up to 0.2 mM); however, there was no significant difference in *CrMTP4* transcript abundance between treatments (Fig. 1b) demonstrating that *CrMTP4* expression is not transcriptionally regulated by Cd or excess Mn status, for the concentrations tested.

The full length *CrMTP4* cDNA was obtained by RT-PCR and metal tolerance function was confirmed by yeast heterologous expression. The cDNA sequence of *CrMTP4*, and the amino acid sequence, was identical to the predicted genome annotation. When expressed in the Mn sensitive yeast mutant strain *pmr1*, *CrMTP4* expression was able to suppress the Mn sensitivity and provide strong Mn tolerance (Fig. 1c). Following expressing in a wild type yeast strain, *CrMTP4* was able to increase tolerance to Cd (Fig. 1c and d), however, there was no change in the ability of *CrMTP4*-expressing yeast to grow in excess concentrations of other metals including Zn, Co, Cu or Al (Fig. 1d). The Mn and Cd tolerance provided by *CrMTP4* expression in yeast appeared to be due to internal sequestration as total cellular

concentration of Mn and Cd was significantly increased in the *CrMTP4* yeast compared to the control, by 2.48-fold for Mn and 2.29-fold for Cd (Fig. 1e).

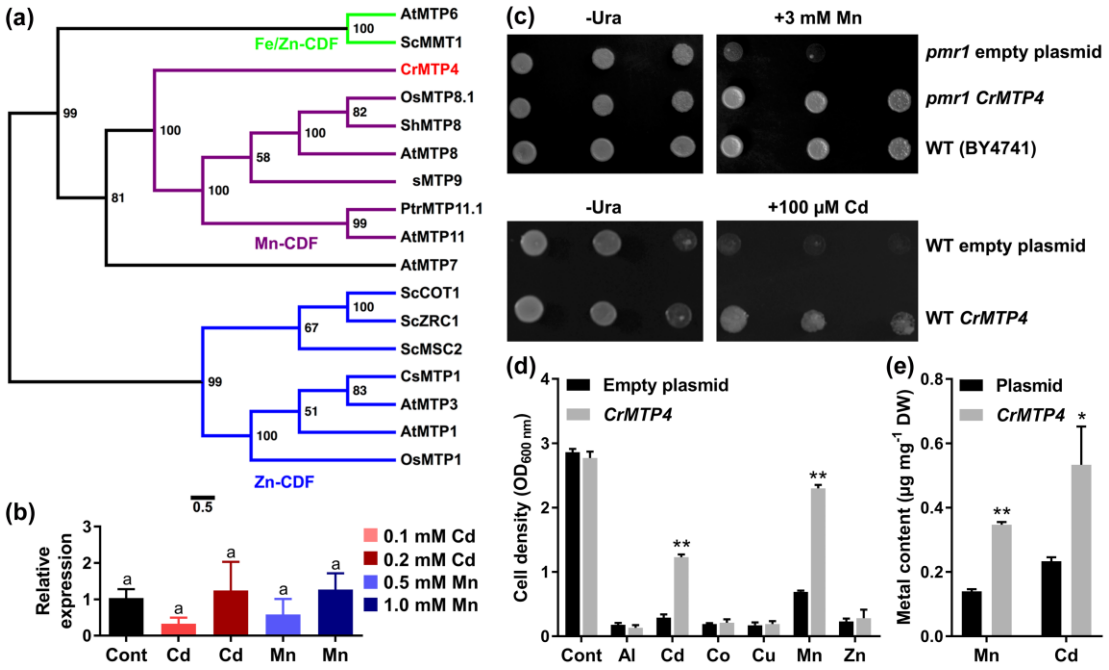

Fig. 1. Cloning of *CrMTP4* and characterization by yeast heterologous expression. (a) Phylogenetic comparison of CrMTP4 with selected Mn-CDF, Zn-CDF and Fe/Zn-CDF proteins from *Arabidopsis thaliana* (At), *Cucumis sativus* (Cs), *Oryza sativa* (Os), *Populus trichocarpa* x *P. deltoides* (Ptr), *Stylosanthes hamata* (Sh) and *Saccharomyces cerevisiae* (Sc). A maximum likelihood tree was derived from full length amino acid sequence alignment. Bootstrap values from 100 replications are indicated and the branch length scale bar indicates the evolutionary distance of 0.5 amino acid substitution per site. (b) Gene expression of *CrMTP4* in wild type cells in response to Cd and Mn treatment. Expression of *CrMTP4* as determined by real-time PCR is shown relative to *CBLP* expression. Data points are means (±SE) calculated from 3 independent biological replicates. Bars sharing the same lowercase letter indicate no significant difference between treatments ($P > 0.05$). (c) Suppression of Mn sensitivity of the *pmr1* yeast mutant, and Cd sensitivity of wild type (BY4741) yeast, both expressing *CrMTP4* in comparison with empty plasmid-containing strains. Liquid cultures of strains were serially diluted then spotted onto SD –Ura medium with or without added Mn or Cd. Yeast growth is shown after 3 d. A representative experiment is shown. (d) BY4741 strains transformed with empty plasmid or *CrMTP4* normalized to an identical starting cell density then grown in liquid YPD medium with or without added metals (0.1 mM Al, 75 µM Cd, 1 mM Co, 1 mM Cu, 5 mM Mn, 5 mM Zn) and grown for 24 h. Cell density was determined by optical density measurement at 600 nm. (e) Mn and Cd uptake in EDTA-washed BY4741 yeast transformed with empty plasmid or *CrMTP4* following growth in 2 mM Mn or 30 µM Cd YPD media. Data points are means (±SE) of 3 independent biological replicates. Bars indicated by asterisks show significant difference between empty plasmid and *CrMTP4* strain within each metal treatment (*, $P < 0.05$; **, $P < 0.01$).

3.2. Over-expression of CrMTP4 in C. reinhardtii enhances Cd tolerance

A genomic construct of *CrMTP4* containing all predicted exons and introns was transformed into the nuclear genome of wild type *C. reinhardtii* CC125 and three lines showing strong hygromycin tolerance and significantly higher expression of *CrMTP4* relative to wild type level of *CrMTP4* expression (Fig. 2a) were chosen for further analysis. There was no significant difference in cell density or chlorophyll concentration in any of the *CrMTP4* over-expression lines compared to wild type under standard growth conditions (Fig. 2b and c). Furthermore, while Mn addition to the medium up to 2 mM inhibited cell growth (Fig. 2b) and chlorophyll concentration (Fig. 2c) there was no increase in tolerance to Mn by any of the *CrMTP4* lines. However, there was evidence of a subtle but significant increase in Mn accumulation in two of the three *CrMTP4* lines (*CrMTP4-OE2* and *CrMTP4-OE3*) compared to wild type, by 1.9- and 2.3-fold, respectively (Fig. 2d).

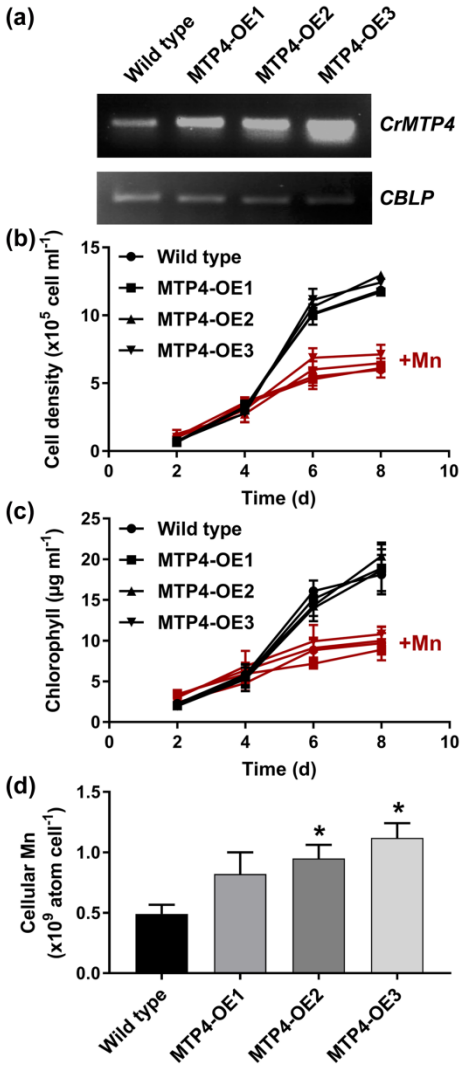

Fig. 2. Generation of transgenic *CrMTP4* over-expression lines with enhanced Mn accumulation. (a) RT-PCR detection of *CrMTP4* mRNA transcript abundance in wild type cells in comparison to three independent *CrMTP4* overexpression (MTP4-OE) lines. RT-PCR was performed using *CBLP* as a normalization control transcript. (b and c) Cell density as determined by cell count measurement (b) and total chlorophyll yield (c) in wild type and MTP-OE lines over time in TAP medium with (red symbols) or without (black symbols) 2 mM Mn addition. (d) Cellular Mn content in EDTA-washed wild type and MTP-OE cells following growth in 2 mM Mn medium after 8 d. Data points are means (±SE) of 3 independent biological replicates. Bars indicated by asterisks show significant difference between wild type and MTP-OE line (*, $P < 0.05$).

In contrast, all three *CrMTP4* lines exhibited significant tolerance to Cd addition (Fig. 3a). While the wild type cells displayed a marked loss in cell density and chlorosis in all Cd treatments, growth and total chlorophyll yield of the *CrMTP4* lines was significantly enhanced but there was no significant difference between the individual *CrMTP4* lines (Fig. 3b and c). In 0.5 mM Cd conditions, the wild type strain was unable to grow while *CrMTP4*

over-expression strains grew strongly (Fig. 3b), although growth was still significantly inhibited compared to treatments without Cd addition (Fig. 3b). To examine whether the increased stress of the strains in response to Cd addition was associated with oxidative stress, the reporter DCFH-DA for intracellular ROS production was used. *C. reinhardtii* strains were grown in TAP medium with 0.4 mM Cd addition and ROS production was measured in day 4 cells relative to cells with no added Cd. There was a 152 ± 26 % increase in ROS production in wild type and a 138 ± 32 % increase in the *CrMTP4* over-expression line but there was no significant difference between the strains. Growth of the *CrMTP4* over-expression lines was compared on other metal treatment conditions including Zn, Cu and Al toxicity but there was no difference in growth compared to wild type, indicating that the *CrMTP4* over-expression metal tolerance trait is specific for Cd.

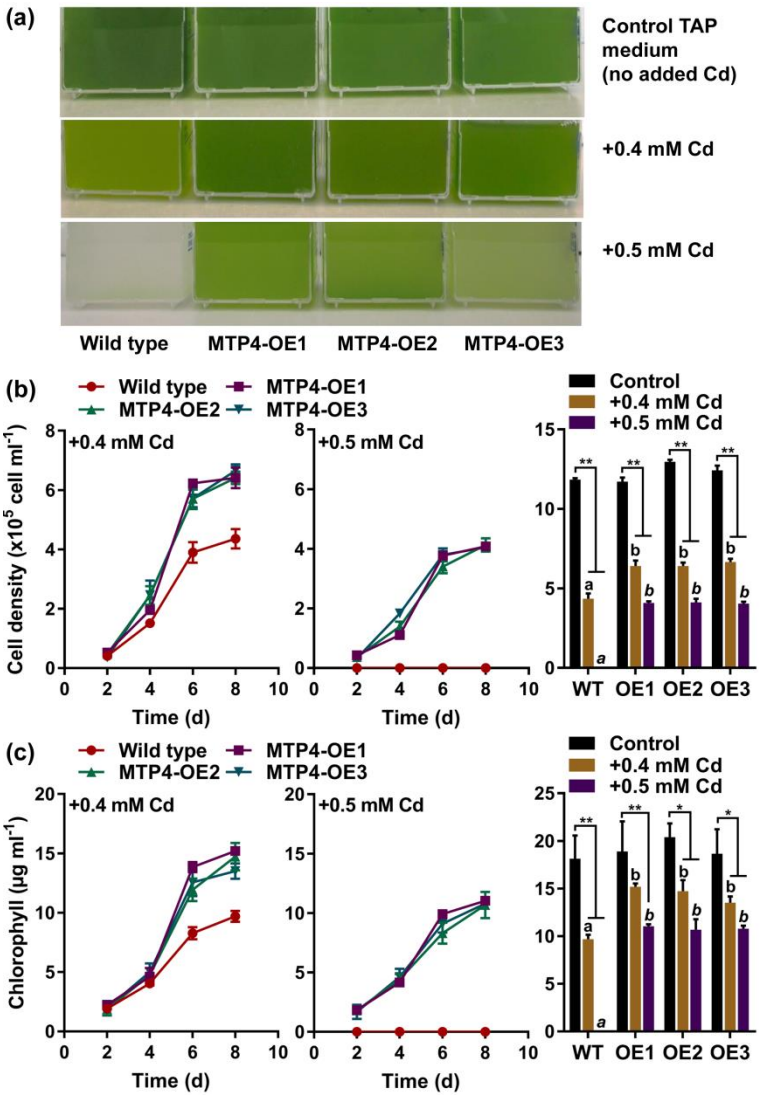

Fig. 3. Cd tolerance of *CrMTP4* over-expression lines. (a) Culture phenotypes of wild type *C. reinhardtii* and *CrMTP4* overexpression (MTP4-OE) lines after 7 d growth in medium with or without Cd. A representative experiment is shown. (b and c) Cell density as determined by cell count measurement (b) and total chlorophyll yield (c) in wild type and MTP-OE lines over time in TAP medium with 0.4 or 0.5 mM Cd addition. Cell density and chlorophyll parameters are shown in comparison to control treatment without Cd addition after 8 d growth (right-hand panels). Data points are means (±SE) of 3 independent biological replicates. Bars indicated by different lower case letters show significant difference ($P < 0.05$) within Cd treatments and between wild type (WT) and MTP-OE lines. Bars indicated by asterisks show significant difference between control and Cd treatments (*, $P < 0.05$; **, $P < 0.01$).

3.3. Natural wastewater adapted microalgae strains provide more substantial Cd tolerance and accumulation than CrMTP4 C. reinhardtii

Microalgae that are found in polluted environments such as in wastewater are adapted over many generations to the pollution conditions and therefore might be expected

to perform better with regard to metal tolerance and bioremediation than non-adapted strains. However, it is unclear how a transgenic line such the *CrMTP4* overexpression strain would compare against an adapted strain. Three microalgae strains of species *C. luteoviridis*, *P. hussii* and *P. kessleri*, were previously isolated from, or artificially acclimated to, municipal wastewater conditions [25, 26]. This secondary-treated wastewater was the outflow from an activated sludge treatment at a facility receiving mainly domestic wastewater and pre-treated effluent from a nearby oil refinery. The primary sedimentation and activated sludge treatment had reduced the concentration of suspended solids, biochemical oxygen demand/chemical oxygen demand, and ammonium and phosphate, but at this stage the wastewater conditions still included exposure to high concentrations of ammonium (22.7 – 30.1 mg L^{-1}) and phosphate (1.4 – 1.7 mg L^{-1}), and bacterial contamination. Trace metals were present in the wastewater although their concentrations were fairly low (0.9 µM Cd; 2.7 µM Cu; 125.2 µM Fe; 16.7 µM Mn; 18.8 µM Zn). Nevertheless, the three strains showed substantial tolerance to multiple metals compared to wild type and non-adapted *C. reinhardtii*. The tolerance to 150 µM Cd addition, as determined by chlorophyll content, for all three natural strains, especially *P. hussii*, was significantly higher compared to wild type *C. reinhardtii* but also significantly higher compared to the *CrMTP4* over-expression strains (Fig. 4a). However, it was seen that even under non-stressed (no added metal) conditions, the three wastewater-adapted strains grew better than the *C. reinhardtii* strains on the basis of total chlorophyll content. Normalized to non-stressed treatments, all three of the wastewater adapted strains still showed significantly greater tolerance than the wild type *C. reinhardtii*, while *P. hussii* showed significantly greater tolerance compared to the *CrMTP4* over-expression strains (Fig. 4a inset). *CrMTP4* over-expression lines were able to accumulate significantly more Cd than wild type (2.81- to 3.06-fold higher) but Cd accumulation was also significantly higher for the wastewater-adapted strains compared to both wild type and *CrMTP4* over-expressing *C. reinhardtii*; *C. luteoviridis*, *P. hussii* and *P. kessleri* displayed 9.7-fold, 8.6-fold, and 7.3-fold higher accumulation compared to wild type *C. reinhardtii*, respectively (Fig. 4b). For this analysis, Cd concentration was determined in

EDTA-washed cells in order to remove cell wall-bound metal and therefore determine internalised metal uptake.

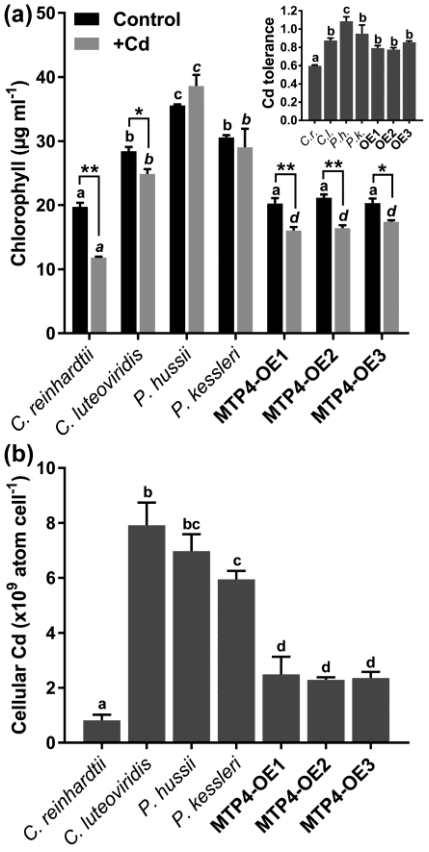

Fig. 4. Cd tolerance and accumulation of *CrMTP4* over-expression lines in comparison to natural wastewater adapted microalgae strains. (a) Total chlorophyll yield of strains after 8 d of growth in TAP medium with or without (control) 150 µM Cd addition. Inset: normalized chlorophyll yield of strains following Cd treatment relative to treatment without Cd. (b) Cellular Cd content in EDTA-washed cells following growth in 150 µM Cd medium after 8 d. Data points are means (±SE) of 3 independent biological replicates. Bars indicated by different lower case letters show significant difference ($P < 0.05$) within treatments, and pairs of bars indicated by asterisks show significant difference between control and Cd treatment (*, $P < 0.05$; **, $P < 0.01$).

While the *CrMTP4* over-expression strains were specifically enhanced for Cd tolerance and accumulation, the three wastewater-adapted strains also displayed increased tolerance to other metals including Al, Cu and Zn compared to wild type *C. reinhardtii*, in terms of cell density (Fig. 5a) and chlorophyll content (Fig. 5b), including when normalized to values without metal addition (Fig. 5a inset and 5b inset). For all metal conditions, on the basis of cell density, *P. hussii* was the most tolerant microalga, while *P. kessleri* showed high metal tolerance on the basis of chlorophyll content. The internalised accumulation of Al, Cu

and Zn was also significantly higher for all three strains, with the exception of Zn accumulation by *C. luteoviridis*, compared to *C. reinhardtii*, and with *P. kessleri* having the highest accumulation of all three metals (Fig. 5c).

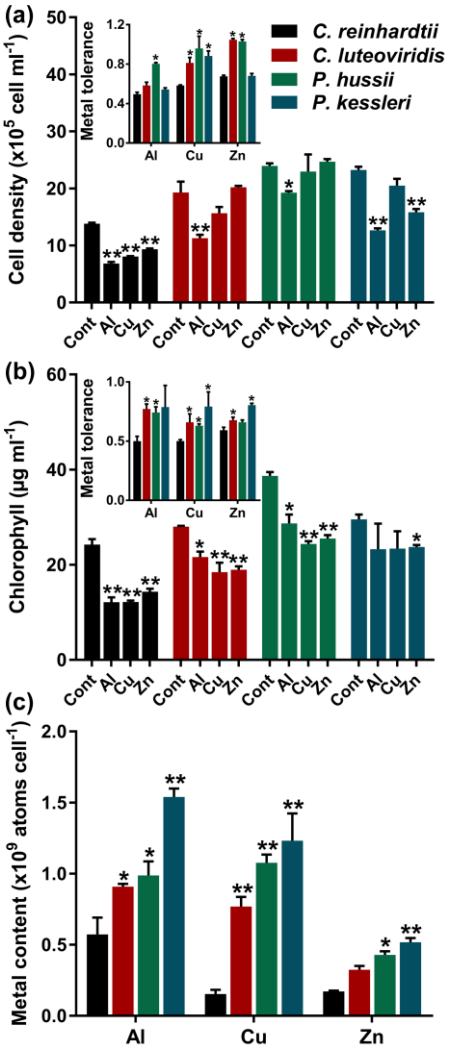

Fig. 5. Metal tolerance and accumulation characteristics of natural wastewater adapted microalgae strains. (a and b) Cell density (a) and total chlorophyll yield (b) of natural strains treated without metal addition (Cont.) or with 100 µM Al, 100 µM Cu, and 225 µM Zn in TAP medium for 8 d. Inset graphs: normalized cell density (a) and chlorophyll yield (b) of strains following metal treatment relative to treatment without metals. (c) Cellular metal content in EDTA-washed cells following growth in 50 µM Al, 50 µM Cu and 150 µM Zn medium after 8 d. Data points are means (±SE) of 3 independent biological replicates. Bars indicated by asterisks show significant difference between metal treatment and control treatment, or between *C. reinhardtii* and the wastewater adapted strain (*, $P < 0.05$; **, $P < 0.01$).

4. Discussion

Mn-CDF genes have been identified and characterized from various higher plant species but have not as yet been examined in much detail from microalgae. Furthermore, there have been very few studies examining the bioremediation potential of MTP gene targets, and none using microalgae. Here we have functionally determined the substrate specificity of a Mn-CDF, CrMTP4 from *C. reinhardtii* and demonstrated the ability to enhance Cd tolerance and accumulation in this species of microalgae through over-expression of this Mn-CDF.

Phylogenetic analysis showed that CrMTP4 groups with other known Mn^{2+} transporting MTP proteins, while previous experiments showing induction of *CrMTP4* mRNA transcript under Mn deficiency conditions had implicated this putative MTP as a Mn^{2+} transporter [24]. Yeast heterologous expression clearly confirmed the ability of CrMTP4 to increase the cellular accumulation of Mn^{2+} as well as to enhance tolerance of Mn in yeast, possibly due to vacuolar sequestration although this could not be confirmed. Furthermore, the yeast assay suggested that CrMTP4 also displayed Cd^{2+} transport and tolerance activity but it could not provide yeast tolerance to other metals tested including Zn^{2+}. Cd^{2+} is a non-essential metal ion for photosynthetic organisms but is readily taken up and transported throughout cells often in competition with essential metal ions such as Zn^{2+} [36], and indeed some Zn-CDF proteins such as OsMTP1 have been implicated in the transport of both Zn^{2+} and Cd^{2+} [37]. Many of the previously studied Mn-CDF proteins such as ShMTP8, AtMTP8, and AtMTP11 appear to be specific for Mn^{2+} [14, 21, 38], but it is possible for Mn^{2+} transporters to also be able to transport Cd^{2+}. For example, the vacuolar Mn^{2+} and Ca^{2+} transporter AtCAX2 is also able to transport Cd^{2+} [16], while the Mn-CDF transporter CsMTP9 was also shown to be able to transport Cd^{2+} [22]. As a Mn^{2+} and Cd^{2+} transporter that was able to increase metal accumulation when expressed in yeast, CrMTP4 was therefore a suitable target to examine bioremediation potential by over-expression in *C. reinhardtii*.

A genomic construct of *CrMTP4* led to increased *CrMTP4* mRNA transcript abundance in *C. reinhardtii*, which had no obvious negative effects to the cells under non-stressed conditions. However, unlike in yeast, increased expression of CrMTP4 was unable to provide tolerance to excess Mn, despite the transgenic cells exhibiting a slight increase in Mn accumulation. Although CrMTP4 was able to promote Mn tolerance in a heterologous system, the Mn^{2+} transport and homeostasis characteristics may be different *in vivo*. Allen *et al.* (2007) [24] demonstrated that out of all five *C. reinhardtii* MTP genes, *CrMTP4* showed the highest induction in response to Mn deficiency, perhaps indicating that this gene encodes a high affinity Mn^{2+} transporter responsible for Mn^{2+} delivery under situations of low Mn availability and therefore is poorly suited to conditions of high Mn stress. In fact we found that *CrMTP4* was not transcriptionally induced by excess concentrations of Mn, or indeed Cd. Alternatively, this may suggest that *CrMTP4* is not directly regulated by toxic concentrations of metals or possibly that regulation is post-transcriptional. While some higher plant MTP genes such as *CsMTP9* are transcriptionally induced by metal excess [22], others such as *AtMTP11* and *OsMTP8.1* are not significantly induced by high concentrations of Mn [21, 39], although OsMTP8.1 protein abundance is moderately enhanced by high Mn supply. It is relevant to note that like for *CrMTP4*, the yeast Zn-CDF gene *ScZRC1* is also transcriptionally induced by metal limitation but shows low expression in response to high metal conditions, despite being an important component in Zn tolerance [40]. It was suggested that this is a proactive protective mechanism against a sudden switch from metal limited to excess conditions. Finally it is worth noting that the sub-cellular localization of CrMTP4 was not determined and the organelle or vesicle on which CrMTP4 resides may provide insufficient internal Mn storage to tolerate sequestration of high concentrations of Mn^{2+}. Unlike higher plants and yeast, *C. reinhardtii* does not possess a large central vacuole but many smaller acidic vacuoles or acidocalcisomes [41, 42]. This potentially argues that internal metal sequestration as a bioremediation mechanism may be less useful for microalgae.

In contrast to the Mn stress response, the MTP4-OE lines all showed increased tolerance to increasing concentrations of Cd, up to 0.5 mM Cd, coupled with increased cellular content of Cd compared to wild type, suggesting internal sequestration of Cd rather than Cd efflux. In comparison to other previously generated transgenic Cd tolerant *C. reinhardtii* strains, the MTP4-OE lines displayed greater tolerance to Cd than the proline-accumulating *P5CS* lines that only showed a 1.5-fold increase in growth compared to wild type grown in 100 µM Cd [12]. The MTP4-OE lines also had greater Cd tolerance compared to *C. reinhardtii* expressing a chicken metallothionein [10]. To date, this is the only example of an MTP gene being genetically engineered in an algal species to enhance potential metal bioremediation characteristics, and there are just two recent examples to compare of an MTP being over-expressed in higher plants. OsMTP1 was ectopically expressed in tobacco to give increased tolerance to 100 µM Cd, by supressing inhibition of growth, lipid peroxidation and cell death compared to wild type tobacco [43]. Furthermore, OsMTP1 expression gave an approximately 2-fold increase in whole plant Cd accumulation compared to wild type, with the tolerance and accumulation trait likely due to increased vacuolar sequestration. Likewise, ectopic expression of CsMTP9 in *A. thaliana* increased plant tolerance to Cd and led to enhanced shoot Cd accumulation relative to the roots [22]. Unlike for CrMTP4, CsMTP4 over-expression also led to increased Mn tolerance, however, it is important to note that CsMTP4 localized to the plasma membrane and provides metal tolerance by cellular efflux.

Despite the increased Cd tolerance and accumulation provided by *CrMTP4* over-expression, the natural wastewater adapted strains all showed substantially greater tolerance and accumulation of Cd, as well as other metals including Al, Cu and Zn. Previous analysis of these natural strains demonstrated that they are able to tolerate the toxic effects of the wastewater environment because of high oxidative stress tolerance activities, which include increased ascorbate peroxidase activity and carotenoid accumulation compared to the non-adapted strains [25, 26]. Prolonged exposure to metals such as Cd induces cellular toxicity in part due to oxidative stress [44, 45]. Indeed it was also demonstrated here that

ROS accumulation was increased in *C. reinhardtii* strains in response to the Cd treatment, however, there was no significant difference in ROS production following *CrMTP4* over-expression, suggesting that the increased Cd tolerance provided by enhanced CrMTP4-mediated Cd transport activity was not due to reduced ROS accumulation. Although it cannot be ruled out that the wastewater-adapted *P. hussii*, *P. kessleri* and *C. luteoviridis* strains have higher abundance and activity of other metal tolerance mechanisms, such as metal transporters, compared to *C. reinhardtii*, these results suggest that oxidative stress tolerance activity is a better target in microalgae than enhanced metal sequestration alone. This also suggests that future genetic engineering strategies for microalgae metal bioremediation should additionally focus on manipulating oxidative stress tolerance. Moreover with potential concerns about the risks of genetically modified microalgae escape into the environment as well as regulatory restrictions [46], there is arguably currently greater attraction for natural strains rather than transgenic strains.

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Figure legends:

Fig. 1. Cloning of *CrMTP4* and characterization by yeast heterologous expression. (a) Phylogenetic comparison of CrMTP4 with selected Mn-CDF, Zn-CDF and Fe/Zn-CDF proteins from *Arabidopsis thaliana* (At), *Cucumis sativus* (Cs), *Oryza sativa* (Os), *Populus trichocarpa* x *P. deltoides* (Ptr), *Stylosanthes hamata* (Sh) and *Saccharomyces cerevisiae* (Sc). A maximum likelihood tree was derived from full length amino acid sequence alignment. Bootstrap values from 100 replications are indicated and the branch length scale bar indicates the evolutionary distance of 0.5 amino acid substitution per site. (b) Gene expression of *CrMTP4* in wild type cells in response to Cd and Mn treatment. Expression of *CrMTP4* as determined by real-time PCR is shown relative to *CBLP* expression. Data points are means (±SE) calculated from 3 independent biological replicates. Bars sharing the same lowercase letter indicate no significant difference between treatments ($P > 0.05$). (c) Suppression of Mn sensitivity of the *pmr1* yeast mutant, and Cd sensitivity of wild type (BY4741) yeast, both expressing *CrMTP4* in comparison with empty plasmid-containing strains. Liquid cultures of strains were serially diluted then spotted onto SD –Ura medium with or without added Mn or Cd. Yeast growth is shown after 3 d. A representative experiment is shown. (d) BY4741 strains transformed with empty plasmid or *CrMTP4* normalized to an identical starting cell density then grown in liquid YPD medium with or without added metals (0.1 mM Al, 75 µM Cd, 1 mM Co, 1 mM Cu, 5 mM Mn, 5 mM Zn) and grown for 24 h. Cell density was determined by optical density measurement at 600 nm. (e) Mn and Cd uptake in EDTA-washed BY4741 yeast transformed with empty plasmid or *CrMTP4* following growth in 2 mM Mn or 30 µM Cd YPD media. Data points are means (±SE) of 3 independent biological replicates. Bars indicated by asterisks show significant difference between empty plasmid and *CrMTP4* strain within each metal treatment (*, $P < 0.05$; **, $P < 0.01$).

Fig. 2. Generation of transgenic *CrMTP4* over-expression lines with enhanced Mn accumulation. (a) RT-PCR detection of *CrMTP4* mRNA transcript abundance in wild type cells in comparison to three independent *CrMTP4* overexpression (MTP4-OE) lines. RT-PCR was performed using *CBLP* as a normalization control transcript. (b and c) Cell density as determined by cell count measurement (b) and total chlorophyll yield (c) in wild type and MTP-OE lines over time in TAP medium with (red symbols) or without (black symbols) 2 mM Mn addition. (d) Cellular Mn content in EDTA-washed wild type and MTP-OE cells following growth in 2 mM Mn medium after 8 d. Data points are means (±SE) of 3 independent biological replicates. Bars indicated by asterisks show significant difference between wild type and MTP-OE line (*, $P < 0.05$).

Fig. 3. Cd tolerance of *CrMTP4* over-expression lines. (a) Culture phenotypes of wild type *C. reinhardtii* and *CrMTP4* overexpression (MTP4-OE) lines after 7 d growth in medium with or without Cd. A representative experiment is shown. (b and c) Cell density as determined by cell count measurement (b) and total chlorophyll yield (c) in wild type and MTP-OE lines over time in TAP medium with 0.4 or 0.5 mM Cd addition. Cell density and chlorophyll parameters are shown in comparison to control treatment without Cd addition after 8 d growth (right-hand panels). Data points are means (±SE) of 3 independent biological replicates. Bars indicated by different lower case letters show significant difference ($P < 0.05$) within Cd treatments and between wild type (WT) and MTP-OE lines. Bars indicated by asterisks show significant difference between control and Cd treatments (*, $P < 0.05$; **, $P < 0.01$).

Fig. 4. Cd tolerance and accumulation of *CrMTP4* over-expression lines in comparison to natural wastewater adapted microalgae strains. (a) Total chlorophyll yield of strains after 8 d of growth in TAP medium with or without (control) 150 µM Cd addition. Inset: normalized chlorophyll yield of strains following Cd treatment relative to treatment without Cd. (b) Cellular Cd content in EDTA-washed cells following growth in 150 µM Cd medium after 8 d. Data points are means (±SE) of 3 independent biological replicates. Bars indicated by

different lower case letters show significant difference ($P < 0.05$) within treatments, and pairs of bars indicated by asterisks show significant difference between control and Cd treatment (*, $P < 0.05$; **, $P < 0.01$).

Fig. 5. Metal tolerance and accumulation characteristics of natural wastewater adapted microalgae strains. (a and b) Cell density (a) and total chlorophyll yield (b) of natural strains treated without metal addition (Cont.) or with 100 µM Al, 100 µM Cu, and 225 µM Zn in TAP medium for 8 d. Inset graphs: normalized cell density (a) and chlorophyll yield (b) of strains following metal treatment relative to treatment without metals. (c) Cellular metal content in EDTA-washed cells following growth in 50 µM Al, 50 µM Cu and 150 µM Zn medium after 8 d. Data points are means (±SE) of 3 independent biological replicates. Bars indicated by asterisks show significant difference between metal treatment and control treatment, or between *C. reinhardtii* and the wastewater adapted strain (*, $P < 0.05$; **, $P < 0.01$).