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SHORT COMMUNICATION

Copper oxide nanoparticles promote the evolution of multicellularity in yeast

Jiaqi Tan[a,b]*, Qixin He[c]*, Jennifer T. Pentz[a], Cheng Peng[d], Xian Yang[a], Meng-Hsiu Tsai[a], Yongsheng Chen[e], William C. Ratcliff[a] and Lin Jiang[a]

[a]School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA, USA; [b]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA; [c]Department of Ecology and Evolution, University of Chicago, Chicago, IL, USA; [d]College of Environmental Science and Engineering, Donghua University, Shanghai, China; [e]School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta, GA, USA

ABSTRACT

Engineered nanomaterials are rapidly becoming an essential component of modern technology. Thousands of tons of nanomaterials are manufactured, used, and subsequently released into the environment annually. While the presence of these engineered nanomaterials in the environment has profound effects on various biological systems in the short term, little work has been done to understand their consequences over long, evolutionary timescales. The evolution of multicellularity is a critical step in the origin of complex life on Earth and a unique strategy for microorganisms to alleviate adverse environmental impacts, yet the selective pressures that favor the evolution of multicellular groups remain poorly understood. Here, we show that engineered nanomaterials, specifically copper oxide nanoparticles (CuO NPs), promote the evolution of undifferentiated multicellularity in Baker's yeast (*Saccharomyces cerevisiae* strain Y55). Transcriptomic analysis suggests that multicellularity mitigates the negative effects of CuO NPs in yeast cells and shifts their metabolism from alcoholic fermentation towards aerobic respiration, potentially increasing resource efficiency and providing a fitness benefit during CuO NP exposure. Competition assays also confirm that the multicellular yeast possesses a fitness advantage when exposed to CuO NPs. Our results, therefore, demonstrate that nanoparticles can have profound and unexpected evolutionary consequences, underscoring the need for a more comprehensive understanding of the long-term biological impacts of nanomaterial pollution.

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Copper oxide nanoparticles; evolution; multicellularity; yeast

Introduction

Engineered nanomaterials, compared to their bulk counterparts, possess many unique physiochemical characteristics, making them desirable for a wide variety of applications (Keller et al. 2010; Hendren et al. 2011). Thousands of tons of nanomaterials are therefore manufactured, used, and subsequently released into the environment annually (Hendren et al. 2011). Commonly used as antimicrobial agents, metallic nanoparticles may exert toxicity towards biological organisms, via damaging cell membrane structure and inducing reactive oxygen species (Lemire, Harrison, and Turner 2013). The presence of these engineered nanomaterials in the environment is known to have significant effects on various biological systems (Ren et al. 2009; Kahru and Dubourguier 2010). However, previous studies on this topic have focused only on short-term, ecological impacts and potential evolutionary responses of organisms over long timescales have not been explored (Chatterjee, Chakraborty, and Basu 2014; Graves et al. 2015).

Our study experimentally examined the effects of copper oxide nanoparticles (CuO NPs)—a commonly used engineered metallic oxide nanomaterial—on species adaptation to novel environmental stressors. CuO NPs have been used in electronic devices to improve thermophysical properties (Yu and Choi 2003), in fertilizers to promote copper availability to crops (Liu and Lal 2015), and in

CONTACT William C. Ratcliff william.ratcliff@biology.gatech.edu 310 Ferst Drive, Atlanta, GA 30332; Lin Jiang lin.jiang@biology.gatech.edu 310 Ferst Drive, Atlanta, GA 30332
*These authors contributed equally to this work.
Supplemental data for this article can be accessed here.

medical and cosmetic products to control microbial growth (Ren et al. 2009). All these usages release CuO NPs into the environment. Like other metallic oxide nanoparticles, CuO NPs show significant cytotoxicity to various organisms, as they increase the number of reactive oxygen species in cells, causing damages to cell membranes and apoptosis (Karlsson et al. 2008; Fahmy and Cormier 2009). The cell-level damage from these NPs may accumulate over generations, resulting in strong selection for stress tolerating phenotypes. If individuals within a population possess heritable variation in traits that affect nano-stress tolerance, the population may adapt over generations in response to nanoparticle exposure. We note that direct experimental demonstrations of species evolution in response to nanomaterial exposure are lacking.

The origin of multicellular organisms from unicellular ancestors is considered a major transition in evolution (Maynard Smith and Szathmary 1999), paving the way for further development in organismal complexity. The first step in the transition to multicellularity is the formation of undifferentiated groups. Also, as a unique life history strategy for microorganisms, undifferentiated multicellularity can provide advantages to microbes in harsh environments by reducing surface-to-volume ratios (Smukalla et al. 2008) and promoting resource utilization efficiency (Pfeiffer and Bonhoeffer 2003; Koschwanez, Foster, and Murray 2011). These benefits, however, may be outweighed by the costs of social conflicts (Hamilton 1964), such as the decrease in growth (Ratcliff et al. 2012). Overall, the ecological mechanisms underlying the establishment and maintenance of multicellularity in microorganisms remain poorly understood. We experimentally investigated how CuO NP exposure influences the evolution of multicellularity in Baker's yeast (*Saccharomyces cerevisiae* Y55). Starting with a unicellular ancestor, we provided an evolutionary incentive for the yeast to form groups by performing daily gravitational selection. Against the backdrop of physical selection for group formation, we compared the evolutionary trajectories of yeast under exposure to CuO NPs, copper ions, or CuO bulk particles for 42 days (~280 generations).

Material and methods

Copper oxide treatments

Our experiment included four treatments: control, ion, bulk, and nano (Figure 1). The CuO NPs used in our experiment were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). These bare NPs with no

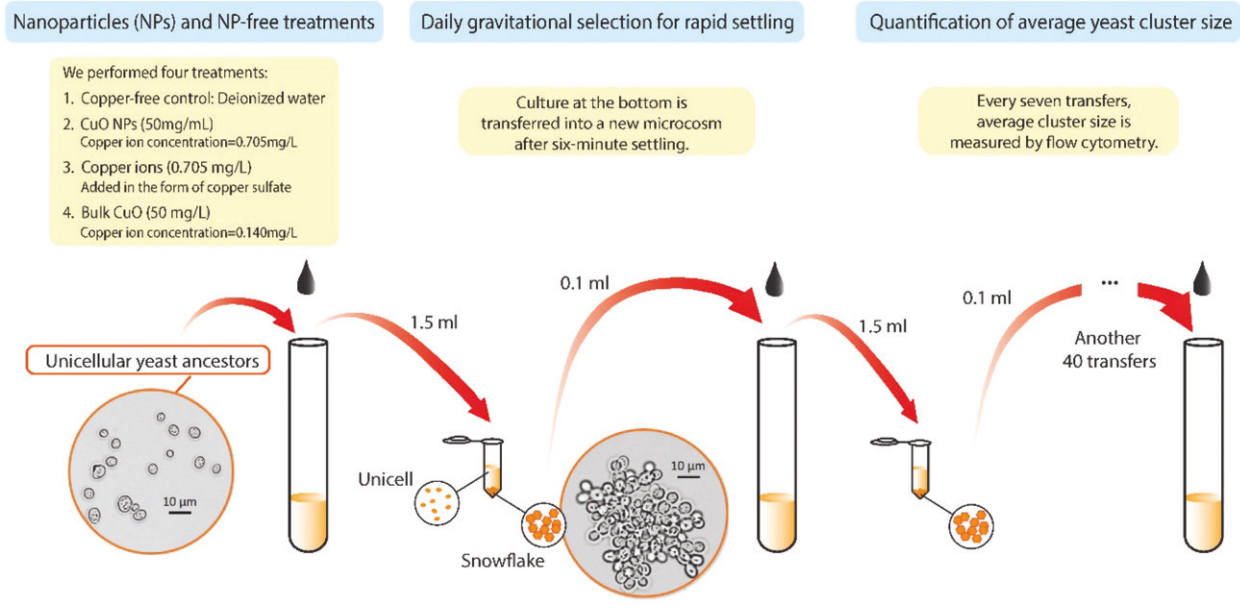

Figure 1. Experimental evolution of simple multicellularity in yeast with copper nanoparticle exposure. Our experiment included four treatments: a copper-free control, copper oxide nanoparticles (CuO NPs), copper ions, and bulk CuO. One genotype of diploid, unicellular yeast served as the ancestor. After 41 rounds of daily gravitational selection, multicellular, snowflake yeast evolved in all populations. The size of multicellular yeast was measured weekly via flow cytometry.

coatings are in a spherical shape with an average particle size of 25 nm (see Supplementary Figure S1). We used 60 ml glass test tubes with open-air culture caps, each of which contained 10 ml 1:10 yeast-extract-peptone-dextrose (YPD) medium (0.2% glucose, 0.2% peptone, 0.1% yeast extract), as the microcosms. We diluted the YPD to 10% of normal strength because it allowed for a more stable suspension of CuO NPs. To suspend the CuO NPs evenly into the culture medium, we first suspended them into deionized water at the concentration of 100 mg/L. The CuO NPs suspension and 1:5 YPD were sterilized separately with autoclave for 40 minutes and then mixed in the ratio of 1:1 in the microcosms. As a result, each microcosm contained 10 ml of CuO NPs and YPD mixture, with 50 mg/L CuO NPs and 1:10 YPD.

Using a Zetasizer-Nano ZS instrument (Malvern Instrument Ltd., UK), we determined that the CuO NPs in the culture medium had 291.6 ± 11.7 (mean ± s.d.) nm of hydraulic diameter and -18.1 mV of zeta potential. We prepared the microcosms with bulk CuO in the same way. To quantify the actual dissolved Cu concentration in the bulk and nano-CuO treatments, we incubated microcosms (without yeast) with either bulk or nano-CuO for 24 h, filtered the samples collected from the microcosms through 0.22 μm glass filters, and measured the concentration of Cu ions in microcosms with an inductively coupled plasma optical emission spectrometer (ICP-OES, iCAP 6300 DUO, Thermo, USA). We found that the copper ions (Cu^{2+}) in the microcosms increased from zero to 0.140 mg/L and 0.705 mg/L in the microcosms with bulk and nano CuO, respectively. We thus included a copper ion treatment with 0.705 mg/L Cu^{2+} added into the medium in the form of copper sulfate ($CuSO_4$, 1.76 mg/L). We replicated each treatment six times.

Experimental protocols

We used *Saccharomyces cerevisiae* strain Y55 (Ratcliff et al. 2012) as the model of evolution in our experiment. We began the experiment with a single genotype of diploid *S. cerevisiae*, strain Y55. At the beginning of the experiment, we streaked out the frozen culture onto YPD agar, randomly selected one colony, and confirmed its unicellular form under the microscope. We propagated the unicellular yeast overnight and introduced it into each experimental microcosm. During the experiment, we incubated the microcosms in a shaker at 250 rpm at 30 °C. Every 24 h, we collected a random 1.5 ml subsample of each 10 ml microcosm for daily gravitational settling selection. We first transferred the 1.5 ml culture to a centrifuge tube, placed this centrifuge tube on bench top for six minutes, and then discarded the top 1.4 ml culture before transferring the remaining 0.1 ml culture at the bottom to a new microcosm with fresh medium and copper materials (Figure 1). We performed this selection experiment for 42 days, measuring the average cluster size of the yeast populations weekly on a Partec Cyflow Cube 8 flow cytometer (Sysmex Partec GmbH, Görlitz, Germany). Average cluster size was quantified as the mean value of forward scatter, based on the screening of at least 20 000 clusters for each sample. At the end of the experiment, we isolated two unicellular genotypes and two multicellular genotypes from each sample. We were unable to retrieve multicellular isolates from one ion and one nano treatment after multiple trials. We propagated these cells and clusters on YPD agar overnight before extracting the genomic DNA and Sanger sequencing their *ACE2* genes.

Competition experiment between the unicellular and multicellular yeast

We performed a competition experiment to determine the relative fitness of isogenic unicellular and multicellular yeast under CuO NPs exposure. Rather than using strains evolved under our treatment conditions, in which treatment-specific compensatory mutations other than multicellularity may have evolved, we created otherwise isogenic unicellular (*ACE2/ACE2*) and multicellular (*ace2::KANMX4/ace2::KANMX4*) lines by completely removing *ACE2* from the unicellular strain (replacing it with *KANMX4* via the lithium acetate-PEG-ssDNA method) (Gietz et al. 1995). We set the initial frequency of the unicellular and multicellular genotypes as 100:1 (resulting in similar initial biomass per strain), allowing them to compete for three days. We replicate each combination six times. These incubation conditions were identical to the main experiment, with the settling selection performed twice (on days 1 and 2). We measured the final frequency of these

two genotypes by flow cytometry, and calculated the fitness of the multicellular yeast relative to the unicellular yeast with the selection rate constant (Lenski et al. 1991), expressed as Equation 1.

$$r = \ln \frac{final\ frequency\ of\ multicellular\ yeast}{initial\ frequency\ of\ multicellular\ yeast} - \ln \frac{final\ frequency\ of\ unicellular\ yeast}{initial\ frequency\ of\ unicellular\ yeast}$$

Transcriptome (RNA) sequencing

To investigate the mechanism underlying the size-related protection, we sequenced the transcriptomes of unicellular (*ACE2/ACE2*) and multicellular (*ace2::KANMX4/ace2::KANMX4*) snowflake yeast. The transcriptomes analysis would identify the genes that are actively expressed in response to the nano stress. We exposed the unicellular ancestor (*ACE2/ACE2*) and isogenic multicellular genotype (*ace2::KANMX4/ace2::KANMX4*) separately to the four experimental conditions for three days. Two biological replicates were performed for each treatment. The protocol of the sample preparation was the same as that of the main evolution experiment, except that we transferred these monocultures of unicellular and multicellular yeast without settling selection. On day 3, 24 h after the last transfer, we collected a 1.5 ml sample from each microcosm, concentrated the yeast cells by centrifuging the sample at 10 000 rpm for two minutes, and snap-froze the yeast cells before shipping them to GeneWiz, LLC. (South Plainfield, NJ, USA), where RNA extractions, library preparations, and sequencing reactions were conducted.

RNA extraction

After bead-based homogenization, total RNA was extracted from each sample with the Qiagen RNeasy Plus Mini Kit (Qiagen, Germantown, MD, USA). The concentration and integrity of extracted RNA were examined using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), respectively.

Library preparations

The RNA library for Illumina sequencing was prepared according to the manufacturer's manual, with the NEBNext Ultra RNA Library Prep Kit (NEB, Ipswich, MA, USA). The mRNA was first enriched with Oligod(T) beads, fragmented for 15 minutes at 94 °C, and conversed to cDNA. Both strands of cDNA were synthesized, end repaired, and adenylated at 3'ends. The universal adapters were further ligated to cDNA fragments, followed by index addition and library enrichment with limited cycle PCR. The sequencing libraries were validated on the Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA), and quantified using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and quantitative PCR (Applied Biosystems, Carlsbad, CA, USA).

Sequencing reactions

The sequencing libraries were multiplexed and clustered onto a flowcell. After clustering, the flowcell was loaded on the Illumina HiSeq 2500 instrument according to the manufacturer's instruction. The 16 samples were pooled and sequenced using a 1 × 50 bp Single-Read (SR) configuration. Image analysis and base calling were conducted by the HiSeq Control Software (HCS) on the HiSeq 2500 instrument. Raw sequence data (.bcl files) generated from Illumina HiSeq 2500 was converted into fastq files and de-multiplexed using Illumina bcl2fastq v 1.8.4 program. One mismatch was allowed for index sequence identification.

Data analysis

We mapped the cDNA sequencing reads of each sample to the transcriptome of yeast R64-1-1 using the software Kallisto (Bray et al. 2016). Kallisto implements a pseudo-alignment algorithm that estimates the likelihood of a transcript generating the reads rather than real alignment, which performs better than or as accurate as existing quantification tools. We then quantified the gene expression

across different treatments, using a method implemented in 'sleuth' package (Pimentel et al. 2017) of R that decouples biological variance from inferential variance by modeling the two sources of variances separately in an additive response error model. This quantification model provided the highest sensitivity at a particular false discovery rate, compared to all the alternative methods. In addition, this method, using bootstrapping with a low number of biological replicates, reduces the number of false positives through accounting for the inferential variance. We calculated divergences among the 16 transcriptomes using Jensen-Shannon index and built a hierarchical clustering tree of expression similarity based on the Jensen-Shannon matrix with Wards' sum-of-squares criterion (Murtagh and Legendre 2014), using 'hclust' function in 'vegan' package of R (Oksanen et al. 2007). The *p*-values of significantly different genes were adjusted with the Benjamini-Hochberg correction for multiple testing (Benjamini and Hochberg 1995) and reported as the false-discovery-rate adjusted *p*-values to control for the Type I error. We then investigated whether genes with significantly different expressions are enriched with certain biological processes in gene ontology terms compared to all genes that had expressions using GOrilla (Eden et al. 2007; Eden et al. 2009). The enriched gene ontology terms are clustered based the similarities measured using the SimRel index (Hsiao and Chen 2017) and visualized as treemaps in REViGO (Supek et al. 2011).

Results and discussion

Under our experimental conditions, multicellular yeast evolved, and their cluster size increased over time in all treatments (see Figure S2 for the picture of a multicellular yeast). However, the multicellular snowflake yeast evolved the largest cluster size when exposed to CuO NPs, compared to other treatments, after day 28 (Figure 2; Tukey's HSD, $p < 0.05$). 16.7%, 40%, and 60% of the sampled multicellular individuals carried nonsynonymous mutations in *ACE2*—a transcription factor necessary for mother-daughter cell separation (Oud et al. 2013)—from the control, ion, and nano treatments, respectively. However, neither the unicellular nor the multicellular individuals from the bulk treatment carried any mutations in *ACE2*. Thus, exposure to CuO NPs changed the genetic basis of adaptation, driving the parallel evolution of mutations in a regulatory element known to produce large snowflake yeast clusters when disabled (Ratcliff et al. 2015).

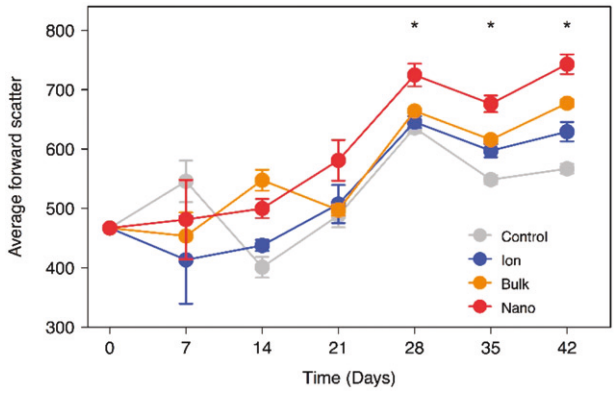

Figure 2. The dynamics of the mean cluster size within our experimental yeast populations (six replicates per treatment) subject to the four experimental treatments. Average cluster size was quantified as the average forward scatter, using flow cytometry. Values are mean ± s.e.m. Asterisks indicate that the average forward scatter in the nano treatment was higher than that of the other three treatments, according to one-way ANOVA, followed by Tukey's HSD tests.

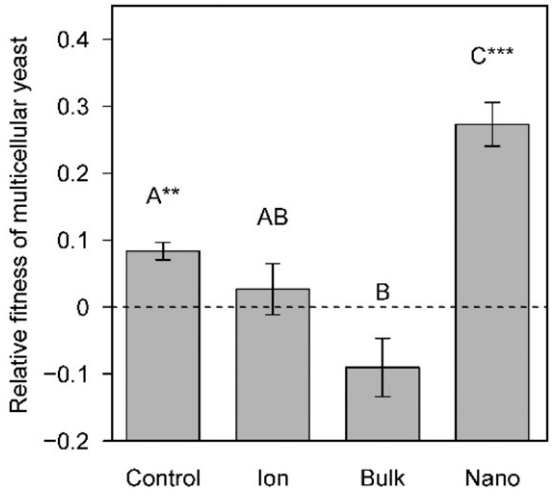

Figure 3. The relative fitness of the multicellular yeast compared to the unicellular ancestor. The relative fitness was quantified as the selection rate constant. The unicellular and multicellular genotypes were initially set as 100:1 (resulting in similar initial biomass per strain). Values are mean ± s.e.m. Treatments sharing the same letter do not differ from each other, according to one-way ANOVA, followed by Tukey's HSD. Asterisks indicate that the fitness values significantly differ from zero, according to the one-sample t-tests (**: $p < 0.01$; ***: $p < 0.001$).

To examine whether multicellularity provides yeast with protection from toxic CuO NPs exposure, we measured the fitness of the multicellular genotypes relative to the unicellular one directly via the

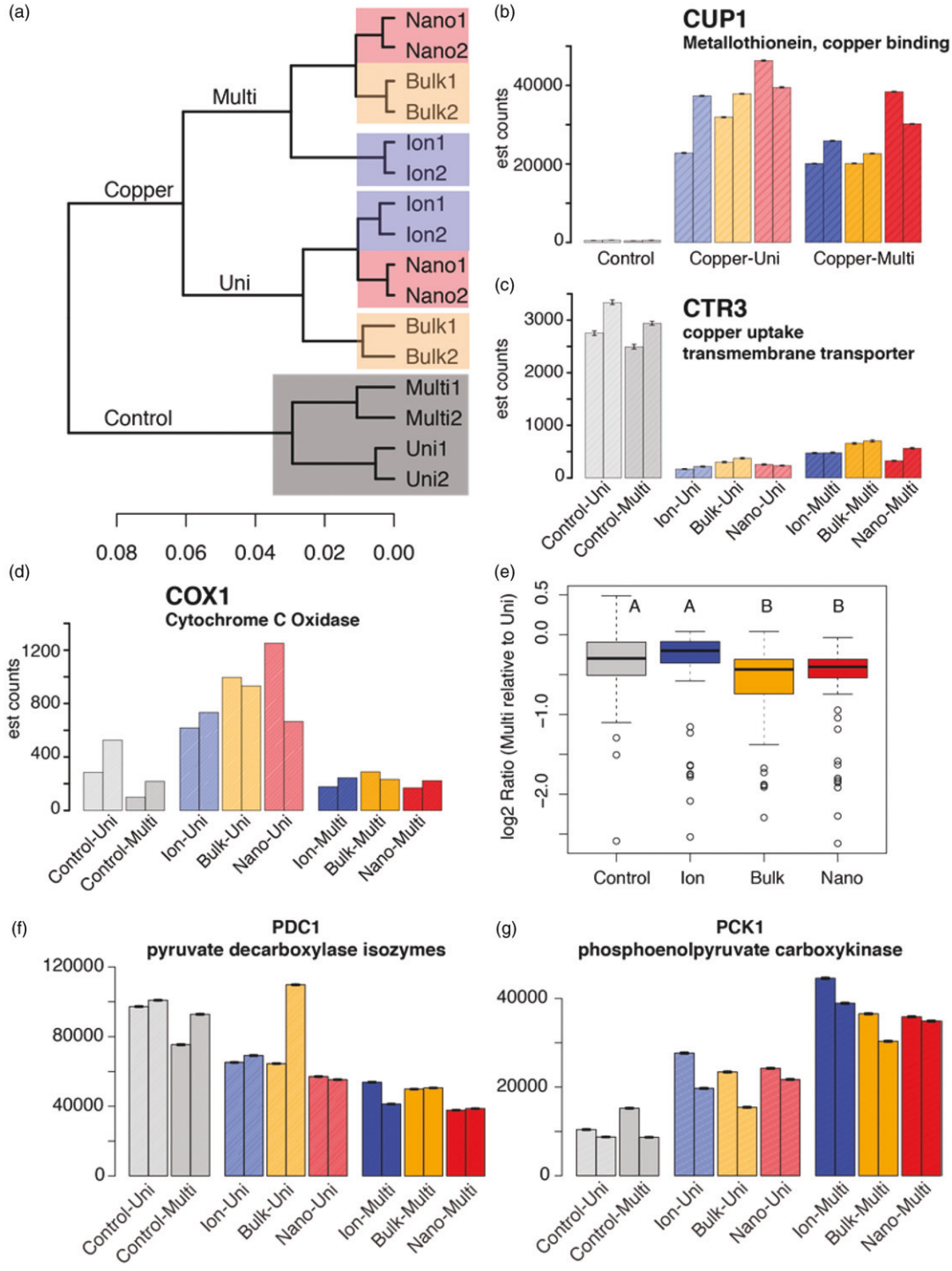

Figure 4. Summary of RNA-seq results. Panel a: Transcriptome similarity between the four copper treatments and two yeast genotypes (Uni: unicellular; Multi: multicellular) was clustered based on the pairwise Jensen-Shannon dissimilarity metrics. Two biological replicates were conducted in each treatment. Yeast genotypic identity is the principal factor determining the overall response to copper stress. Panel b: Levels of *CUP1* (metallothionein) expression. Under copper stress, metallothionein was increasingly expressed, particularly under the nano treatment; metallothionein expression was lower in the multicellular genotype, suggesting they experienced less copper stress (also see Supplementary Figure S3). Panel c: Levels of *CTR3* (copper uptake membrane transporter) expression. Copper uptake membrane transporter was increasingly suppressed under all copper treatments though these were more highly expressed in the multicellular yeast (and closer to the control), suggesting they were less overwhelmed by excess copper. Panel d: Levels of *COX1* (cytochrome C oxidase) expression. The multicellular genotype possessed downregulated redox processes, ATP and carbohydrate metabolism (also see Supplementary Figure S4), with significantly greater log2-fold changes of expression between the two genotypes in the bulk and nano, compared to the ion and control treatments (shown in Panel e by the paired t-test with Benjamini-Hochberg correction for multiple comparisons; treatments sharing the same letter do not differ from each other). Panel f: *PDC1*, the key enzyme in alcoholic fermentation, was downregulated in the multicellular genotype; Panel g: *PCK1*, the key enzyme in the gluconeogenesis, was upregulated in the multicellular genotype. Error bars on the gene est counts are first and third quantile of the bootstrap distributions from 'sleuth.'

competition experiment. Multicellular snowflake yeast increased in frequency and attained the highest fitness in the nano treatments (Figure 3; ANOVA: $F_{3,20}$=19.973, $p < 0.001$; Tukey's HSD, $p < 0.05$). The increase in the multicellular yeast cluster size and fitness under nano exposure supports the idea that simple, undifferentiated multicellularity protects microorganisms from environmental stressors through size-related benefits (Smukalla et al. 2008).

Furthermore, the transcriptome analysis indicated that yeast genotype and copper environment interactively determined the overall transcriptome pattern (Figure 4). First, both genotypes responded to copper stress systematically, creating a distinct expression profile in the three copper treatments, compared to the copper-free control (Figure 4(a); 654 differentially expressed genes; false-discovery-rate adjusted-$p < 0.05$; see the Supplementary tables and Figure S3). To alleviate Cu toxicity, yeast in the copper treatments had at least a 55-fold increase in the expression of *CUP1*, metallothionein that sequesters Cu ions (Figure 4(b)) (Winge et al. 1985), and a six-fold decrease in the expression of *CTR3*, a high-affinity copper transporter to reduce copper uptake (Figure 4(c)) (Ishida et al. 2002). Notably, changes in gene expression were most significant in the nano treatment (Figure 4(b,c)). Second, yeast genotype determined the overall expression patterns within the three copper treatments, with the multicellular genotype less affected by copper stress than the unicellular genotype (Figure 4(a) and S4). In the unicellular genotype, 551 genes (e.g. *COX1*) were upregulated, enriched with processes related to redox reactions and ADP/ATP metabolism (Figure 4(d,e)); only 364 genes of multicellular yeast were upregulated, enriched with transmembrane transporting processes. Changes in *CUP1*, *CTR3*, *COX1* expressions and other metabolism-related genes (Supplementary tables and Figure S4), for example, were higher in the unicellular than multicellular genotype within each treatment (Figure 4(b–d)). Together, these results suggest that undifferentiated multicellularity provided a protective barrier during copper exposure, maintaining metabolism and copper homeostasis as the copper-free treatment.

Previous simulation (Pfeiffer and Bonhoeffer 2003), analytical (Pfeiffer, Schuster, and Bonhoeffer 2001), and experimental studies (Koschwanez, Foster, and Murray 2011) suggested that efficient resource use resulting from cooperative behavior in undifferentiated cell clusters may facilitate the establishment of multicellular organisms. Yeast can regulate ATP yield and rate to adapt to varying environments. While yeast often rapidly produces ATP by fermenting glucose, it also respires ethanol and yields more ATP, albeit with a lower production rate (Gasmi et al. 2014). In the multicellular genotype found in the three copper treatments, *PDC1*, a key alcoholic fermentation enzyme, was downregulated (Figure 4(f)), while *PCK1*, a gluconeogenesis enzyme, was upregulated (Figure 4(g)). This finding suggests that the multicellular yeast, under nano stress, may have shifted their metabolism from fermentation to respiration, allowing them to utilize limited resources more efficiently.

Conclusions

Our study demonstrates that engineered nanomaterials can modulate evolutionary dynamics. Nanoparticles promote the evolution of undifferentiated multicellularity in yeast, as multicellularity mitigates the cellular stresses associated with nanoparticle exposure and favors the switch to a more efficient energy-generating pathway. Engineered nanomaterials, therefore, can precipitate fundamental shifts in species life history evolution. Given that novel engineered nanomaterials with poorly characterized ecological and evolutionary consequences may have significant impacts on microbial communities in various environments, further care should be taken during their manufacture, usage, and disposal. It is also worth noting that many nanoparticles in the environment, such as colloids or macromolecules, are naturally occurring, not artificially manufactured (Hough et al. 2008; Jimenez et al. 2011). Further work is needed to determine the extent to which these natural nanoparticles affect the ecological and evolutionary dynamics of environmental microorganisms.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCID

Jiaqi Tan http://orcid.org/0000-0001-8111-295X
Qixin He http://orcid.org/0000-0003-1696-8203
Cheng Peng http://orcid.org/0000-0002-7123-0714

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