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A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*

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Summary

Interest in exploiting algae as a biofuel source and the role of inorganic nutrient deficiency in inducing triacylglyceride (TAG) accumulation in cells necessitates a strategy to efficiently formulate species-specific culture media that can easily be manipulated. Using the reference organism *Chlamydomonas reinhardtii*, we tested the hypothesis that modeling trace element supplements after the cellular ionome would result in optimized cell growth. We determined the trace metal content of several commonly used Chlamydomonas strains in various culture conditions and developed a revised trace element solution to parallel these measurements. Comparison of cells growing in the revised supplement versus a traditional trace element solution revealed faster growth rates and higher maximum cell densities with the revised recipe. RNA-seq analysis of cultures growing in the traditional versus revised medium suggest that the variation in transcriptomes was smaller than that found between different wild-type strains grown in traditional Hutner's supplement. Visual observation did not reveal defects in cell motility or mating efficiency in the new supplement. Ni^{2+}-inducible expression from the *CYC6* promoter remained a useful tool, albeit with an increased requirement for Ni^{2+} because of the introduction of an EDTA buffer system in the revised medium. Other advantages include more facile preparation of trace element stock solutions, a reduction in total chemical use, a more consistent batch-to-batch formulation, and long-term stability (tested up to 5 years). Under the new growth regime, we analyzed cells growing under different macro- and micronutrient-deficiencies. TAG accumulation in N deficiency is comparable in the new medium. Fe and Zn deficiency also induced TAG accumulation, as suggested by Nile Red staining. This approach can be used to efficiently optimize culture conditions for other algal species to improve growth and to assay cell physiology.

Introduction

Physiologically diverse algae are being developed as potential biofuel feedstocks because of their ability to accumulate triacylglycerides (TAG) in the form of "lipid bodies" under stress conditions (Wijffels and Barbosa, 2010). The large-scale culturing of these diverse strains requires a strategy to efficiently develop growth media that are species-specific and amenable to manipulation. These media should maximize cell density per volume and/or

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TAG accumulation while minimizing the cost and resources required to provide cells with essential mineral nutrients.

Chlamydomonas reinhardtii serves as a reference organism for studies of algal physiology, including photosynthesis (Levine, 1960; Grossman, 2000; Dent et al., 2001; Eberhard et al., 2008), sexual reproduction (Quarmby, 1994; Lee et al., 2008; Nishimura, 2010), ciliary biology (Silflow and Lefebvre, 2001; Cole, 2003; Snell et al., 2004), micronutrient deficiency (Merchant et al., 2006), ecological metal accumulation (Lustigman et al., 1995) and algal biofuel production (Moellering and Benning, 2010; Radakovits et al., 2010). Chlamydomonas has a sequenced genome (Merchant et al. 2007), and there is a rigorous genetic system developed, making it amenable to metabolic manipulation through techniques such as gene knock-down or overexpression (Grossman et al., 2007; Merchant et al., 2007; Grossman et al., 2010; Wijffels and Barbosa, 2010).

The composition of growth media in which Chlamydomonas is cultured affects the overall viability and physiology of cells, especially in the case of micronutrient concentrations, where optimal health is attained only in a narrow concentration range for each nutrient (Sunda et al., 2005; Merchant et al., 2006). In standard practice, the trace element solution usually used to culture Chlamydomonas was derived from a formulation proposed by Hutner and colleagues based on the growth of pseudomonads (Hutner et al., 1950). Hutner's trace element solution is problematic, as Fe precipitation occurs during a 2-week equilibration period. The precipitate must be filtered out, leading to batch-to-batch variation, not only in Fe concentration, but in other metal ion concentrations as a result of their adsorption to the Fe precipitates. Such changes in Fe and other metal ion bioavailability can have dramatic effects on photosynthetic activity and respiration (Moseley et al., 2002; Naumann et al., 2007; Terauchi et al., 2010). A more ideal supplement would be modeled after the specific needs of Chlamydomonas, accounting for metal ion concentrations, chelation strategies, bioavailability, and metal speciation. Previous optimization of ammonium, phosphate, and sulfur concentrations in the growth medium resulted in improved growth and increased hydrogen production in Chlamydomonas (Jo et al., 2006). Variations in ammonium concentration leading to nitrogen depletion have also been used to induce cell differentation in preparation for mating and genetic studies and for the accumulation of lipid bodies, a stress response that could potentially be exploited for developing algae as a biofuel source (Wang et al., 2009; Moellering and Benning, 2010; Radakovits et al., 2010). To date, however, a systematic optimization of the trace element supplement used to culture Chlamydomonas has not been attempted.

Here, we test the hypothesis that a trace element supplement modeled after the ionome of Chlamydomonas will improve its growth. Our revised trace element supplement avoids the pitfalls of traditional recipes. This strategy can be applied to the culturing of other algae to quickly assess the nutritional requirements of each species and optimize growth conditions.

Results

Analysis of Hutner's trace element solution

Multiple trace element recipes are presently in use for culturing Chlamydomonas (Table I) (Hutner et al., 1950;Sager and Granick, 1953;Kuhl, 1962;Bischoff and Bold, 1963). Concentrations of Fe and Cu in some of these recipes are now known to induce symptoms of deficiency even though overall growth is not affected (Merchant et al., 2006). A commonly-used protocol for making a standard trace element supplement for Chlamydomonas was derived from a recipe presented by Hutner and colleagues for the general culturing of photosynthetic prokaryotes (Hutner et al., 1950;Harris, 2009). The preparation of Hutner's stock solution requires the sequential pooling and heating of trace metals followed by an

equilibration and oxidation process that can take up to two weeks. The resulting solution is filtered to remove precipitates, which results in batch-to-batch variation in the final composition, an important consideration given that even mild differences in Fe impact photosynthesis and, therefore, data interpretation in both laboratory to laboratory comparisons and year to year comparisons in the same laboratory (Moseley et al., 2002). Measurement of metal concentration in a batch of Hutner's supplement immediately upon dissolving the trace elements and two weeks later, after filtration of the sample, revealed slight reductions in Zn and Cu, and a 50% decrease of Fe (Table II). These measured final concentrations also varied from those measured in a batch of the mix made 3 years prior in 2007.

Based on these observations, we sought to develop a new medium supplement by determining the metal ion content of healthy wild-type Chlamydomonas cells growing in the traditional recipe and iteratively modifying the supplement to reduce medium concentrations without affecting intracellular stores.

Metal quotas within wild-type cells

We determined the metal quota of three commonly used wild-type Chlamydomonas strains, CC-3269 (2137), CC-125 (137c), and CC-1690 (21gr), as well as CC-425 (an *arg2 cw15* strain derived from CC-125) grown in Tris-Acetate-Phosphate (TAP) medium (Gorman and Levine, 1965), promoting photoheterotrophic growth, and TP (without acetate), promoting phototrophic growth, media supplemented with Hutner's trace element solution. Cells in exponential growth phase were collected by centrifugation, washed with EDTA to remove extracellular bound metals, and analyzed for metal content using ICP-MS (Figure 1). Over a broad range, metal profiles were similar among all four strains, although a statistically significant difference was calculated for the Fe content in strains CC-425 and 2137, as well as for the Mn and Cu content for 21gr relative to the other strains. Fe is the most abundant metal ion in cells, followed by Mn, Zn and Cu ions, respectively. Generally, accumulation of Cu and Fe ions was higher in cells grown photoheterotrophically relative to cells grown phototrophically. In all instances Co and B ions were below detection limits in the cell samples but not in the medium. Comparison of relative metal ion concentrations in the media with relative intracellular metal ion profiles suggests that a dramatic excess of Co, B, and Zn ions may be present in the media, as well as an excess of Mn ions.

Development of a revised trace element supplement

Based on the intracellular metal quotas measured in TAP medium, a new trace element supplement was formulated to provide cells with Cu, Fe, Mn, and Zn ions to support a cell density of 2×10^7 cells/mL, representing the typical cell density of a healthy culture at stationary phase, in 3-fold excess (Table I and Supplemental File F1). The excess amount was meant to address late phase culture conditions by ensuring that cells did not experience trace nutrient deficiency in stationary phase. The excess also accommodates changes in nutritional demand under different physiological conditions, such as those that occur during the transition between respiratory growth and photosynthetic growth (Terauchi et al., 2010). Extreme nutrient excess is not beneficial, as optimum concentrations for some nutrients like Se and Fe cover only a narrow range in humans (~5 to 6-fold) (O'Dell and Sunde, 1997;Merchant et al., 2006). Excess nutrient availability may also cause unwanted metal interactions (Allen et al., 2007;Baxter, 2010). As molybdate concentration did not significantly affect final cell density in TAP-grown cultures (Supplemental Figure S1), it was provided in 2-fold excess, corresponding to concentrations used by Fernández and co-workers who grew CC-1690 cells with nitrate as a sole nitrogen source (Tejada-Jiménez et al., 2007). Boric acid and cobalt ions were excluded completely as they did not impact final cell density (Supplemental Figure S1), and no boron or cobalt could be measured inside the

cells (Figure 1). We also do not know of any biochemical requirement for cobalt or boron for Chlamydomonas (see Discussion). Selenate was added to the supplement to account for the known presence of selenoproteins present in Chlamydomonas (Novoselov et al., 2002;Grossman et al., 2007;Merchant et al., 2007).

The components of the revised trace elements were formulated as individual stock solutions. EDTA was included with Cu, Fe, Zn, and Mn ion stocks to prevent precipitation of the salts. Additionally, 25 μM EDTA was added to the final medium to serve as a trace metal ion buffer (Sunda et al., 2005). The EDTA supplement resulted in more reproducible growth curves when we tested the impact of variation in trace mineral concentrations. Solutions were stored in the dark at room temperature. Cu^{2+}, Mn^{2+}, Fe^{3+}, Zn^{2+}, and Se^{6+} solutions from an early iteration of the recipe were found to be stable over a five year period under these conditions (Figure 2).

Physiological comparison of cells

To compare growth rate and maximum cell density of cultures grown in medium supplemented with either the traditional trace element solution or the revised solution, CC-3269 was inoculated into bioreactors containing TAP medium with the two trace element supplements. Cells were initially followed during growth to stationary phase without the input of additional medium. During this time course, cells were collected at various optical densities (ODs) and analyzed for cell density, size, and metal content. Under these conditions, cells supplemented with the revised trace elements showed an increase in growth rate and in maximum cell density (Figure 3A and 3B). Measurement of intracellular metal content at each time point during the initial growth curve showed no significant differences between the two supplements (Figure 3CDEF). Cell diameter averaged 9.1 μm with a range of 4.4 to 11.8 μm for cells grown with the traditional supplement and 8.5 μm with a range of 3.7 to 11.1 μm for cells grown with the revised trace element solution during early time points. The differences were not statistically significant. The pH did not differ significantly in the two conditions, ranging on average from 7.3 to 8.4 (as acetate was consumed by the cells) for medium supplemented with the revised trace elements and 7.4 to 8.4 for medium supplemented with Hutner's trace elements over the course of the experiment. In a second approach, cultures were maintained in turbidostat mode with 5% tolerance, producing a regular oscillating turbidity pattern introduced by cell growth (an increase in OD) and by medium input (a decrease in OD). Rates of growth were measured over these oscillating patterns for 16-hour intervals. From this, a doubling time of 8.8 hours was calculated for Hutner's solution versus 7.9 hours for the revised supplement. All experiments were done in experimental duplicate and biological triplicates and cell counts were performed concurrently by two individual researchers without prior knowledge of the trace element supplement identity of the samples. Further analysis revealed that mating efficiency was similar for cells supplemented with either trace element solution. The changes in medium metal composition also did not result in differences in cell motility, phototaxis or presence of cilia.

Pairwise comparisons of RNA abundance levels from CC-3269 (2137) cells growing in traditional medium (H_{2137}), 2137 cells grown in an early iteration of the revised medium (mK_{2137}), or D66 cells growing in traditional medium (H_{D66}) (González-Ballester et al., 2010) were performed to evaluate gene expression in the various media (Figure 4). D66 is similar to 2137 except that it lacks a cell wall and contains a mutation in the *NIT2* gene encoding a positive regulator of the nitrate assimilation pathway (Schnell and Lefebvre, 1993). Variation between identical strains growing in the different media (H_{2137} vs. mK_{2137}) is less pronounced than variation between different strains grown in the same traditional medium (H_{2137} vs. H_{D66}). Of the twelve genes encoding selenoproteins in the Chlamydomonas genome, transcript abundance of three genes, encoding MsrA, a functional

methionine-*S*-sulfoxide reductase, SelW1 and SelH, whose functions are unknown (Kim et al., 2006; Grossman et al., 2007), increased in the revised medium relative to traditional medium. The increased abundance of these transcripts likely results from the introduction of selenate ions in the revised supplement.

Because a 3-fold excess of nutrients was supplied in the revised recipe, we characterized cells growing in the first round of a 1 to 3-fold dilution of the supplement, representing a sufficient but not excessive nutrient condition. No difference in cell density was observed relative to the normal supplement, and at late exponential phase, genetic markers for Fe deficiency (*FRE1*), Mn deficiency (*NRAMP1*), and Zn deficiency (*ZRT3*) were also not upregulated relative to cells grown with the normal supplement, showing that cells did not reach deficient conditions at the end of growth phase (Supplemental Figure S2).

Chlamydomonas is a reference organism for fundamental studies of chloroplast and cilia biology, and a metal-responsive promoter has been developed to drive ectopic gene expression (Quinn et al., 2003; Surzycki et al., 2007; Ferrante et al., 2008). The promoter was developed for cells growing in traditional media (Quinn et al., 2003; Ferrante et al., 2008), and we therefore tested how it would work in the revised supplement. A standard concentration of 25 μM Ni ions was required for induction in medium supplemented by the traditional Hutner recipe, while 50 μM Ni ions was required to elicit the response in medium supplemented with the new recipe (Figure 5). This increase corresponds to the 25 μM EDTA added to the medium to serve as a divalent metal ion buffer. A modification of the revised recipe with 25 μM less EDTA resulted in *CYC6* induction at the standard concentration of 25 μM Ni ions. Because of the known toxicity of Ni ions, using the variation of the new supplement with decreased EDTA is recommended for experiments using this promoter.

Characterization of deficient cell phenotypes

There have been many studies of nutrient stress in Chlamydomonas, including S-stress (Wykoff et al., 1998; Laurinavichene et al., 2004; Fouchard et al., 2005), P-stress (Wykoff et al., 1999; Yehudai-Resheff et al., 2007; Moseley et al., 2009), N-stress (Peltier and Schmidt, 1991; Beck and Acker, 1992; Abe et al., 2004), and our studies on micronutrient deficiencies (Merchant and Bogorad, 1986; Moseley et al., 2002; Allen et al., 2007; Haas et al., 2009). For macronutrient stresses such as N-deficiency, the accumulation of triacylglycerides (TAG) has been observed and is presently of great interest because of its utility as an energy source (Wang et al., 2009; Moellering and Benning, 2010; Radakovits et al., 2010).

To characterize the physiology of nutrient deficient cells under the new supplement regime, medium components were individually excluded and cells were grown in deficient conditions into stationary phase (Figure 6). We observed that the new medium allows for the study of individual nutrient deficiencies in a similar manner to our previous studies using the traditional trace element mix, and we see lipid body accumulation under N deficiency as predicted. Nile Red staining of cells to visualize lipid body accumulation (Cooksey et al., 1987) revealed an increase in lipid bodies in Fe and Zn deficient conditions as well. As these two conditions coincide with conditions that also inhibit growth, these results suggest that lipid body accumulation may be a general stress response. Cell size or morphology also appeared significantly different in several deficiencies, including Zn, Mn, and N. As was previously reported, Fe deficiency resulted in a chlorotic phenotype (La Fontaine et al., 2002).

Discussion

Traditionally, the growth of Chlamydomonas relied on a trace element recipe developed for prokaryotes. We present a revised recipe based on wild-type Chlamydomonas ionomic data that reduces the time and metal salts required to make the medium and results in faster growth and higher biomass production. Most importantly, the composition is reproducible and stable for 5 years. This general strategy, outlined in Figure 7, for developing species-specific media for algal culturing can potentially be applied to novel strains and mutants, including large-scale cultures used for biofuel development.

The Chlamydomonas ionome provides insight into its physiology. Marine algae *Bangia* and *Porphyra* and the freshwater green alga *Chlorella* reportedly require boron (Henkel, 1952; McIlrath and Skok, 1958), and boron deficiency in the cyanobacterium *Nostoc* was shown to lead to chlorosis (Eyster, 1952). The lack of a boron requirement in *C. reinhardtii* may be due to the fact that it was isolated from soil, a typically boron-poor environment (Li et al., 2008). Chlamydomonas also lacks rhamnogalacturonan II in the cell wall, a component that requires boron for dimer formation in plants (Kobayashi et al., 1996). Although Chlamydomonas secretes biological compounds that interfere with the AI-2 furanosyl borate diester-mediated quorum sensing system of *Vibrio* spp., the chemical structures of these “mimics” have yet to be identified (Chen et al., 2002; Teplitski et al., 2004).

The lack of a cobalt requirement is consistent with our current understanding of the absence of vitamin B_{12} biosynthesis in eukaryotic cells (Croft et al., 2006). Cobalt has been shown to inhibit chlorophyll synthesis and induce iron deficiency in photosynthetic organisms (Csatorday et al., 1984). Cobalt toxicity in Chlamydomonas was observed at 10 ppm cobalt nitrate and was significant at 20 ppm (Lustigman et al., 1995). The elimination of non-essential trace elements decreases the likelihood of contamination of algal cultures by microbes that may require these nutrients to grow.

Se^{6+} was included in our revised supplement to account for known selenoproteins present in Chlamydomonas (Novoselov et al., 2002). Though it was hypothesized that selenium atoms may cause toxicity by substituting for sulfur atoms in sulfoproteins, exposure of 0.1 μM selenate was not toxic at 8 or 80 μM sulfate for Chlamydomonas (Fournier et al., 2010), and exposure of Se^{6+} to Chlamydomonas in a synthetic fresh water medium did not inhibit growth at 0.1 μM (Geoffroy et al., 2007). These data suggest that no adverse effects should arise from the inclusion of a low amount of selenate in our supplement. Comparison of gene expression levels of cultures supplemented with Hutner’s trace element recipe versus the revised recipe revealed the increased accumulation of transcripts encoding three of the twelve previously identified selenoproteins in the Chlamydomonas genome associated with the revised recipe. In each case, the Sec-encoding codon, UGA, is upstream of an intron. In the absence of a Sec-charged tRNA, the corresponding genes would be subject to nonsense mediated decay (Moriarty et al., 1998). In previous work on the *PCY1* locus, we confirmed that nonsense mediated decay operated in Chlamydomonas (Li et al., 1996). Frameshift mutations leading to premature stop codons at the 5′ end (upstream of an intron) in *pcy1-ac208* and *pcy1-2* resulted in reduced mRNA abundance. The addition of Se^{6+} may be beneficial under conditions of stress, when some members of the Chlamydomonas selenoproteome are thought to be required (Grossman et al., 2007).

We predict that no symptoms of sulfur deficiency should arise from the revised medium, though the final sulfate concentration decreased from 496 μM to 402.5 μM due in part to the decrease in added $ZnSO_4$. The inclusion of excess EDTA as a trace metal ion buffer resulted in more reproducible growth curves, suggesting that growth of Chlamydomonas cells are sensitive to small fluctuations in mineral nutrient availability. This excess EDTA is not

essential, and under situations where accurate accounting of metal nutrients is not critical, or in experiments relying on the Ni^{2+}-inducible gene promoter, may be unnecessary.

We note that the determination of metal quotas does not distinguish between absolute nutritional requirements and apparent requirements affected by the kinetics of nutrient uptake and bioavailability. Although lower absolute metal ion concentrations may be sufficient to allow the proper function of all required metalloenzymes in the cell, the rates of uptake by transporters may not be fast enough to provide optimal health, and the intracellular sequestration of metal ions may also affect nutrient requirements. The potential for interaction between elements also increases the complexity of nutritional studies (Merchant, 2010). A multivariable study of the effect of NO_3^-, PO_4^{3-}, K^+, Na^+, and Cl^- concentrations on *C. vulgaris* and *P. cinctum* growth determined that multiple nutrient profiles could result in similar growth rates (Evens and Niedz, 2010). In Chlamydomonas, our previous studies have documented mineral nutrient interactions. For instance, manganese deficiency results in secondary phosphorous and iron deficiency, and deletion of the C-terminal cysteine-rich region of the Copper response regulator Crr1 in Chlamydomonas disrupts Zn homeostasis (Allen et al., 2007; Sommer et al, 2010).

Studies of nutrient deficiency contributed to the discovery of acclimation mechanisms in Chlamydomonas (Merchant et al., 2006). Here, we characterized cells growing under various deficient conditions and determined that there are no dramatic differences in acclimation responses due to the supplement revision. It is well-established that stress caused by N and S deprivation or drastic pH changes leads to a change in lipid profile and an accumulation of total lipid content in microalgae, a physiological response that may be useful in the development of biofuels (Cooksey et al., 1987; Guckert and Cooksey, 1990; Sheehan et al., 1998; Sato et al., 2000; Wang et al., 2009). A hypothesis to explain this observation proposed that accumulation was a passive result of cell division inhibition caused by stress combined with the steady production of new lipids (Sheehan et al., 1998). Consistent with this hypothesis, we observed that iron-deficient cells accumulated TAG in parallel with inhibition of cell division (Urzica et al., unpublished), and a recent study noted that salt stress also resulted in growth inhibition and TAG accumulation in Chlamydomonas strain CC-124 (Siaut et al, 2010). Here, we broadened our study to investigate intracellular lipid accumulation response under conditions of other trace metal deficiencies. Increased lipid accumulation in N-, Fe-, and Zn-deficient situations all corresponded to growth inhibition (Figure 6).

The revised medium and the work presented here establishes baseline conditions for future studies focused on the interaction of multiple deficiencies and/or toxicities that require the ability to measure intracellular metal quotas and compare them between experiments and between laboratories.

Materials and methods

Culturing and strains

C. reinhardtii wild-type strains CC-3269, CC-425, CC-125, and CC-1690 were cultured under 50 – 100 μmol m^{-2} s^{-1} illumination in Tris-Acetate-Phosphate (TAP) and Tris-Phosphate (TP) media with the specified trace element supplements. These strains may be obtained from the Chlamydomonas Resource Center at the University of Minnesota. For metal-free studies, all glassware was freshly washed in 6N hydrochloric acid and medium was made in Milli-Q (MILLIPORE) water (Quinn and Merchant, 1998).

Trace element solutions

Hutner's Trace Element solution was made according to the protocol described in Harris et al. (2009). A detailed protocol for the revised trace element supplement is available as a supplemental file. Briefly, stock solutions of 25 mM EDTA-Na_2, 28.5 μM $(NH_4)_6Mo_7O_{24}$, 0.1 mM Na_2SeO_3, 2.5 mM $ZnSO_4$ in 2.75 mM EDTA, 6 mM $MnCl_2$ in 6 mM EDTA, 20 mM $FeCl_3$ in 22 mM EDTA, and 2 mM $CuCl_2$ in 2 mM EDTA were made individually in Milli-Q water and stored away from direct sunlight at room temperature. Each stock solution was diluted 1 to 1000 in the final growth medium.

Monitoring growth

Cells were inoculated to a density of 1×10^4 cells/mL into Tris-acetate-phosphate (TAP) medium in a Photobioreactor FMT150 device (Photon Systems Instruments) with a cultivation chamber volume of 450 mL. The culture was kept at a stable temperature of 24 °C and a light intensity of 190 $\mu E \cdot m^{-2} \cdot s^{-1}$ (split equally between red and blue wavelengths). Cells were mixed by aeration. Cell size, growth rate, and metal content were determined during the course of growth. Cell size was calculated by using the arithmetic average of the length and width of individual cells measured on a Cellometer (Nexcelom) as a proxy for cell diameter. Growth rate was measured both by counting under a microscope with a hemocytometer and by automated counting with a Cellometer after correcting for cell clusters. At each time point, counts were performed by two independent researchers who did not have prior knowledge of the identity of the trace element mix in the culture. Growth rate was also calculated for cultures grown in turbidostat mode set for an OD of 0.7 (approximately 3×10^6 cells/ml) with 5% tolerance at 680 nm. Peaks in OD values created by this tolerance were used to compute doubling time using formulas $k = \log_2(O_t/O_0)/dt$ and $T = 1/k$, where k is the number of doublings per h, O_t, and O_0 are the OD at the end and beginning of the time interval, dt is duration of the interval in hours and T is doubling time in h (Adl et al., 2005). Data for doubling time computation were collected for 16 h from the time when OD 0.7 was reached.

Measurement of intracellular metal content

Cells were collected by centrifugation at 1700 ×g for 5 min. Pellets were washed once in 1 mM EDTA to remove cell surface-associated metals and once in Milli-Q water. The washed cell paste was overlaid with nitric acid corresponding to a final concentration of 24% in 1 mL and digested at 65 °C. To obtain a corresponding blank, the volume of the cell paste was replaced by deionized water and treated as described above. Total metal and phosphorous content was measured by ICP-MS.

Mating efficiency

Fresh mt^+ and mt^- cells from TAP medium supplemented with Hutner's trace elements were plated onto nitrogen deficient agar plates supplemented with either Hutner's trace elements or the revised formulation for 3 d. Cells were resuspended into sterile water and allowed to shake for 1 h prior to mixing. Upon mixing, cells were allowed to mate for 1 h or overnight. Cell density before and after mixing as well as clumping of cells was used to estimate mating efficiency.

Motility

Motility in cells was verified by microscopic observation of exponentially growing cells from triplicate cultures for their ability to move away from a light source and by the presence of cilia.

RNA Analysis

Total Chlamydomonas RNA was prepared as described by Quinn and Merchant (1998).

Pairwise Transcriptomic Comparisons

Probability plots were generated as described in González-Ballester et al. (2010). In brief, RNA-Seq data was mapped to the genome, filtered to keep unambiguous alignments and assigned to annotated genes (Au5 gene models, 2007 Chlamydomonas genome assembly). For each experiment *i*, we compute a whole-transcriptome vector of *normalized expression* E_{ij} with units of alignment hits per million reads. This vector is used to compute, for each pairwise comparison (*i*,*i*′) and each gene *j*, *expression fold-changes* ($E_{ij} / E_{i'j}$) and *overall expression measures* ($\sqrt{E_{ij}/E_{i'j}}$).

Quantitative Real-Time PCR (RT-PCR)

Genomic DNA was removed from the total RNA preparation by treatment with Turbo DNase (Ambion) according to manufacturer's instructions with the following modifications: 3 U of enzyme was used per 10 μg nucleic acid, and incubation at 37°C was held for 90 min. Complementary DNA, primed with oligo(dT), was generated with reverse transcriptase (Invitrogen) according to manufacturer's instructions. Amplification was carried out with reagents from the iQ SYBR Green Supermix qPCR kit (Bio-Rad Laboratories). Each reaction contained the vendor's master mix, 0.3 μM of each primer, and cDNA corresponding to 20 ng input RNA in the reverse transcriptase reaction. The reaction conditions for the Opticon 2 from MJ Research were: 95 °C for 5 min, followed by cycles of 95 °C for 10 s, 65 °C for 30 s, and 72 °C for 30 s, up to 40 cycles. The fluorescence was measured at each cycle at 72 °C and 83 °C. The $2^{-\Delta\Delta CT}$ method was used to analyze the database on the fluorescence at 83°C (Livak and Schmittgen, 2001). Melting curves were performed after the PCR reaction to assess the presence of a unique final product.

Ni^{2+}-induced *CYC6* expression

CC-125 was grown in TAP medium supplemented with the revised trace elements, a modification of the revised recipe containing approximately 22.5 μM less EDTA, or Hutner's trace elements. When the culture reached late exponential phase, $NiCl_2$ was added. RNA was extracted 5 hours later. Expression of *CYC6* was determined by RT-PCR. Transcript abundance was normalized to *EIF5A*, and all data are shown relative to time 0, immediately prior to Ni^{2+} addition.

Fluorescence and light microscopy

Cells were grown to stationary phase in replete medium, media lacking Cu, Zn, Mn, Mo, Se ions, or media containing low levels of Fe (0.4 μM) or N (0.7 mM). Cells were stained with Nile Red (10 μg/ml final concentration, Sigma) for 15 min at room temperature, collected by centrifugation at 500 × g for 3 min. Cells were mixed with low melting agarose (Bethesda Research Laboratories) in phosphate buffer and viewed by confocal microscopy on a Leica TCS SPE with an ACS APO 63x oil objective lens (numerical aperture 1.30). The Nile Red signal was captured using a laser excitation line at 488 nm; and emission was collected between 554–599 nm (gain: 700; offset: 0). Chlorophyll autofluorescence was excited at 635 nm and captured between 620 and 700 nm. DIC images were acquired in the PM Trans channel (gain: 371; offset: 0). Images were colored using Leica confocal software.

A Zeiss AxioImager Z1 microscope equipped with a color camera (AxioCam HRc) and a 63x oil immersion objective lens was used in DIC mode to capture morphological changes induced by various micronutrient deficiency regimes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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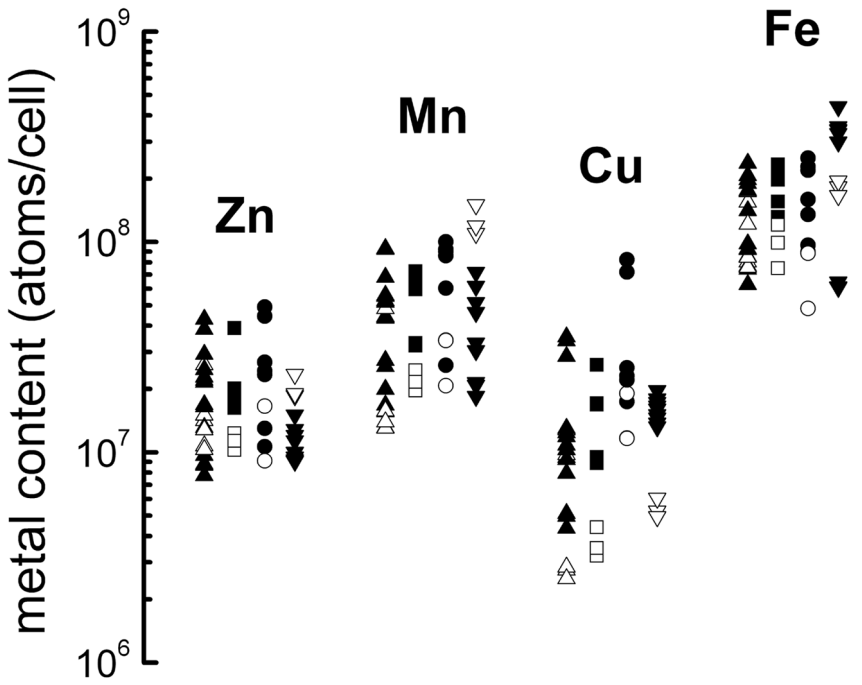

Figure 1. Intracellular micronutrient content

Cells of strains CC-3269 (upright triangles), CC-125 (squares), and CC-1690 (circles), and CC-425 (inverted triangles) Chlamydomonas cells were grown in TAP (filled symbols) and TP (open symbols) media supplemented with Hutner's mineral nutrient supplement and collected by centrifugation. The cell pellet was washed with 1 mM EDTA to remove extracellular bound metal and dissolved in 30% nitric acid by incubation in 65 °C. The metal content was measured by ICP-MS. The *y*-axis plots the range of metal content on a per cell basis under the various conditions or for different strains.

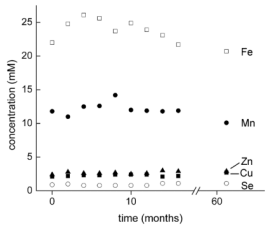

Figure 2. Stock solutions are stable over 5 years

The concentrations of Cu (filled squares), Fe (open squares), Mn (filled circles), Se (open circles), and Zn (filled triangles) in stock solutions from a primary iteration of the revised trace element composition were measured by ICP-MS over a period of 61 months. Each solution was stored at room temperature in the dark. Before each sampling, the solution was filtered through a 0.22 μm filter nto a new container. Each metal solution was diluted to an appropriate concentration prior to injection into the ICP-MS.

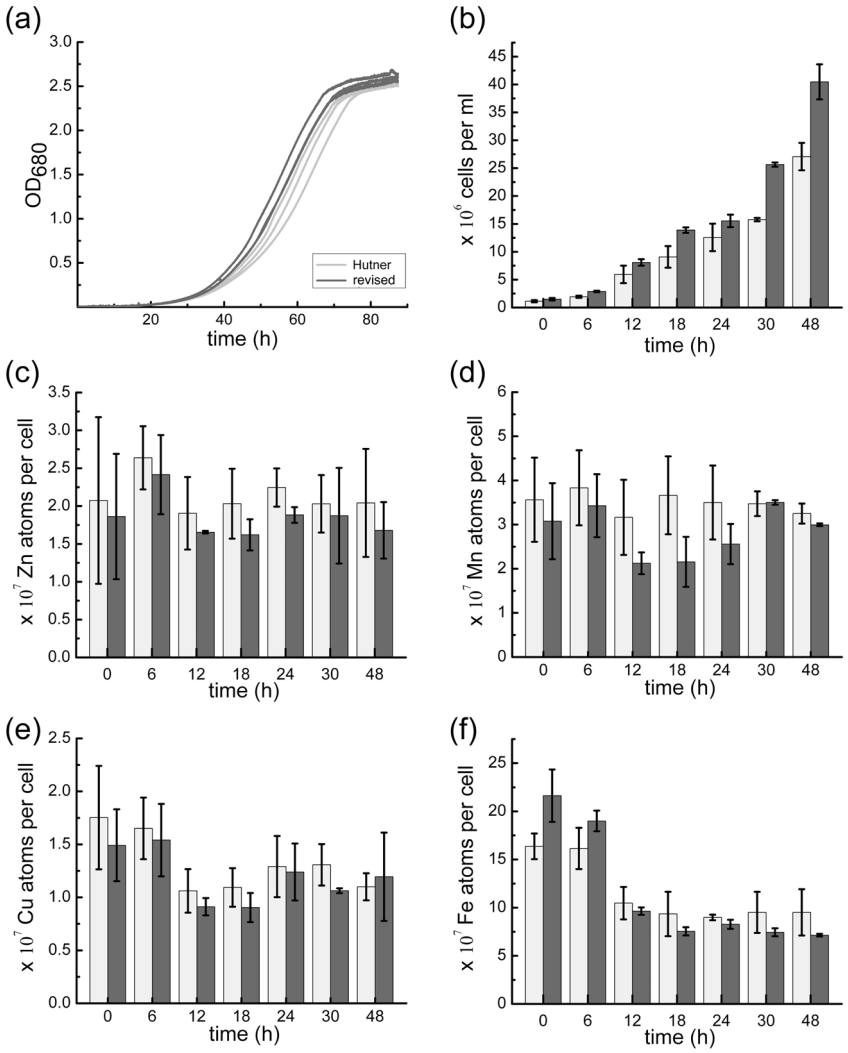

Figure 3. Supplementation with a revised trace element composition improves cell growth

Strain CC-3269 was grown in TAP medium supplemented with Hutner's trace elements (light gray) or the revised composition (dark gray). Cell growth was measured by monitoring optical density at 680 nm (A) and by counting cells using a hemocytometer (B). Intracellular Zn (C), Mn (D), Cu (E), and Fe (F) were measured by ICP-MS as described in Figure 1. The average of biological triplicates is shown and is representative of experimental duplicates. Cell counts were performed by two independent researchers for each duplicate without prior knowledge of the source of the supplement.

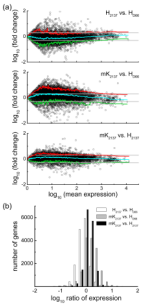

Figure 4. Pairwise comparison of transcriptomes from cells supplemented with different trace element recipes

A. Logarithmic mean-difference scatterplots of normalized expression fold-change (y axes) vs. mean expression (x axes). The plots were generated from pairwise transcriptome comparisons of wild-type strain 2137 supplemented with Hutner trace element recipe (H_{2137}) (Castruita et al. unpublished), wild-type strain D66 supplemented with Hutner trace element recipe (H_{D66}) (González-Ballester et al., 2010), and wild type strain 2137 supplemented with an early iteration of the revised trace element mix (mK_{2137}). Cyan lines in scatter plots represent the average fold change. Red and green lines represent the 95th and 5th percentile of fold changes, respectively. Gray lines represent a 2-fold difference in fold change. B. Histogram of expression fold-changes for each pairwise comparison grouped into clusters of $\log_{10}$ 0.2.

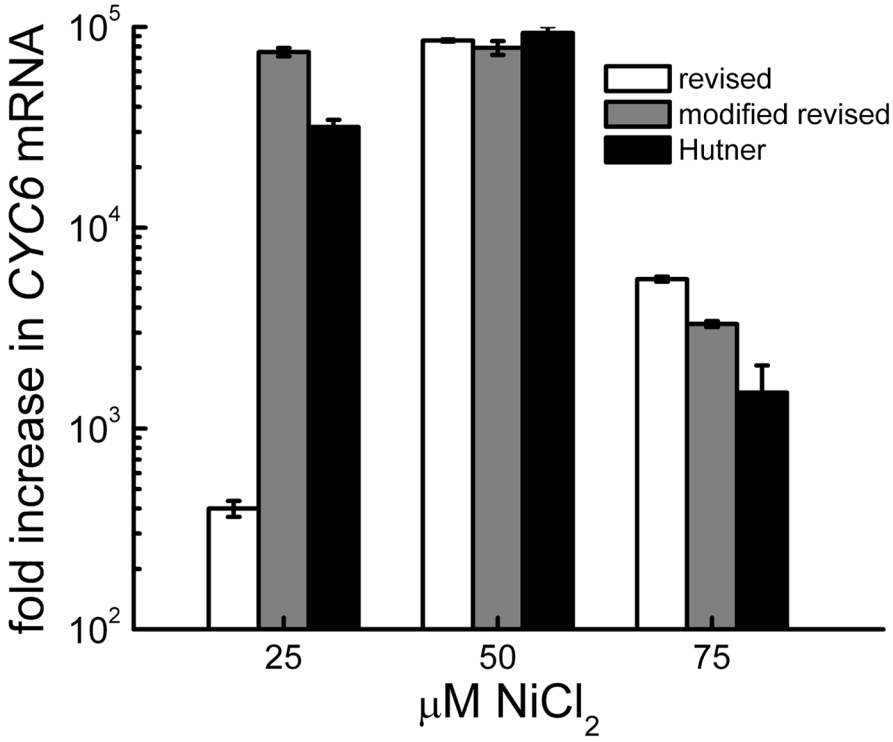

Figure 5. Optimization of the amount of Ni^{2+} required to induce *CYC6* expression in the revised trace elements

RNA was isolated from cells grown in TAP medium supplemented with the revised mineral nutrient mix, a modification of the revised recipe with EDTA reduced so that the molar amount of free species was comparable to the amount in Hutner's (2.5 μM), or Hutner's trace elements 5 hours after addition of the indicated amount of $NiCl_2$. Transcript abundance was measured by RT-PCR and normalized to *EIF5A* expression. Average C_T value was calculated from technical triplicates. Expression was calculated by the $2^{-\Delta\Delta CT}$ method. The average of experimental triplicates is shown.

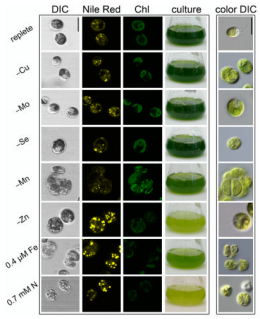

Figure 6. Comparison of lipid body formation, cell morphology, and culture appearance of different growth conditions

Cells were grown under replete and deficient nutrient regimes as indicated in the figure. Confocal micrographs of Nile Red stained cells in Photomultiplier Tube Trans and Photomultiplier Tube 1 (Nile Red fluorescence) channels are shown in columns 1 and 2, respectively. Chlorophyll autofluorescence is shown in column 3. The overall appearance of the cultures is shown in the column 4. Additionally, cells were imaged on a Zeiss AxioImager Z1 microscope equipped with a color camera (AxioCam HRc) in DIC mode (column 5). Representative cells shown. Scale bars represent 10 μm.

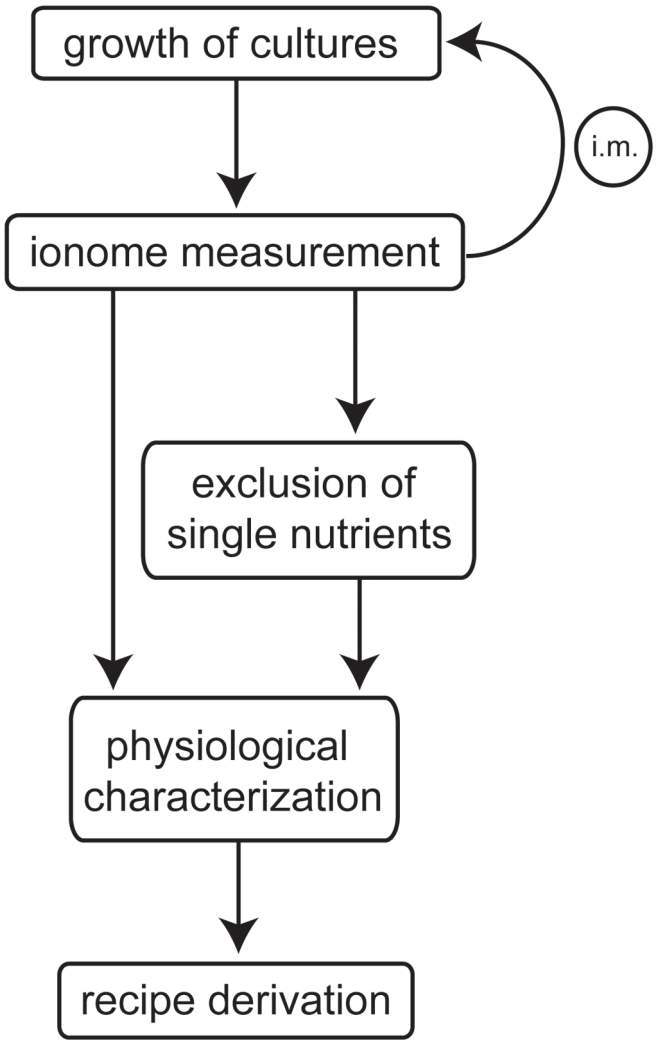

Figure 7. A strategy for deriving species-specific culture media
i.m. = iterative modification of nutrient concentrations to accommodate the cell ionome.

Table I

Comparison of trace metal supplements

	Hutner	Sager-Granick	Kuhl	Bold	Kropat
Fe^{2+}	17.9	0	25	17.9	0
Fe^{3+}	0	370	0	0	20
Zn^{2+}	76.5	3.5	1.0	30.7	2.5
Cu^{2+}	6.3	0.25	0.01	6.3	2
Co^{2+}	6.8	0.84	0	1.7	0
Mn^{2+}	25.6	2.0	1.0	7.3	6
Mo^{6+}	6.2	0.82	0.07	4.9	0.2
BO_3^{3-}	184	16	1.0	184	0
Se^{4+}	0	0	0	0	0.1
EDTA	134	0	25	171.1	57.8

Comparison of various trace metal supplements used to culture Chlamydomonas, adapted from Harris et al., 2009. Values shown are the final concentration (in μM).

Table II

Metal concentrations in Hutner's trace element solution

	Calculated	Measured		
		April 2010		October 2007
		Fresh	> 2 weeks	> 2 weeks
Fe	17.9	22	12	9.6
Zn	76.5	76	68	78
Cu	6.3	6.6	5.8	6.4
Co	6.8	7.2	6.4	6.7
Mn	25.6	27	24	24
Mo	6.2	6.5	5.7	6.8
B	184	ND	ND	ND

Metal concentrations in Hutner's trace element stock solution (mM) as described in the protocol and as measured before and after the 2-week equilibration period from a batch made in April 2010 and one made previously in October 2007.