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Basis of genetic adaptation to heavy metal stress in the acidophilic green alga *Chlamydomonas acidophila*

Fernando Puente-Sánchez[a], Silvia Díaz[b], Vanessa Penacho[c], Angeles Aguilera[d], Sanna Olsson[e,f,*]

[a] Systems Biology Program, Centro Nacional de Biotecnología (CNB-CSIC), Calle Darwin 3, 28049, Madrid, Spain
[b] Department of Physiology, Genetics and Microbiology, Complutense University of Madrid (UCM), Calle José Antonio Novais 12, 28040 Madrid, Spain
[c] Bioarray, S.L. Parque Científico y Empresarial de la UMH, Edificio Quorum III, Avenida de la Universidad s/n, 03202 Elche, Alicante, Spain
[d] Centro de Astrobiología (CSIC-INTA), Carretera de Ajalvir Km 4, 28850 Torrejón de Ardoz, Madrid, Spain
[e] INIA Forest Research Centre (INIA-CIFOR), Department Forest Ecology and Genetics, Carretera de la Coruña km 7.5, 28040 Madrid, Spain
[f] Department Agricultural Sciences, P.O. Box 27, 00014 University of Helsinki, Finland

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ABSTRACT

To better understand heavy metal tolerance in *Chlamydomonas acidophila*, an extremophilic green alga, we assembled its transcriptome and measured transcriptomic expression before and after Cd exposure in this and the neutrophilic model microalga *Chlamydomonas reinhardtii*. Genes possibly related to heavy metal tolerance and detoxification were identified and analyzed as potential key innovations that enable this species to live in an extremely acid habitat with high levels of heavy metals. In addition we provide a data set of single orthologous genes from eight green algal species as a valuable resource for comparative studies including eukaryotic extremophiles.

Our results based on differential gene expression, detection of unique genes and analyses of codon usage all indicate that there are important genetic differences in *C. acidophila* compared to *C. reinhardtii*. Several efflux family proteins were identified as candidate key genes for adaptation to acid environments. This study suggests for the first time that exposure to cadmium strongly increases transposon expression in green algae, and that oil biosynthesis genes are induced in *Chlamydomonas* under heavy metal stress. Finally, the comparison of the transcriptomes of several acidophilic and non-acidophilic algae showed that the *Chlamydomonas* genus is polyphyletic and that acidophilic algae have distinctive aminoacid usage patterns.

1. Introduction

Cd is a widespread environmental pollutant which is even at low concentrations extremely toxic to aquatic microorganisms, in particular microalgae (Brayner et al., 2011; Wang et al., 2013). In spite of its harmfulness there exist very few studies on transcriptomic alterations caused by increased levels of this or other heavy metals in green algae, (Hutchins et al., 2010; Jamers et al., 2013; Zhang et al., 2014). Cd binds to organic molecules by forming bonds with sulfur and nitrogen, thereby inactivating proteins causing a broad range of adverse effects. It is easily absorbed and bio-accumulated by lower organisms and transferred to higher trophic levels in food chain. It has been shown to inhibit growth (Okamoto et al., 1996), chlorophyll and chloroplast synthesis (Lamai et al., 2005), cause disintegration of the cell wall as well as induce a large increase in superoxide dismutase (SOD) activity, indicative of oxidative stress (Okamoto et al., 1996). Additionally, Cd replaces zinc and selenium at the active sites of enzymes, competes with other ions in membrane transport, andecreases RNA and DNA synthesis as well as photosynthetic pigments and proteins (Prasad and Strzalka, 1999; Wang et al., 2013).

The extremophilic green alga *Chlamydomonas acidophila* grows in very acidic environments (pH 2.3–3.4). Metal sequestration in vacuoles seems to be an important mechanism in cadmium tolerance and detoxification in *C. acidophila* (Aguilera and Amils, 2005) but there is evidence that also unique genetic features in *C. acidophila* contribute to its high heavy metal tolerance (Olsson et al., 2015; Olsson et al., 2017). The strain analyzed in this work was isolated from Río Tinto (SW Spain), one of the most extensive examples of natural extreme acidic environments (Fernández-Remolar et al., 2003). The river has a low pH (ranging from 0.8 to 2.5) buffered by ferric iron and with high concentrations of heavy metals (Aguilera et al., 2006). These extreme conditions are produced by the metabolic activity of chemolithotrophic prokaryotes that are found in high numbers in its waters (González-Toril et al., 2003). Despite these extreme environmental conditions, Río

* Corresponding author at: INIA Forest Research Centre (INIA-CIFOR), Department Forest Ecology and Genetics, Carretera de A Coruña km 7.5, E-28040 Madrid, Spain.
E-mail address: sanna.olsson@helsinki.fi (S. Olsson).

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Tinto shows an unexpected degree of eukaryotic diversity (Amaral-Zettler et al., 2011). Cd was chosen for this study due to its toxicity and also because it is found in very high concentrations in Río Tinto, with local average amounts that can reach ca. 40 mg/L (Aguilera et al., 2007).

Research on extremophilic organisms significantly contribute to our understanding of the evolution of heavy metal tolerance in plants and algae. The results enable detection of novel genes potentially useful for biotechnology and phytoremediation of contaminated water resources. In spite of this, there is very limited genetic data available for *C. acidophila* while the genome of *C. reinhardtii* has been sequenced and annotated (Merchant et al., 2007; Manichaikul et al., 2009). For *C. reinhardtii*, there also exist several physiological, molecular, and genetic studies including experimental verification of the functionality of the predicted ORFs (Ghamsari et al., 2011). To increase genomic resources in *C. acidophila* we assembled an improved transcriptome for this non-model species. We compared it to the transcriptomes of the model microalga *Chlamydomonas reinhardtii* from the same genus and other publicly available green algal transcriptomes. To explain how *C. acidophila* is able to survive extreme environments we used transcriptomic sequencing and qRT-PCR to detect transcriptional changes caused by high Cd concentrations in *C. reinhardtii* and *C. acidophila* and identified possible adaptive key genes. The high level of genes with unknown function as well as lack of an annotated genome assembly makes the identification of important genes involved in heavy metal detoxification in *C. acidophila* challenging. In spite of these difficulties we provide new information on heavy metal tolerance in this organism, extremophiles and green algae in general.

2. Material and methods

2.1. Sample collection, cultivation and exposure to Cadmium

Chlamydomonas acidophila strain RT46 was collected from water samples taken in 2010 at the CEM sampling station of Río Tinto (SW Spain) (Aguilera et al., 2006), and isolated to grow in the presence of antibiotics, vancomycin 50 μg/mL, cefotaxime 100 μg/mL and chloramphenicol 15 μg/mL (Sigma), on agar plates made with 0.22 μm-filtered river water. Individual colonies were transferred into K medium (Keller et al., 1987), pH 2. A strain of *Chlamydomonas reinhardtii* (CC-1374, SAG 77.81) was purchased from the Chlamydomonas Resource Center (University of Minnesota) and grown in K medium, pH 7. The K medium was supplied with the same antibiotics as the ones used for cell isolation (vancomycin 50 μg/mL, cefotaxime 100 μg/mL and chloramphenicol 15 μg/mL).

The algae were grown under ca. 70 $\mu E\, s^{-1}\, m^{-2}$ irradiance provided by day-light fluorescent tubes, 16:8 h LD cycle and 22 °C of temperature. The cultivations were refreshed every two weeks in corresponding growth media and cells undergoing exponential growth were grown to be treated with metalloid solutions. To reach exponential growth 5 ml of *Chlamydomonas* cultivate was transferred into an 1 l Erlenmeyer bottle with 500 ml medium. After 10 days of growth 15 ml of cultivate was transferred into three 2 l Erlenmeyer bottles with 980 ml medium in each.

For the transcriptomic sequencing a Cd solution ($CdCl_2 \times 2\ ½\ H_2O$) with a final concentration of 245 μM was used. Earlier studies on *Chlamydomonas* showed a peak of gene expression between three and four hours in genes involved in cadmium tolerance (Hanikenne et al., 2005; Olsson et al., 2017). Therefore time points for cell collection were set before the treatment, at 3 h and 6 h after Cd exposure. The cells were collected in 50 ml Falcon tubes, centrifuged for 10 min in 5000 rpm, the supernatant was discarded and the pellets frozen at −80 °C until RNA extraction. For qRT-PCR cultures were treated with following solutions: 1 μM Cd solution ($CdCl_2 \times 2\ ½\ H_2O$), 1 mM Cu ($CuSO_4 \times 5H_2O$), 10 mM Fe ($FeSO_4 \times 7H2O$), 1 mM As (III) ($AsNaO_2$) or 5 mM As(V) (Na_2HAsO_4) and cells were collected at 1, 3 or 24 h after exposure.

2.2. RNA extraction and sequencing

Total RNA was extracted with TRI Reagent® Solution (Ambion, Life Technologies, CA, USA) following manufacturer's protocol. RNA quality and quantity were estimated using an Agilent 2100 bioanalyzer (Agilent Technologies). RNA library preparation and high-throughput sequencing were carried out in the NGS sequencing Unit (Scientific Park Foundation, Madrid, Spain) using Illumina GAiix sequencing platform. One full lane of 75 basepair long reads for each sample was sequenced to provide sufficient coverage for a representative overview of the expression profile. The generated transcriptome library was non-normalized to allow detection of differences on the gene expression level between the different treatments and untreated cultures.

2.3. Data preprocessing de novo hybrid assembly

All raw transcriptomic reads were filtered and trimmed with PRINSEQ lite (version 0.18.3 (Schmieder and Edwards, 2011) in order to remove duplicates and low quality reads (using default parameters except for the following: –min qual mean 25 –derep 12, –ns max p 1–derep 12–lc method dust −lc threshold 7–trim tail left 6–trim tail right 6–trim ns left 2–trim ns right 2–trim qual left 25–trim qual right 25).

The single-end Illumina reads from *C. acidophila* obtained in this study were combined with the 454 reads obtained in Olsson et al. (2015). Paired-end Illumina reads were simulated from 454 reads by using the *run_simulate_illuminaPE_from_454ds.sh* script included in the Trinity suite. The resulting reads were subsequently normalized in silico with the *normalize_by_kmer_coverage.pl* included in Trinity. The paired-end normalized reads coming from the 454 dataset were pooled together with the single-end Illumina reads obtained in this study, and assembled with Trinity (release 2013_08_14) (Grabherr et al., 2011) using Jellyfish (Marcais and Kingsford, 2011) for k-mer counting with a maximum memory of 40G, minimum contig length of 200, paired fragment length of 350 and a maximum butterfly heap space of 20G. Contigs with a BLASTn identity of more than 90% to the *E. coli*, *C. reinhardtii* and human transcriptomes were discarded.

2.4. Abundance estimation and transcriptome coverage analysis

The RSEM software package (version 1.1.18.modified) (Li and Dewey, 2011) was used to estimate the gene and isoform expression values. For *C. acidophila*, a reference transcriptome was generated from the Trinity assembly by using the RSEM commands e*xtract-transcript-to-gene-map-from-trinity* and *rsem-prepare-reference* with default parameters. For *C. reinhardtii,* the reference transcriptome v4.0 (Merchant et al., 2007) available from Phytozome (http://www.phytozome.net/) was used as a reference for estimating transcript expression. Reads from the six samples were aligned separately to the reference transcriptomes by using Bowtie (version 0.12.7) (Langmead et al., 2009) and expression values for each sample were obtained with RSEM. The resulting expression counts were normalized with the trimmed mean of M-values method, as implemented in the edgeR package (version 2.15.0) (Robinson et al., 2010). The transcripts with a log2 fold change higher than 6 and FPKM (Fragments Per Kilobase Million) of more than 20 in at least one sample were selected for further analysis. For *C. acidophila*, only the longest transcript per Trinity subcomponent was reported.

In order to assess the coverage of each sequence in our *C. acidophila* assembly, reads from the three *C. acidophila* samples were pooled and aligned against the reference transcriptome. We used the *align-reads.pl* script included in the Trinity package (release 2013_08_14), resorting to Bowtie (version 0.12.7) to perform the alignment. The script also utilized Samtools (version 0.1.18) (Li et al., 2009) for SAM-format alignment manipulations. The output file bowtie out.coordSorted.bam, which contains both properly mapped pairs and single unpaired fragment reads, was used as input f*or Quali*map (version 0.6) (García-

Alcalde et al., 2012) in order to estimate transcript coverage.

2.5. Taxonomic and functional annotation

All transcripts were annotated via BLASTx searches (Altschul et al., 1997). For taxonomic annotation GenBank's non-redundant protein database (nr) was used. For functional annotation two other major databases, Uniprot's Swiss-Prot and TrEMBL protein databases were used in addition to the nr database to get more accurate information on genetic functions. Taxonomic and functional information from the multiple databases for each differentially expressed contig was collected into a table preferring the most accurate functional annotation from Swiss-Prot when available using the methods and scripts modified from De Wit et al. (2012).

2.6. Protein prediction and orthology search with OrthoFinder across green algal transcriptomes

To identify orthologous gene groups among green algae, representative transcriptome files were downloaded from Phytozome v.11 (http://www.phytozome.net/) for six available species: *Chlamydomonas reinhardtii*, *Coccomyxa subellipsoidea*, *Micromonas pusilla*, *Micromonas* sp. RCC299, *Osterococcus lucimarinus* and *Volvox carteri*. The *de novo* assemblies of *C. acidophila* and *D. acidophila* (Puente-Sánchez et al., 2016) were translated to amino acids with TransDecoder (v. 3.0.0, The Broad Institute). Orthologous sequences from these eight species were grouped with the clustering software OrthoFinder (Emms and Kelly, 2015). The resulting alignments were filtered to contain only the longest isoform of *C. acidophila* and *D. acidophila* when several isoforms of the same gene (belonging to same component and subcomponent in the *de novo* assembly built with Trinity) were present in the same orthologous group. Orthologous groups related to heavy-metal tolerance were subject to further analyses while orthologous groups representing putative single-copy nuclear genes (an orthologous group with exactly one gene/species) present in all species were used to build a phylogeny.

2.7. Genes present in C. acidophila but not in C. reinhardtii

To find an explanation for the different responses to heavy metals in extremophiles and neutrophiles, two approaches to identify genes that are present in *C. acidophila* but not *C. reinhardtii* were employed. First, screening for genes related to heavy metal tolerance and detoxification was done based on keywords in the annotation of the *C. acidophila* transcriptome. Only transcripts that had other organisms than *C. reinhardtii* as first BLAST match were included. To verify that the identified candidate genes are not present in *C. reinhardtii*, a local BLASTn search against *C. reinhardtii* transcripts was performed. Reciprocal BLAST was performed to confirm the matches and confirmed isoforms were used in downstream analyses.

Secondly, to identify genes specific to acidophiles, orthologous groups containing both of the extremophiles (*C. acidophila* and *D. acidohila*) but not *C. reinhardtii* were extracted. As a precaution to exclude contaminant sequences, in the absence of reference genomes for the extremophiles, only the orthologous groups containing at least one additional green algal species were kept. In addition transcripts with organellar annotations (mitochondrial or chloroplast) were excluded. Phylogenetic analyses were made for the transcripts with an annotation related to heavy metal tolerance and detoxification after confirming their absence in *C. reinhardtii* by a BLASTx against nr database with a cut-off E-value of $\leq 10-3$.

2.8. Phylogenetic analyses

Sequences were aligned with Mafft (Katoh et al., 2002). For individual genes the alignments were manually edited in PhyDE® v1.0 (Müller et al., 2005) by excluding ends of the alignments which could not be confidently aligned due to length differences and ambiguities in homology assessment. The concatenated data matrix of 488 single orthologous groups was trimmed with Trimal (Capella-Gutiérrez et al., 2009) using the option −gappyout. Bayesian analyses were performed with MrBayes v3.2.1 (Ronquist et al., 2012), applying the suggested search strategies for amino acids (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). For the individual genes four runs with four chains (1×10^6 iterations each) were run simultaneously while for the concatenated matrix of 488 single orthologous groups four runs with two chains (1×10^6 iterations each) were run. Chains were sampled every 1000 generations and the respective trees written to a tree file. Calculations of the consensus tree and of the posterior probability of clades were performed based upon the trees sampled after the chains converged. The concatenated matrix was also analyzed using RAxML (Stamatakis, 2006; Stamatakis et al., 2008) defining the used model automatically with the option −m PROTGAMMAAUTO. Consensus topologies and support values from the different methodological approaches were compiled and drawn using TreeGraph2 (Stöver and Müller, 2010).

2.9. Quantitative reverse transcription PCR (qRT-PCR)

For qRT-PCR protocols established by Díaz et al. (2007) were followed, applying the modifications detailed in Olsson et al. (2017). Actin (ACT1) and 18S were used as endogenous control genes. All qRT-PCR reactions were carried out in an iQTM5 multicolor Real-Time PCR detection System (Bio-Rad) apparatus with the following cycling conditions: (i) 5 min at 95 °C to denature reverse transcriptase, (ii) 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C after 20 s. Both NTC (no template control) and RT minus control were negative. The real-time dissociation curve was used to check primer specificity and to confirm the presence of a unique PCR product. Standard curves were obtained using 10-fold serial cDNA dilutions and determining the Ct (cycle threshold) values. The standard line parameters (amplification efficiency, slope and correlation coefficient) are reported in Table 1. Analysis of relative gene expression was carried out according to the Standard-curve quantification method (Larinov et al., 2005) from, at least, four independent experiments (each performed in duplicates). Primers for qRT-PCR were designed using the program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with default settings. All primers used in this study are listed in Table 2.

2.10. Codon usage bias and GC content analyses

Complete CDSs (coding DNA sequences) were extracted from the eight algal transcriptomes (*Chlamydomonas acidophila, Dunaliella acidophila, Chlamydomonas reinhardtii*, *Coccomyxa subellipsoidea*, *Micromonas pusilla*, *Micromonas* sp. RCC299, *Osterococcus lucimarinus* and *Volvox carteri*) by using the Transdecoder software included in the Trinity suite. GC content and codon and aminoacid usage for each CDS were calculated with GCUA (General Codon Usage Analysis; McInerney,

Table 1
Quantitative real-time RT-PCR standard-curve parameters for selected transcripts present in C. *acidophila* but not in *C. reinhardtii* and the expression control (housekeeping) genes 18S rNA and actin. S = slope, R^2 = correlation coefficient, E = amplification efficiency.

Gene	S	R2	E
ACR3	−2.858	0.99	2.24
Arsenite transporter	−2.885	0.97	2.22
AcrB	−3.04	0.99	2.13
Glutathione-regulated potassium-efflux family	−2.823	1	2.26
MATE efflux protein	−3.079	0.99	2.11
Arsenite-antimonite efflux family	−2.96	0.96	2.18

Table 2
Primers used for quantitative real-time RT-PCR used in this study. For each region, forward (F) and reverse (R) primers are indicated, as well as product size.

Gene	Primer name	5′ Sequence 3′	F/R	Product size (bp)
Multidrug efflux transporter AcrB comp_16471	comp16471_AcrB-F	GTAGGCATTCCCTTGCTGTC	F	89
	comp16471_AcrB-R	CCAAGGACCAAAACAAGCAT	R	
ACR3 comp_14907	comp14907_ACR3-F	ACTTTTGGCTTCTGGGAGGT	F	106
	comp14907_ACR3-R	TTTCACCATAAGCCCAGACC	R	
Arsenite transporter comp_15936	comp15936_ArsB-F	AATGTTACGGCAAAGCGAAC	F	100
	comp15936_ArsB-R	CAGTCACTGGCGAGCTCATA	R	
MATE efflux protein comp_12911	comp12911_MATE-F	ACTTTGGGTTCATGGCTTTG	F	98
	comp12911_MATE-R	CACTCCTGCCAGTCCTAACC	R	
Arsenite-antimonite efflux family comp_15332	comp15332_MATE-F	CTAACACTCCTGTGGCAGCA	F	125
	comp15332_MATE-R	CAGCCTGTTAAGCCCTTTTG	R	
Glutathione-regulated potassium-efflux family comp_16013	comp16013_K-efflux-F	CGCTAGAAATTCCCAACCAG	F	87
	comp16013_K-efflux-R	GCATTTCTTTGCACCTCCAT	R	

Table 3
Sequence statistics on A) Illumina sequencing and for comparison, statistics on 454 reads from Olsson et al. (2015) are also shown. B) transcriptome assembly with Trinity using a hybrid assembly strategy combining 454 reads with Illumina reads.

A

Illumina sequencing

Library	Condition	Raw reads	Input bases (Gb)	Trimmed reads	Discarded sequences (including duplicates)
J1	Reinhardtii-0h	21243002	1.61	451530	9504620
J2	Reinhadtii-3h	21819884	1.66	459756	10645037
J3	Reinhadtii-6h	19474228	1.48	438115	9709139
J4	Acidophila-0h	23656624	1.80	477750	11356978
J5	Acidophila-3h	22928585	1.74	503621	11287054
J6	Acidophila-6h	22006149	1.67	466332	11948336

454 data from Olsson et al. (2015)

Input 454 reads	**Simulated Illumina reads**	**Normalized simulated pairs**
1021062	458306001	7717263

B

Hybrid assembly

Input pairs	7717263
Input SE reads	33998990
Bases in assembly (Mb)	293
Trinity genes	47411
Trinity isoforms	151449
Isoform median length	1398
Isoform mean length	1936.66
Range of isoform lengths	201–19360
Isoform N50	3212
Isoform mean coverage	54.62X
Isoform std coverage	107.15X
Isoforms after filtering	129188
Isoforms after filtering with nr BLAST matches	87676

1998). For each gene, only the longest transcript was included in the analysis.

2.11. GO-terms enriched/depleted in particular aminoacids in acidophiles versus non-acidophiles

In order to detect Gene Ontology (GO) terms with a significant enrichment/depletion of particular aminoacids in acidophiles versus non-acidophiles, the following procedure was followed. Firstly, we selected the proteins with i) less than 2% of glutamate, ii) less than 2% of aspartate, iii) more than 4% of cysteine, iv) more than 15% of serine. These proteins will henceforth be referred to as “extreme” proteins. The particular aminoacids and the percentage cutoff values were selected after inspection of the aminoacid utilization profiles shown in the figure obtained in the previous section and shown in Additional File 6. For each of the four aminoacids, we then counted the number of appearances of each GO-term in the “extreme” proteomes and in the “non-extreme” proteomes of the two acidophilic species and the six non-acidophilic species, respectively. This was information used to build the following contingency table for each GO-term, which was subjected to the Fisher’s Exact test in order to assess whether that particular GO-term was significantly enriched ($p < 0.05$) in the extreme fraction of the proteome in acidophiles versus non-acidophiles, that is, was significantly enriched/depleted in that particular aminoacid in acidophiles versus non-acidophiles.

# GO-term appearances in the extreme proteome of acidophiles	# GO-term appearances in the non-extreme proteome of acidophiles
# GO-term appearances in the extreme proteome of non-acidophiles	# GO-term appearances in the non-extreme proteome of non-acidophiles

3. Results and discussion

3.1. High-throughput sequencing, assembly and taxonomic annotation of C. acidophila transcripts

Six single-end Illumina Hi-Seq libraries were sequenced in order to monitor the transcriptomic response of *Chlamydomonas reinhardtii* and *Chlamydomonas acidophila* to cadmium stress right after cadmium exposure, three hours after exposure and six hours after exposure. A total of 131,128,472 raw reads were generated, of which 66,677,308 passed quality filtering, with the duplication level being consistent with that found in other studies (Gómez-Álvarez et al., 2009).

In order to obtain a high quality draft transcriptome for *C. acidophila*, the reads obtained in this study were pooled together and co-assembled with the reads obtained in Olsson et al. (2015). This yielded 151449 transcripts of unique isoforms corresponding to 47411 unique Trinity subcomponents (which can be interpreted as distinct genes), with a N50 of 3212 nucleotides, and average isoform coverage of 54.62X. The pre-processing and assembly statistics are summarized in Table 3A and B. The hybrid assembly significantly improved the assembly results and genome fraction coverage over the existing assembly from the earlier study (GenBank accession GBAH00000000) for which only 454 reads were used.

3.2. Differential expression analysis of transcripts

For both species, the gene expression after 3 h and 6 h of cadmium exposure was compared to the gene expression right before cadmium exposure. H43 (Rubinelli et al., 2002) and Cds1 (Hanikenne et al., 2005) are among the few genes that have been identified to be induced by cadmium in *C. reinhardtii*. In addition a novel phytochelatin synthase CaPCS2 was recently showed to be strongly induced by Cd in *C. acidophila* (Olsson et al., 2017). Transcripts homologous to these genes were not found to be differentially expressed in this study, possibly due to the strict cutoff values applied. The time and concentration of the exposure might also greatly affect the transcriptomic response of green algae to Cd. Hanikenne et al. (2005) observed a peak of expression in the half-size ABC transporter gene *Cds1* at 4 h after 200–400 μM Cd exposure and argued that the transcript levels of this gene were too low to be detected under the experimental conditions (2 h exposure to 25 μM cadmium) used earlier by Rubinelli et al. (2002). On the other hand, Olsson et al. (2017) reported a very strong induction of the gene CaPCS2 in as low concentration as 1 μM. Furthermore, different isoforms might result in different expression values.

In this study we focused on genes showing differential gene expression when exposed to very strong Cd exposure. To complement the gene expression profile of selected candidate genes qRT-PCR was performed using different concentrations and time points.

3.3. Differentially expressed genes in C. reinhardtii

The low number of transcripts detected to be differentially expressed in *C. reinhardtii* (Additional File 1) is likely due to the high amount of Cd used in the experiment, which was chosen to give a visible effect on the transcriptomic expression in *C. acidophila*.

The transcripts with highest increase in expression after Cd exposure between control and one of the cadmium treated samples include transcripts coding for an apoptosis-inducing factor, NSG6 protein, NifU-like protein 5, and a vacuolar protein sorting-associated protein, in addition to transcripts with unknown function. Induction of stress related genes, as well as genes operating in metal uptake and export as a response to cadmium has been observed previously in *C. reinhardtii* (Jamers et al., 2013) as well as in cyanobacteria (Houot et al., 2007). Other differentially expressed genes could not be directly linked to heavy metal detoxification but can nonetheless be related to stress responses. For example, NSG6 is involved in gametogenesis and induced under nitrogen starvation (Abe et al., 2004). Interestingly, gametogenesis in *C. reinhardtii* leads to an increased production of lipids with use as biofuels (Miller et al., 2010). Here we show that, apart from nitrogen starvation, this process can also be induced by heavy metal stress, opening the way for novel engineering strategies in the search for high oil yields

3.4. Differentially expressed genes in C. acidophila

The top fifty up regulated higher transcripts are summarized in Additional File 2, and included several transposable elements. Significant upregulation was observed in a transcript annotated as retrotransposon copia (FPKM in 0 h 0, 3 h 240.83, 6 h 840.66 in comp17295_c0_seq16), a transcript annotated as retrovirus-related Pol polyprotein from transposon TNT 1–94 (the annotation varies according to isoform, highest FPKM in comp17071_c1_seq27: 0 h 1.84, 3 h 156.11, 6 h 676.71), retrovirus-related Pol polyprotein from transposon 297 (comp18064_c0_seq15) and Transposon Ty3-I Gag-Pol polyprotein (comp16440_c0_seq16). Retrotransposons are assumed to be a major driving force for genome evolution through genome organization and gene regulation in plants (Flavell et al., 1992), some being transferred horizontally (Cheng et al., 2009 and references therein). There are indications that retrovirus and retrotransposons are involved in gene regulation and detoxification of heavy metals. Retrovirus-related Pol polyprotein from transposon TNT 1–94 has been shown to alter its methylation status in *Populus alba* when grown on heavy metal contaminated soil (Cicatelli et al., 2014). Castrillo et al. (2013) showed that heavy-metal stress induced transposon activity in plants. Exposure to Cd strongly increased transposon expression in *C. acidophila*, which suggests for the first time that heavy-metal stress induces transposon activity also in green algae.

There are several transcripts with the annotation arsenite resistance protein ArsB among the differentially expressed genes (comp14907_c0 or comp15936_c0). The annotations are partly incongruent, comp14907_c0 getting annotated as arsenite resistance protein ArsB or ubiquitin-like modifier-activating enzyme ATG7, while the annotations for comp15936_c0 are arsenite resistance protein ArsB or arsenate reductase. The automated annotation is complicated by the fact that the nomenclature of the ACR3 family ArsB protein overlaps with ArsB of *E.coli* belonging to the ArsB family (Wit Wu et al., 1992). It was verified from the alignments including all isoforms (data not shown) that all isoforms of one component belong together, and the different annotations are due to lack of highly similar sequences in GenBank of some sequence parts. To avoid confusions in this manuscript the isoforms of comp14907_c0 are referred to arsenical-resistance protein ACR3 and isoforms of comp15936_c0 as ACR3 family arsenite transporter based on the annotation of the consensus sequences of these isoforms. Most differentially expressed transcripts of both comp15936_c0 and comp14907_c0 are strongly induced by Cd, with the exception of comp15936_c0_seq52. However, according to qRT-PCR analyses the ACR3 family arsenite transporter comp_15936_c0 is down-regulated by Cd (Table 4). The incongruent results between the measures based on the gene expression data and the qRT-PCR are likely due to the differences in the used Cd concentrations.

An oil globule associated protein (comp13235_c0_seq1) was detected to be induced by copper by Olsson et al. (2015), and is now shown to be also induced by Cd (FPKM in 0 h 0, 3 h 10.058, 6 h 30.682). This again highlights the role of heavy metals as inductors of oil production in *Chlamydomonas* (see previous section). Furthermore, the ability of *C. acidophila* to tolerate extreme acidity and heavy metal concentrations might help it avoid the contamination issues that commonly hamper microalgal biodiesel production (Siaut et al., 2011; Wang et al., 2016). While a detailed study of the oil production potential of *C. acidophila* is beyond the scope of this manuscript, our findings warrant further investigation on its biotechnological applications.

Table 4
Results of gene expression analysis by qRT-PCR of selected genes present in *C. acidophila* but not in *C. reinhardtii*. The cells were collected at 1, 3 or 24 h after exposure to 1 μM Cd solution ($CdCl_2 \times 2\ ½\ H_2O$), 1 mM Cu ($CuSO_4 \times 5H_2O$), 10 mM Fe ($FeSO_4 \times 7H2O$), 1 mM As (III) ($AsNaO_2$) or 5 mM As(V) (Na_2HAsO_4). The relative mRNA expression levels of target genes were normalized against the levels of actin and 18S. The fold induction and SD for each target gene is shown. ACR3 comp14907 = arsenical-resistance protein ACR3, ACR3 comp15936 = ACR3 family arsenite transporter, MATE comp12911 = MATE efflux protein, Arsenite-antimonite comp15332 = Arsenite-antimonite efflux family, AcrB comp16471 = Multidrug efflux transporter AcrB, MATE comp12911 = MATE efflux protein. Nd = Not defined.

	ACR3 comp14907	ACR3 comp15936	MATE comp12911	Arsenite-antimonite comp15332	AcrB comp16471	MATE comp12911
Cd 1h	−12,98 ± 1,97	−513 ± 126,1	−675,6 ± 79,1	−2327 ± 574	−761 ± 112	−8,1 ± 2,5
Cd 24h	−6,36 ± 0,8	−14,3 ± 3,6	−20,62 ± 3,0	−2862 ± 599	−609 ± 113	−12,5 ± 3,3
As(III) 1h	0,52 ± 0,06	−1,19 ± 0,09	−1,04 ± 0,3	−10,2 ± 1,4	−13,5 ± 2	−0,7 ± 0,1
As(III) 3h	−293,79 ± 36,59	−1824 ± 138	−731,7 ± 135,4	−15158 ± 1135	−3142 ± 378	−4,2 ± 1,2
As(III) 24h	−11,7 ± 1,45	−3445 ± 261	−314,9 ± 46,1	−3531 ± 864	−476 ± 70	−18 ± 5,4
Cu 1h	4,15 ± 0,52	−3687 ± 1012	3,38 ± 1,1	−3,8 ± 1,2	−6639 ± 786	−55 ± 11,7
Cu 3h	4,65 ± 0,5	−6358 ± 2424	5,6 ± 0,4	−11,48 ± 3,3	−690 ± 81	−1,5 ± 0,4
Cu 24h	1,04 ± 0,12	−170,2 ± 35,7	−26,02 ± 3,9	−554,9 ± 89	−207 ± 30	−47 ± 12
Fe 3h	Nd	Nd	1,31 ± 0,2	−819,8 ± 94,7	Nd	Nd
Fe 24h	0,36 ± 0,05	1,18 ± 0,2	0,8 ± 0,1	−2,2 ± 0,3	Nd	Nd

3.5. Species phylogeny based on orthologous sequences

We identified 488 single orthologues present in all eight species (Additional File 3), which were used to build a species phylogeny. According to the phylogenomic analyses the genus *Chlamydomonas* is not monophyletic (Fig. 1). This is not so surprising since *Chlamydomonas* is known to be polyphyletic and in need for revision, first shown by Buchheim et al. (1990) and confirmed by several later studies (e.g. Leliaert et al., 2012; Nakada et al., 2016). However, earlier phylogenies have been based on few molecular markers and now the polyphyly of *Chlamydomonas* is shown for the first time on a phylogenomic level. *Micromonas pusilla* was resolved as best root in the species tree. All clades got full support both with MrBayes and RAxML.

3.6. Identification of genes unique to C. acidophila

Some genes can be important in heavy metal tolerance and metal homeostasis even if their expression is not altered in the presence of the metal. Most phytochelatin synthases, for example, are known to be constitutively expressed but post-translationally activated by heavy metals in plants (Cobbett and Goldsbrough, 2002; Rea et al., 2004). To better understand the mechanisms that enable *C. acidophila* to live in its extremely acid environment we therefore identified genes involved in heavy metal tolerance and detoxification that are present in *C. acidophila* but do not have an orthologue in *C. reinhardtii*, irrespective of their expression. Two approaches were employed.

First we identified thirteen candidate genes based on annotations of the transcripts and verified by reciprocal Blast searches as explained in material and methods (Table 5). Of these, in addition to the ACR3 family members discussed above, transcripts with following annotations

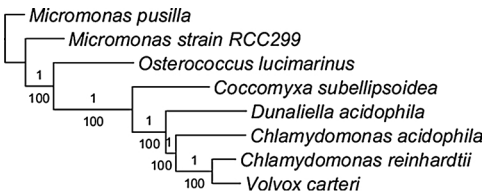


Fig. 1. Phylogenetic relationships based on 488 nuclear single orthologous genes clustered with OrthoFinder and present in all eight species (*Chlamydomonas acidophila, Dunaliella acidophila, Chlamydomonas reinhardtii, Coccomyxa subellipsoidea*, *Micromonas pusilla*, *Micromonas* sp. RCC299, *Osterococcus lucimarinus* and *Volvox carteri*). The trees represent the majority consensus of trees sampled after stationarity in the Bayesian analysis. Posterior probability values from the Bayesian inference are indicated above, the corresponding bootstrap values of the maximum likelihood analysis below the branches.

were up-regulated: several isoforms of mitochondrial carrier protein MTM1 (comp10226_c0), which carries manganese for the mitochondrial superoxide dismutase, and of the MATE efflux family protein DETOXIFICATION 44 (comp12911_c0). To test for changes in gene expression caused by a low Cd exposure and metal specificity, qRT-PCR was performed on a selection of these candidate genes unique to *C. acidophila* (Table 4). Cells were collected for qRT-PCR at 1, 3 or 24 h after exposure to Cd (1 μM), Cu (1 mM), Fe (10 mM), As (III) (1 mM) or As(V) (5 mM). Due to degraded cDNA Cd and Fe are represented by only two time points each.

Cd was noted to somewhat affect the expression of 18S and therefore the relative mRNA expression levels of target genes were normalized against the levels of actin. Surprisingly, none of the tested transcripts were detected to be significantly induced by any of the added metals but significant down-regulation can be observed in most of them (Pair Wise Fixed Reallocation Randomisation test, $p < 0.01$). These incongruences could be due to known methodological caveats in RNA-seq including different expression of the different isoforms, gene duplications or artifacts in assembly and annotation (Conesa et al., 2016). They demonstrate the importance of detailed functional studies of individual genes, although automated studies with massive input can offer useful information about general trends and serve as first step for further studies.

Secondly, we employed OrthoFinder to cluster genes and detect those unique to acidophiles (*C. acidophila* and *Dunaliella acidophila*). Eighteen genes present in both extremophilic species and at least one further algal species were extracted from the resulting orthologous groups (Table 6). Three of them (phytochelatin synthase CaPCS2, Arsenical-resistance protein ACR3 and multidrug efflux transporter AcrB) were detected both by the first method based on key word search for metal tolerance from the annotations and the second method based on filtering of orthologous groups.

Some of the key candidate genes highlighted in this study have been shown to enhance heavy metal tolerance in *C. acidophila* or other organisms. The phytochelatin synthase CaPCS2 was shown to be strongly induced by Cd in *C. acidophila* and cloning and expression of the gene in *Escherichia coli* clearly improved its cadmium resistance (Olsson et al., 2017). Cobalamin has been shown to protect against oxidative stress in the acidophilic iron-oxidizing bacterium *Leptospirillum* (Ferrer et al., 2016). Arsenical-resistance protein ACR3 is suggested to be a key trait to its arsenic tolerance in the arsenic hyperaccumulator *Pteris vittata* (Indriolo et al., 2010) and it might similarly enhance the tolerance to heavy metals in *C. acidophila*. Arsenite resistance efflux pump ArsB, which pumps arsenite and antimonite, but not arsenate or cadmium, was first described in *E. coli* (Wit Wu et al., 1992. Our results suggest that these genes could be key traits for heavy-metal hypertolerance in *C. acidophila*. It has been proposed in other extremophiles as well that

Table 5
Transcripts coding for genes that are involved in heavy metal tolerance present in *C. acidophila* but not in *C. reinhardtii* based on transcript annotations.

Contig name	Putative function	BLAST top match organism	BLAST match accession	E-value
comp10128_c0_seq1	Peroxisome isogenesis	*Coccomyxa subellipsoidea*	XP_005647114	3.01E-39
comp10226_c0_seq5	Mitochondrial carrier	*Coccomyxa subellipsoidea*	XP_005652123	3.95E-27
comp11852_c0_seq1	Phytochelatin synthase	*Calothrix* sp.	YP_007140091	6.44E-30
comp12911_c0_seq40	Protein DETOXIFICATION 44	*Chlorella variabilis*	EFN56963	6.62E-22
comp13602_c0_seq11	NRAMP family protein	*Volvox carteri*	XP_002947173	5.89E-153
comp14042_c0_seq2	ABC-ATPase	*Coccomyxa subellipsoidea*	XP_005643834	1.62E-92
comp14907_c0_seq53	Arsenical-resistance protein ACR3	*Coccomyxa subellipsoidea*	XP_005649016	1.99E-29
comp15241_c0_seq3	Cobalamin biosynthesis CobW	*Chlamydomonas reinhardtii*	XP_001699037	7.26E-60
comp15241_c0_seq6	Cobalamin biosynthesis CobW	*Burkholderia vietnamiensis*	YP_001117931	1.28E-80
comp15332_c0_seq3	Arsenite-antimonite efflux family	*Guillardia theta*	EKX52062	3.20E-66
comp15936_c0_seq52	ACR3 family arsenite transporter ArsB	*Coccomyxa subellipsoidea*	XP_005649501	6.10E-54
comp16013_c0_seq1	Glutathione-regulated potassium-efflux system	*Volvox carteri*	XP_002953483	8.63E-37
comp16471_c0_seq3	Multidrug efflux transporter AcrB	*Zea mays*	AFW59203	4.15E-58
comp17557_c1_seq9	Multidrug resistance-associated protein	*Coccomyxa subellipsoidea*	XP_005651467	1.59E-144

just a few key genes would be responsible for their hypertolerance to heavy-metals, for example Fer1 in the acidophilic archaeon *Ferroplasma acidarmanus* (Baker-Austin et al., 2007).

3.7. Phylogenetic distribution of candidate key genes involved in heavy-metal hyper-tolerance in C. acidophila

Most of the transcripts not present in *C. reinhardtii* with an annotation related to heavy metal tolerance are most closely related to genes in other green algae or vascular plants (Fig. 2A and Additional File 4). However, some transcripts get a first Blast hit in other algae, fungi, prokaryotes and amoebozoa. The phylogenetic distribution patterns in these genes can be explained by ancient gene duplications, loss in some lineages or horizontal gene transfer, and according to our results there are more than one explanation for the origin of these genes.

The mitochondrial carrier MTM1 (comp_10226, Fig. 2B), DETOXIFICATION 44 protein (comp_12911, Additional File 4) and arsenite-antimonite efflux family (comp_15332, Additional File 4) include both green algae and Chromalveolata among the most closely related genes. The phytochelatin synthase CaPCS2 (comp 11852, Additional File 4), which is located within a clade of prokaryotic genes, has been functionally characterized and shown to likely originate from horizontal gene transfer from bacteria (Olsson et al., 2017). The cobalamin biosynthesis protein CobW contains two gene copies in *C. acidophila* (comp15241_c0_seq3 and comp15241_c0_seq6), of which one is similar to *C. reinhardtii* but the other is more similar to bacterial homologues (Fig. 2C).

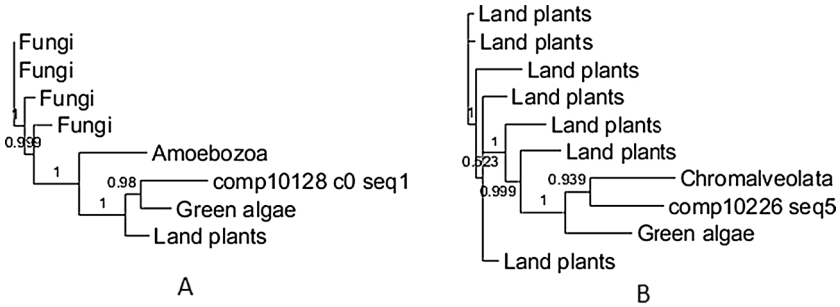
Similarly, the genes not present in *C. reinhardtii* extracted with OrthoFinder have variable phylogenetic distribution patterns (Fig. 3). Some of the genes are nested in a clade containing mainly bacteria (e.g. dioxygenase) and could be horizontally transferred. But for others, like transmembrane protein comp9629_c0_seq1 are closely related only to green algae and gene loss is a more likely explanation for their presence in *C. acidophila* but absence in *C. reinhardtii*.

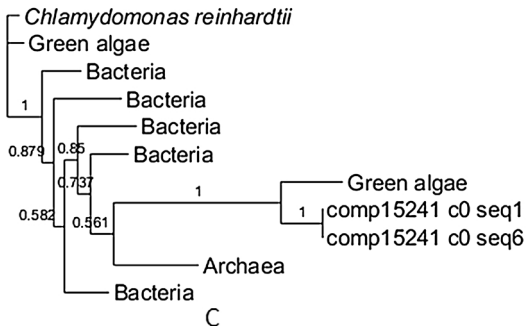
3.8. Codon code and aminoacid usage analysis

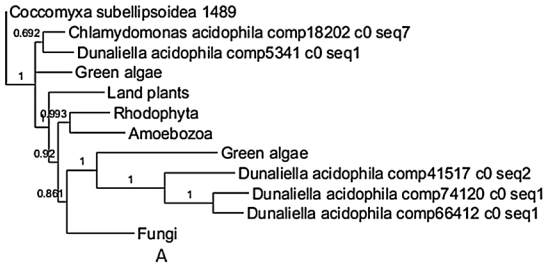
The transcripts belonging to each of the analyzed species (*Chlamydomonas acidophila*, *Chlamydomonas reinhardtii*, *Coccomyxa subellipsoidea*, *Dunaliella acidophila*, *Micromonas pusilla*, *Micromonas sp. RC299*, *Ostreococcus lucimarinus* and *Volvox carteri*) clearly clustered together with regards to their Relative Synonymous Codon Usage (Fig. 4a), showing the presence of distinct codon usage biases, even within phylogenetically close species. For the most part, those differences did not result in different aminoacid usage (Fig. 4b). The majority of the transcripts clustered together regardless of their source organism, except for a large set of transcripts from *C. reinhardtii* and the two *Micromonas* species, which clustered independently. Both *C. acidophila* and *D. acidophila* showed similar utilization profiles for several aminoacids, particularly an enrichment in serine and cysteine, and a

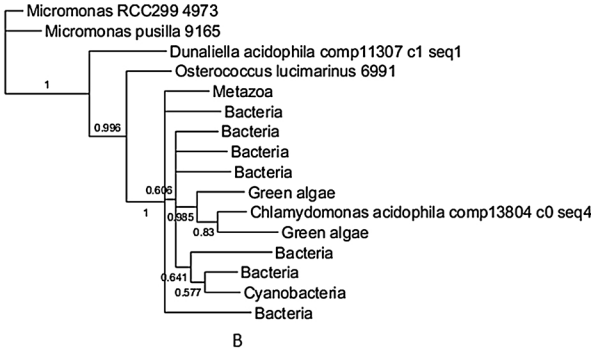
Table 6
Transcripts coding for genes that are involved in heavy metal tolerance present in *C. acidophila* but not in *C. reinhardtii* filtered from orthologous groups defined with OrthoFinder. Orthologous groups with annotations related to heavy metal tolerance and detoxification are marked with *. In the case of groups including several transcripts, the Blast hit organism and accession refers to the first one.

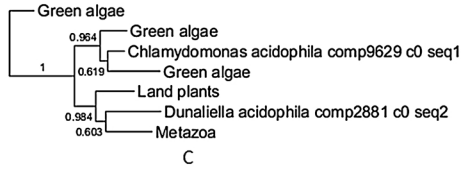
Orthologous group	Contig name	Putative function	BLAST top match organism	BLAST match accession	E-value
OG0001276	comp13064_c0_seq2, comp15004_c0_seq1	Tripeptidyl-peptidase 1	*Polysphondylium pallidum*	EFA84081	1.38e-17
OG0001752	comp12567_c0_seq1	Amino acid permease 2	*Capsella rubella*	EOA20485	3.33e-60
OG0001782	comp15790_c0_seq1	Alpha-1,3-glucosyltransferase	*Coccomyxa subellipsoidea*	XP_005651392	9.52e-92
OG0003420	comp16380_c0_seq1	Metal-nicotianamine transporter	*Amborella trichopoda*	ERN09450	5.88e-32
OG0003495	comp18202_c0_seq7	2-hydroxyacyl-CoA lyase	*Galdieria sulphuraria*	XP_005708092	0.0
OG0004374	comp18062_c0_seq1	Abhydrolase domain-containing protein	*Dictyostelium purpureum*	XP_002957250	2.33e-05
OG0004475*	comp11852_c0_seq1	Phytochelatin synthase	*Calothrix* sp.	YP_007140091	6.44E-30
OG0005070	comp16077_c0_seq9	G-box-binding factor 1	*Brassica napus*	CAA58774	1.33e-10
OG0005487*	comp14907_c0_seq15	Arsenical-resistance protein ACR3	*Coccomyxa subellipsoidea*	XP_005649016	1.45e-85
OG0005928*	comp13804_c0_seq4	dioxygenase	*Volvox carteri*	XP_002957190	2.32e-53
OG0006489	comp13735_c0_seq3	Snurportin-1	*Physcomitrella patens*	XP_001763666	5.34e-49
OG0006590*	comp16471_c0_seq1	multidrug efflux transporter AcrB	*Arabidopsis thaliana*	OAP00250	5.35e-63
OG0007003	comp14473_c0_seq1	Ankyrin-1	*Aegilops tauschii*	EMT31987	3.47e-56
OG0007890*	comp9629_c0_seq1	Transmembrane protein 230	*Physcomitrella patens*	XP_001772694	2.89e-17
OG0008459*	comp3348_c0_seq1	Cocaine esterase	*Achromobacter xylosoxidans*	WP_006387564	1.35e-80
OG0009876*	comp14433_c0_seq1	SDR-family protein with acetoacetyl-CoA reductase activity	*Sphingobium japonicum*	YP_003545425	9.92e-41
OG0010052	comp17871_c0_seq9	Hisitidine kinase	*Synechocystis* sp.	WP_009631601	1.298e-39

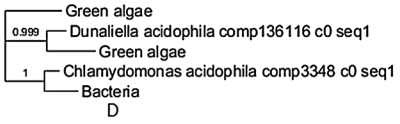












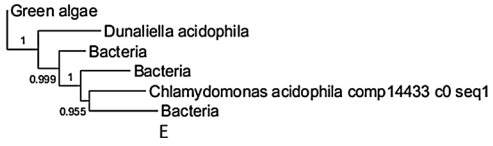


Fig. 2. Simplified phylogenetic analyses of transcripts coding for genes with an annotation related to heavy metal tolerance and present in *C. acidophila* but not in *C. reinhardtii*. The phylograms represent the majority consensus of trees sampled after stationarity in the Bayesian analysis. PP values equal or greater than 0.50 are shown above branches. The scale bar indicates relative distance between different sequences based on mutation rate. A) peroxisome isogenesis protein comp_10128 B) mitochondrial carrier comp 10226C) cobalamin biosynthesis protein CobW comp_15241.

Fig. 3. Simplified phylogenetic analyses of transcripts coding for genes that are involved in heavy metal tolerance and are present in *C. acidophila* but not in *C. reinhardtii* extracted from the results from the search for orthologous genes with OrthoFinder. The phylograms represent the majority consensus of trees sampled after stationarity in the Bayesian analysis. PP values equal or greater than 0.50 are shown above branches. The scale bar indicates relative distance between different sequences based on mutation rate. A) 2-hydroxyacyl-CoA lyase comp18202_c0_seq7 B) Dioxygenase comp13804_c0_seq4C) Transmembrane protein 230 comp9629_c0_seq1 D) Cocaine esterase comp3348_c0_seq1 E) SDR-family protein with acetoacetyl-CoA reductase activity comp_14433_c0_seq1.

depletion in glutamic and aspartic acids when compared to the non-acidophilic species (Fig. 5a, Additional File 5). This depletion in Glu and Asp in acidophiles was also observed by Goodarzi et al. (2008), but their study only included bacterial and archaeal genomes. To the best of our knowledge, this is the first study which proposes that the same can also be true in eukaryotes. We further calculated which GO-terms were significantly depleted in Glu and Asp, or enriched in Cys and Ser, in the acidophilic species when compared to the non-acidophilic species (Fig. 5b, Additional File 6). In the four cases, the significant GO-terms chiefly belonged to the binding, catalytic activity, and transporter activity base categories. These four modifications (lower Glu, lower Asp, higher Cys and higher Ser contents) are likely related to optimizations for acidic environments. For example, Glu and Asp are negatively charged in neutral conditions, but become neutral at lower pHs, which cancels their ability to stabilize proteins via salt bridges (Anderson et al., 1990). On the other hand, the higher content of cystein could contribute to metal detoxification and provide extra stability via disulfide bonds.

While the differences in total amino acid usage between organisms in acidophilic environments are usually caused mostly by a limited number of amino acids (Goodarzi et al., 2008), the observed differences in codon usage might be due to stronger selection pressure for codon

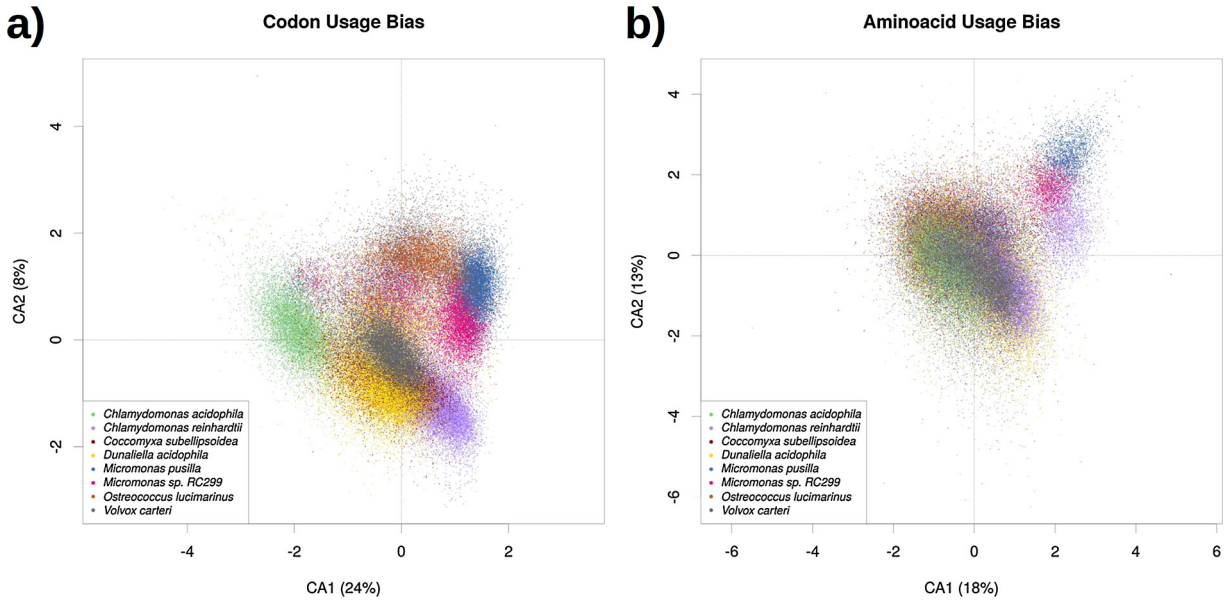


Fig. 4. a) Correspondence analysis showing the distribution of transcripts (points) according to their Relative Synonymous Codon Usage distribution and b) Correspondence analysis showing the distribution of transcripts (points) according to the aminoacid usage bias of their predicted ORFs. The percentage of inertia explained by each axis is indicated in the axis caption. Transcripts are coloured by their source genome.

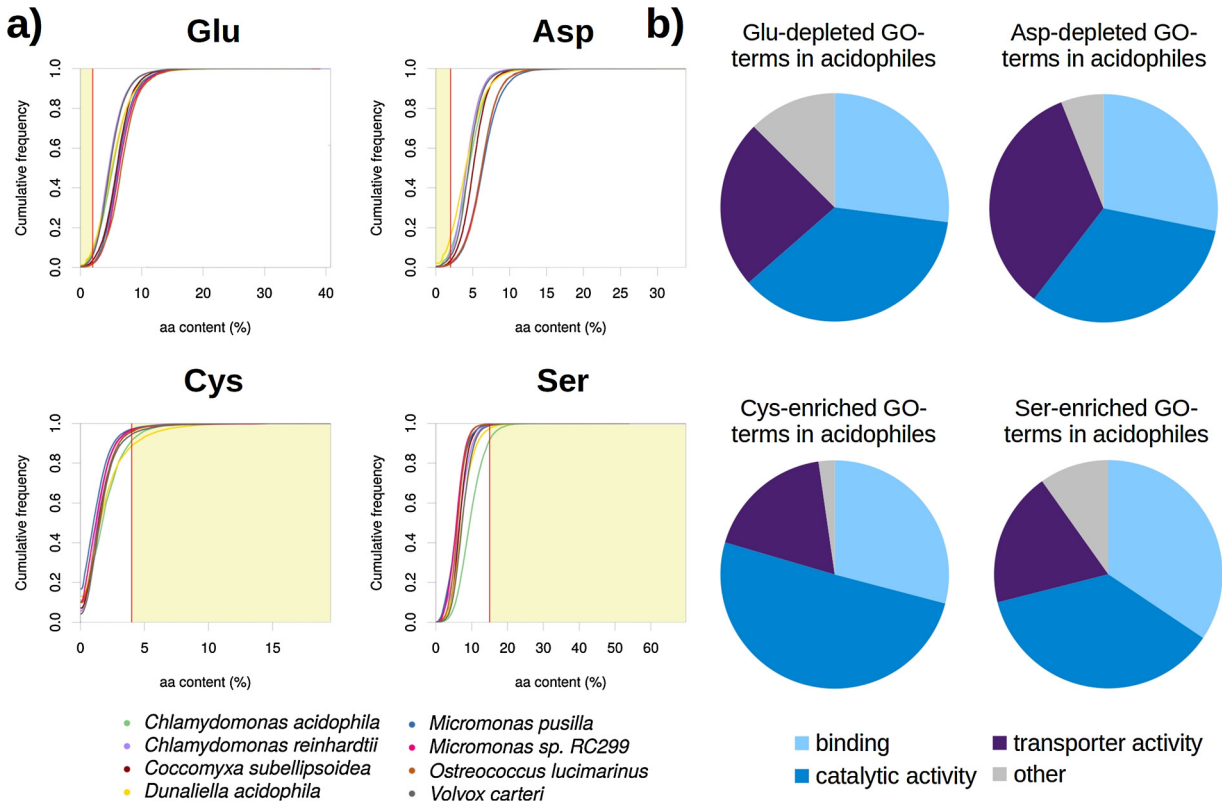


Fig. 5. a) Distribution of Glu, Asp, Cys and Ser contents in the predicted ORFs from the eight species analyzed in this study. The ORFs included in the green area (low Glu, low Asp, high Cys and high Ser) were subjected to a GO-term enrichment analysis between the two acidophilic species and the rest. b) Summary of the Molecular Function GO-terms found to be significantly enriched ($p < 0.05$) in acidophiles versus non-acidophiles, when focusing in the low Glu, low Asp, high Cys, and high Ser fractions of the proteomes. Full results are provided in Additional File 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

optimization in extreme environments. Natural selection acting through external environmental factors can shape the genomic pattern of synonymous codon usage in extremophilic prokaryotes (Lynn et al., 2002; Zeldovich et al., 2007), and our study is the first to suggest this to be true also in eukaryotes.

The GC contents of a large amount of transcripts in all codon positions are different (Fig. 6) and can't be explained by, for example, GC-content differences in a few horizontally transferred genes. Differences in the first codon position affect the amino acid usage while differences in the third position are likely due to preferences for different

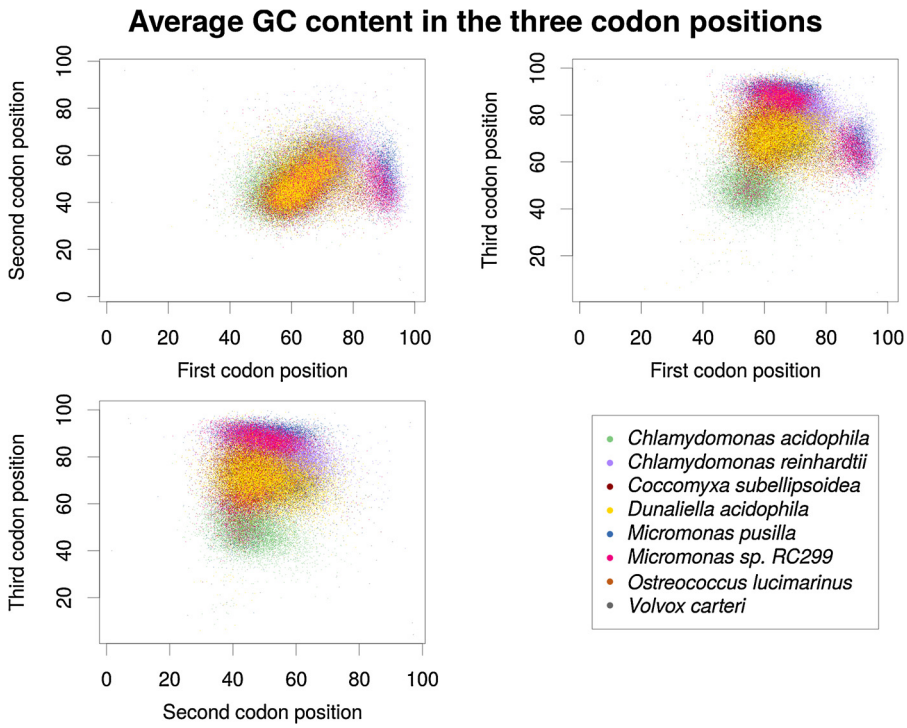


Fig. 6. Scatterplots showing the GC content on each transcript (points) in the three different codon positions. Transcripts are coloured by their source genome.

synonymous codons. Our results confirm that the overall genome-wide GC content is the most significant parameter in explaining codon bias differences between organisms, suggested by Hershberg and Petrov (2008).

3.9. Conclusions

The results of this study, including the most complete published transcriptome of *C. acidophila* and a set of identified orthologous genes between eight green algae, increase the genomic information available on green algae and extremophilic eukaryotes, highlight the adaptations mechanisms used by algae to thrive in acidic environments, and provide a valuable resource for comparative studies on green algae from different habitats. Further work should focus on detailed analyses of individual genes and applied exploitation of the results, including engineering heavy metal tolerance in green algae for environmental and economic interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aquatox.2018.04.020.

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